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Karen Fulan Discola

"Caracterização estrutural e funcional das glutarredoxinas ditiólicas de *Saccharomyces cerevisiae*"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutora em Biologia Funcional e Molecular, na área de Bioquímica.

Orientador: Prof. Dr. Luis Eduardo Soares Netto

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BANCA EXAMINADORA

Prof. Dr. Luis Eduardo Soares Netto (Orientador)

Assinatura

Prof. Dr. Jörg Kobarg

Assinatura

Prof. Dr. Richard Charles Garratt

Assinatura

Prof. Dr. Fabio Ceneviva Lacerda Almeida

Assinatura

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Assinatura

Profa. Dra. Maria Cristina Nonato

Assinatura

Prof. Dr. João Alexandre Ribeiro Gonçalves Barbosa

Assinatura

Profa. Dra. Lúcia Rossetti Lopes

Assinatura

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LISTA DE ABREVIAÇÕES

BSA: albumina de soro bovino;

CHP: hidroperóxido de cumeno;

Cys_{GS}: resíduo de cisteína da molécula de glutationa;

DTT: ditiotreitol;

***Ec*Grx**: glutarredoxina de *Escherichia coli*;

EDTA: ácido etilenodiamino tetra-acético;

EROs: espécies reativas de oxigênio;

γ-Glu_{GS}: resíduo de γ-glutamato da molécula de glutationa;

Gly_{GS}: resíduo de glicina da molécula de glutationa;

GR: glutationa redutase;

Grx: glutarredoxina;

Grx-SG: glutarredoxina glutatiolada;

Grx_{ox}: glutarredoxina na forma oxidada;

Grx_{red}: glutarredoxina na forma reduzida;

GSH: glutationa reduzida, γ-glutamil**-**cisteinil**-**glicina;

GSSG: glutationa oxidada;

GST: glutationa-S-transferase;

HED: β-hidroxi-etil-dissulfeto;

***Hs*Grx**: glutarredoxina humana;

IAM: iodoacetamida;

IPTG: isopropil Δ-D-tiogalactosídeo;

β-ME-SG: dissulfeto misto formado entre HED e glutationa;

MBB: monobromobimano;

NADPH: nicotinamida adenina dinucleotídeo fosfato reduzida;

PDI: proteína dissulfeto isomerase;

PMSF: fluoreto de fenil-metil-sulfonil*;*

Proteína-SG: proteína glutatiolada;

Prx: peroxirredoxina;

Sc*Grx:* glutarredoxina de *Saccharomyces cerevisiae;*

***Sc*Trr**: tiorredoxina redutase de *Saccharomyces cerevisiae;*

RS^-: tiolato;

$RS^\bullet$: radical tiila;

RSNO: nitroso-tiol;

RSOH: ácido sulfênico;

RSO_2H: ácido sulfínico;

RSO_3H: ácido sulfônico;

***t*-BOOH**: *tert*-butil hidroperóxido;

Trx: tiorredoxina;

Trr: tiorredoxina redutase.

RESUMO

Glutarredoxinas (Grxs) são pequenas oxidorredutases que possuem pelo menos um resíduo de cisteína conservado em seus sítios ativos e têm atividade dissulfeto redutase dependente de tiol. Embora Grxs estejam envolvidas em diversos processos celulares, como enovelamento protéico e proteção contra espécies reativas de oxigênio, poucos substratos biológicos dessas enzimas são conhecidos. Na levedura *Saccharomyces cerevisiae*, oito Grxs foram identificadas (*Sc*Grx1-8); destas *Sc*Grx1-2 são ditiólicas e possuem o motivo Cys-Pro-Tyr-Cys em seus sítios ativos. Ambas Grxs ditiólicas são citosólicas, embora *Sc*Grx2 também seja encontrada na mitocôndria. Neste trabalho, mostramos que *Sc*Grx2 possui atividade específica como oxidorredutase quinze vezes maior do que *Sc*Grx1, embora estas enzimas compartilhem 64% de identidade e 85% de similaridade de seqüência. A análise cinética bi-substrato mostrou que *Sc*Grx2 possui tanto um menor K_M para glutationa quanto um maior *turnover* que *Sc*Grx1. Com o intuito de compreender melhor estas diferenças bioquímicas, determinamos os valores de pK_a da cisteína N-terminal (Cys27) dos sítios ativos destas duas proteínas e demonstramos que estes parâmetros não justificam a diferença de atividade observada. Tentando identificar características estruturais relacionadas a essa diferença de atividade, determinamos as estruturas cristalográficas de *Sc*Grx2 na forma oxidada e do mutante *Sc*Grx2-C30S ligado à glutationa a 2.05 e 1.91 Å de resolução, respectivamente, e comparamos estas estruturas com as estruturas de *Sc*Grx1 descritas por Håkansson & Winther, 2007. As análises estruturais nos permitiram formular a hipótese de que substituições dos resíduos Ser23 e Gln52 de *Sc*Grx1 por Ala23 e Glu52 em *Sc*Grx2 poderiam modificar a capacidade da cisteína C-terminal do sítio ativo de atacar o dissulfeto misto formado entre a cisteína N-terminal e glutationa. Nossa hipótese foi testada através de ensaios enzimáticos com proteínas mutantes. Acreditamos que as diferenças funcionais e estruturais observadas entre *Sc*Grx1 e *Sc*Grx2 possam refletir em variações na especificidade por substratos e indicam que estas enzimas possuem funções biológicas não redundantes em *S. cerevisiae*.

ABSTRACT

Glutaredoxins (Grxs) are small thiol-dependent oxidoreductases with disulfide reductase activity endowed by at least one cysteine at their active sites. Although Grxs are implicated in many cellular processes, including protein folding and protection against reactive oxygen species, few of their targets are known. In the yeast *Saccharomyces cerevisiae*, eight Grxs isoforms were identified (*Sc*Grx1-8). Two of them (*Sc*Grx1-2) are dithiolic, possessing a conserved Cys-Pro-Tyr-Cys motif. Both dithiol glutaredoxins are cytosolic, however *Sc*Grx2 is also located at the mitochondria. In spite of the fact that *Sc*Grx1 and *Sc*Grx2 share 85% of amino acid sequence similarity, we have shown that *Sc*Grx2 is fifteen times more active as oxidoreductase than *Sc*Grx1. Further characterization of the enzymatic activities through two-substrate kinetics analysis revealed that *Sc*Grx2 possesses both a lower K_M for glutathione and a higher turnover than *Sc*Grx1. To better comprehend these biochemical differences, the pK_a of the N-terminal active site cysteines (Cys27) of these two proteins were determined. Since the pK_a values of *Sc*Grx1 and *Sc*Grx2 Cys27 residues are very similar, these parameters cannot account for the difference observed between their specific activities. In an attempt to better understand the mechanisms and differences between yeast dithiol Grxs activities, we elucidated the crystallographic structures of *Sc*Grx2 in the oxidized state and of the *Sc*Grx2-C30S mutant with a glutathionyl mixed disulfide at resolutions of 2.05 and 1.91 Å, respectively. Comparisons among these structures and those of *Sc*Grx1 (Håkansson & Winther, 2007) provided insights into the remarkable functional divergence between these enzymes. We hypothesize that the substitutions of Ser23 and Gln52 in *Sc*Grx1 by Ala23 and Glu52 in *Sc*Grx2 can modify the capability of the active site C-terminal cysteine to attack the mixed disulfide between the N-terminal active site cysteine and the glutathione molecule. Mutagenesis studies supported this hypothesis. The observed structural and functional differences between *Sc*Grx1 and *Sc*Grx2 may reflect variations in substrate specificity and non-redundant biological functions.

I - INTRODUÇÃO

I.1 - Trxs e Grxs

O meio intracelular é geralmente um ambiente redutor em grande parte devido a altas concentrações de glutationa *(γ-glutamylcistenyglycine*), da ordem de milimolar (Penninckx, 2002). Entretanto, espécies reativas de oxigênio (EROs) e outros oxidantes podem alterar o balanço redox celular. A glutationa atua como um tampão do estado redox da célula, já que este tiol de baixa massa molecular é prontamente oxidado por diversas enzimas antioxidantes e radicais livres. Por isso, a razão glutationa reduzida/glutationa oxidada (GSH/GSSG) tem sido largamente utilizada como indicador do estado de oxidação celular (Schafer & Buettner, 2001).

O balanço redox também é mantido por sistemas antioxidantes dependentes de tiorredoxina (Trx) e glutarredoxina (Grx) (Garrido & Grant, 2002). Grx e Trx são oxidorredutases de baixa massa molecular (9-12 kDa), que possuem resíduos de cisteína altamente conservados em seus sítios ativos (Holmgren, 1989). O sítio ativo de Trx é composto pelo motivo Cys-Gly-Pro-Cys, enquanto o de Grxs clássicas é composto por Cys-Pro-Tyr-Cys (Garrido & Grant, 2002). Ambas as proteínas são amplamente distribuídas entre os seres vivos e são reduzidas por nicotinamida adenina dinucleotídeo fosfato (NADPH), mas por diferentes vias metabólicas. A Trx oxidada é reduzida por NADPH em reação catalisada pela flavo-enzima tiorredoxina redutase (Trr) (Figura 1). Por outro lado, a Grx oxidada é reduzida pela GSH, gerando glutationa oxidada (GSSG). GSSG, por sua vez, é reduzida por NADPH em uma reação catalisada pela flavo-enzima glutationa redutase (GR) (Trotter & Grant, 2003; Holmgren, 1989) (Figura 1).

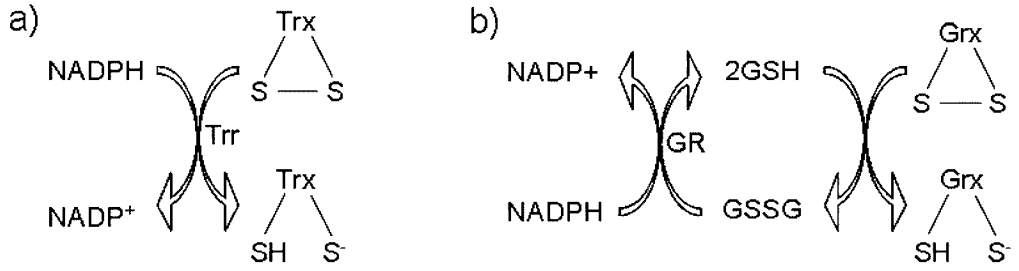

Figura 1: Redução de tiol/dissulfeto oxidorredutases. (a) Redução de Trxs: a Trr catalisa a redução por NADPH da Trx oxidada. (b) Redução de Grxs: Grxs são reduzidas por duas moléculas de GSH, gerando GSSG, a qual é reduzida por NADPH em uma reação catalisada pela GR.

A função clássica atribuída a Trx e Grx é como fonte de elétrons para processos que envolvem a redução de pontes dissulfeto em proteínas alvos. Como pontes dissulfetos são ligações químicas importantes para a estrutura, estabilidade e função de várias proteínas (Riestch & Beckwith, 1998), Trx e Grx têm papel muito central na fisiologia celular. O mecanismo de ação de Trx e Grx envolve a interconversão tiol-dissulfeto entre seus sítios ativos e os sítios ativos de proteínas alvos (Ritz & Beckwith, 2001).

Grxs foram descobertas inicialmente como doadores de elétrons para a enzima ribonucleotídeo redutase em um mutante de *Escherichia coli* sem Trxs (Holmgren, 1985). Neste caso, a linhagem mutante sem Trxs é viável porque Grxs atuam como redutores na conversão de ribonucleotídeos a desoxirribonucleotídeos, possibilitando a síntese de DNA.

O fato de os sistemas Grx e Trx apresentarem funções similares na manutenção do estado redox celular de proteínas é suportado pela observação de que a função de glutationa redutase é essencial para o crescimento celular do duplo mutante Δ*trx1trx2* de *Saccharomyces cerevisiae* (Muller, 1996). Isso provavelmente devido ao acúmulo de GSSG no triplo mutante Δ*trx1trx2glr1*, já que, na ausência de Trxs, as Grxs estão reduzindo substratos e gerando GSSG, a qual não pode ser reduzida por GR. Porém, a perda das Grxs ditiólicas não afeta o estado de oxidação ou os níveis de GSH na célula (Muller, 1996).

Além da atividade de oxidorredutase, outras atividades bioquímicas foram atribuídas às Grxs: peroxidase dependente de tiol, redutor da forma oxidada do ascorbato

(deidroascorbato) e glutationa S-transferase (Collinson *et al.*, 2002; Wells *et al.*, 1990; Collinson & Grant, 2003).

Outra atividade bioquímica de Grxs pode estar relacionada com a prevenção da oxidação irreversível de cisteínas em proteínas. Resíduos de cisteína estão entre os que são mais facilmente oxidados em proteínas. O mecanismo de oxidação de cisteínas pode envolver a formação de ácido sulfênico (Cys-SOH). Cys-SOH pode reagir com outras moléculas de peróxidos, originando ácido sulfínico (Cys-SO_2H) ou sulfônico (Cys–SO_3H), os quais não podem ser reduzidos por ditiotreitol (DTT), ou Trxs e Grxs (Poole *et al.*, 2004). Dessa forma, enzimas com Cys–SO_2H ou Cys–SO_3H, em geral, são inativadas oxidativamente (Poole *et al.*, 2004). Alternativamente, Cys–SOH pode reagir com outra sulfidrila (Cys-SH), originando um dissulfeto que pode ser reduzido por DTT, Trxs e Grxs.

Estas oxidações irreversíveis podem também ser evitadas através da glutatiolação de proteínas (Figura 2), na qual os grupos -SOH de proteínas formam dissulfetos mistos com GSH (Proteína-SG, proteína glutatiolada). A reação de glutatiolação é reversível, e a desglutatiolação pode ocorrer pela redução direta de GSH, ou em reação catalisada por Grxs (Gallogly & Mieyal, 2007). Diversos outros mecanismos foram propostos para a glutatiolação protéica (revisado por Shelton *et al.*, 2005), alguns deles são mostrados na Figura 2.

$$GSSG + Proteína\text{-}SH \rightarrow Proteína\text{-}SSG + GSH$$

$$GSH + Proteína\text{-}SOH \rightarrow Proteína\text{-}SSG + H_2O$$

$$GS^{\cdot} + Proteína\text{-}S^{\cdot} \rightarrow Proteína\text{-}SSG$$

$$GSOH + Proteína\text{-}SH \rightarrow Proteína\text{-}SSG + H_2O$$

$$GSNO + Proteína\text{-}S^{\cdot} \rightarrow Proteína\text{-}SSG + NO^{\cdot}$$

$$GSNO + Proteína\text{-}S^{-} \rightarrow Proteína\text{-}SSG + NO^{-}$$

Figura 2: Possíveis mecanismos para a glutatiolação de proteínas. Esta figura foi feita baseando-se no esquema apresentado por Shelton *et al.*, 2005.

A glutatiolação afeta a função de diversas proteínas, de modo que a glutatiolação reversível de proteínas específicas tem sido implicada na regulação da homeostase celular (revisado por Gallogly *et al.*, 2009). É importante ressaltar que a redução de substratos glutatiolados é a principal atividade atribuída às Grxs (revisado por Lillig *et al.*, 2008). A manipulação dos níveis de Grxs tem demonstrado afetar o *status* da glutatiolação protéica (revisado por Gallogly *et al.*, 2009).

I.2 - Mecanismos de catálise de Grxs

Grxs podem catalisar reações por dois mecanismos. Um deles é conhecido como mecanismo monotiólico, pois apenas um dos resíduos de cisteína do sítio ativo está envolvido: a cisteína N-terminal (Cys27 em *Sc*Grx1 e *Sc*Grx2). O outro é conhecido como mecanismo ditiólico, pois ambos os resíduos de cisteína do sítio ativo de Grx são necessários. Reações de redução de dissulfetos mistos entre glutationa e proteínas, ou entre glutationa e tióis de baixa massa molecular (exemplo: HED), se processam pelo mecanismo monotiólico de Grxs, enquanto reações de redução de ligações dissulfeto em proteínas se processam pelo mecanismo ditiólico (Fernandes & Holmgren, 2004).

É importante ressaltar que todas as Grxs ditiólicas (que possuem dois resíduos de Cys em seus sítios ativos) estudadas até o momento catalisam reações de desglutatiolação pelo mecanismo monotiólico, mas nem todas elas catalisam a redução de dissulfetos em proteínas através do mecanismo ditiólico (revisado por Lillig *et al.*, 2008).

No mecanismo ditiólico, a cisteína N-terminal do sítio ativo, que se encontra mais exposta ao solvente na superfície da estrutura protéica, inicia o ataque sobre um dos átomos de enxofre da ligação dissulfeto da proteína alvo (Figura 3, reação a). Conseqüentemente, um dissulfeto misto é formado entre a cisteína N-terminal de Grx e a proteína alvo. A cisteína C-terminal do sítio ativo é desprotonada e ataca a cisteína N-terminal de Grx (Figura 3, reações b, c), gerando Grx oxidada (com uma ligação dissulfeto intramolecular) e a proteína alvo na forma reduzida (Fernandes & Holmgren, 2004). A regeneração da forma reduzida de Grx, como descrito anteriormente, se dá através de reações seqüenciais com duas moléculas de GSH (Figura 3, reações d, e).

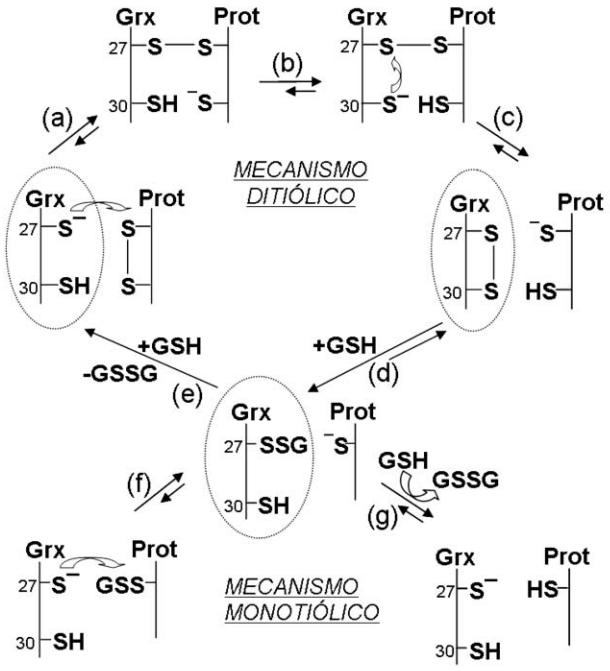

Figura 3: Mecanismos de catálise de Grxs. No mecanismo de ação ditiólico, a Cys N-terminal de Grxs atacam o dissulfeto da proteína alvo (reação a), formando um dissulfeto misto entre Grx e a proteína alvo. Este dissulfeto misto é reduzido pelo ataque da Cys C-terminal desprotonada (reação c), liberando a proteína reduzida e Grx oxidada com um dissulfeto intramolecular. Esta forma de Grx oxidada é reduzida por duas reações consecutivas com moléculas de glutationa (reações d, e). No mecanismo de ação monotiólico, o dissulfeto misto Proteína-SG é reduzido por Grx, ocorrendo, então, a formação do intermediário Grx-SG (reação f), o qual é reduzido por outra molécula de GSH (reação g).

No mecanismo monotiólico, Grx utiliza apenas a cisteína N-terminal de seu sítio ativo, interagindo especificamente com o motivo glutationa da proteína ou tiol glutatiolado, através do seu sítio de ligação para glutationa (Figura 3, reação f) (Bushweller *et al.*, 1992: Bushweller *et al.*, 1994). Forma-se um intermediário que consiste em um dissulfeto misto entre Grx e glutationa (Grx-SG), e a molécula alvo é liberada na forma reduzida. Então uma segunda molécula de GSH reduz o intermediário Grx-SG, produzindo Grx reduzida e glutationa oxidada (GSSG, Figura 3, reação g), a qual é reduzida pela GR, à custa de elétrons do NADPH (Fernandes & Holmgren, 2004).

Como pode ser observado na Figura 3, o intermediário Grx-SG é comum a ambos os mecanismos mono e ditiólico. A redução do dissulfeto misto Grx-SG por uma segunda molécula de GSH (Figura 3, reações e, g) é a etapa lenta e determinante da velocidade da reação global (Srinivasan *et al.*, 1997). Se o ataque nucleofílico da cisteína C-terminal sobre Grx-SG é favorecido (Figura 3, reação d), a reação se processa pelo mecanismo ditiólico, mas se a redução de Grx-SG por um nucleófilo externo (como GSH) é favorecida (Figura 3, reação g), a reação se processa pelo mecanismo monotiólico (Nordstrand *et al.*, 1999; Jao *et al.*, 2006).

Em reações de desglutatiolação, que se processam pelo mecanismo monotiólico, a ocorrência da reação secundária de ataque da cisteína C-terminal do sítio ativo sobre o intermediário Grx-SG (Figura 3, reação d) leva a um decréscimo na velocidade da reação global, provavelmente por que parte das moléculas de Grx e de glutationa fica envolvida em reações de intercâmbio tiol-dissulfeto desnecessárias (Yang *et al.*, 1998).

A especificidade da primeira etapa da reação do mecanismo monotiólico (Figura 3, reação f) é dependente da ligação não-usual formada pela cadeia lateral do γ-carboxilato do Glu N-terminal e o grupo α-amino da Cys presente na molécula de GSH (Peltoniemi *et al.*, 2006). A reação de Grx1 de *Escherichia coli* (*Ec*Grx1) com um peptídeo glutatiolado resulta apenas na formação do dissulfeto misto Grx-SG, enquanto que a reação de *Ec*Grx1 com um peptídeo ligado a ECG (glutamil**-**cisteinil**-**glicina, análogo da GSH com ligação peptídica normal entre Glu e Cys) produz apenas o dissulfeto Grx-peptídeo (Peltoniemi *et al.*, 2006). Essa especificidade para o motivo glutationa na primeira etapa da reação também foi descrita para Grx1 humana (*Hs*Grx1) e Grx de porco (Yang *et al.*, 1998; Rabenstein & Millis, 1995).

I.3 - Grxs de *Saccharomyces cerevisiae*

A levedura *Saccharomyces cerevisiae* possui oito Grxs. As glutarredoxinas *Sc*Grx1 e *Sc*Grx2 são ditiólicas clássicas, enquanto *Sc*Grx3-7 são isoformas monotiólicas. *Sc*Grx8 foi caracterizada recentemente, é uma isoforma ditiólica, porém apresenta características bioquímicas e estruturais que a diferem de *Sc*Grx1 e *Sc*Grx2. A Tabela 1 resume algumas informações sobre as Grxs de levedura.

Tabela 1: Localização celular, massa molecular e resíduos dos sítios ativos das Grxs de *S. cerevisiae.*

	Localização Celular	Massa Molecular (kDa)	Sítio Ativo
*Sc*Grx1	Citosol	12,38	CPYC
*Sc*Grx2	Citosol/Mitocôndria	11,96/ 15,86	CPYC
*Sc*Grx3	Núcleo	32,48	CGFS
*Sc*Grx4	Núcleo	27,49	CGFS
*Sc*Grx5	Mitocôndria	16,93	CGFS
*Sc*Grx6	Retículo endoplasmático/*cis*-Golgi	25,78	CSYS
*Sc*Grx7	Retículo endoplasmático/*cis*-Golgi	22,56	CPYS
*Sc*Grx8	Citosol	12,52	CPDC

As Grxs monotiólicas de levedura apresentam extensões na região N-terminal, mas têm similaridade significativa com as Grxs ditiólicas na região C-terminal (Figura 4). *Sc*Grx6 e *Sc*Grx7 são mais similares às Grxs ditiólicas do que *Sc*Grx3-5 o são, uma vez que não possuem uma inserção antes da cisteína N-terminal do sítio ativo, ou o motivo WP, anterior ao sítio TXP de ligação à glutationa (Mesecke *et al.*, 2008b).

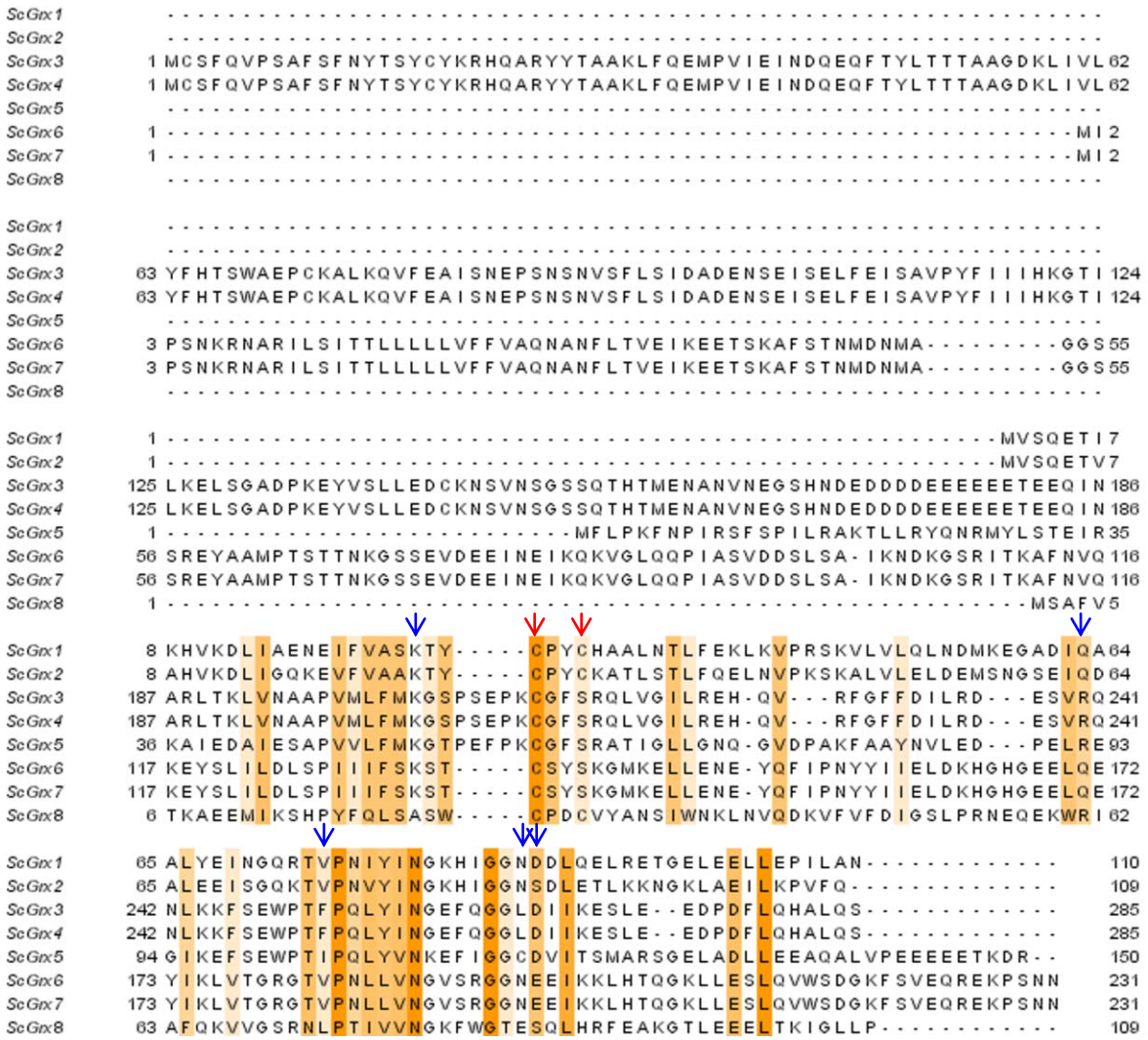

Figura 4: Alinhamento das seqüências das Grxs de *S. cerevisiae*. Em laranja escuro, estão destacados os aminoácidos mais conservados entre as Grxs de levedura; e, à medida que diminui o grau de conservação, o tom de laranja se torna mais claro. As cisteínas dos sítios ativos de *Sc*Grx1 e *Sc*Grx2 (isoforma de 11.9 kDa) estão indicadas pelas setas vermelhas, e os resíduos destas proteínas que interagem com glutationa estão indicados pelas setas azuis. O alinhamento foi feito utilizando o programa Multalin versão 5.4.1 (Corpet, 1988), e a representação, com o programa Jalview (Clamp *et al.,* 2004).

I.3.1 - Grxs ditiólicas *S. cerevisiae*

Na levedura *S. cerevisiae,* foram identificados dois genes de Grxs ditiólicas, denominados *Scgrx1* e *Scgrx2*, que apresentam 40-52% de identidade e 61-76% de similaridade com Grxs de bactérias e mamíferos (Luikenhuis *et al.*, 1998). *Sc*Grx1 e *Sc*Grx2 fazem parte da subfamília das Grxs clássicas que possuem dois resíduos de cisteína no sítio ativo. Apesar do alto grau de identidade (64%) e similaridade (85%) entre *Sc*Grx1 e *Sc*Grx2, ensaios com extrato de leveduras mutantes Δ*grx1*, Δ*grx2 e* Δ*grx1grx2* mostraram que *Sc*Grx2 é a maior responsável pela atividade como oxidorredutase na célula, sendo que uma de suas principais funções pode ser a redução de dissulfetos mistos formados como resultado de danos causados por EROs (Luikenhuis *et al.*, 1998). Além disso, a diferença de atividade como oxidorredutase observada por estes autores não é devida a uma diferença na expressão dos dois genes.

*Sc*Grx1 é citosólica, enquanto *Sc*Grx2 apresenta duas diferentes isoformas com pesos moleculares de 11.9 e 15.9 kDa que se localizam ambas na mitocôndria, porém a menor isoforma de *Sc*Grx2 também é encontrada no citosol (Pedrajas *et al.*, 2002). Neste projeto, estudamos a isoforma de 11.9 kDa de *Sc*Grx2. As isoformas de *Sc*Grx2 (11.9 e 15.9 kDa) são sintetizadas a partir de dois códons de iniciação de transcrição localizados *in-frame*. A isoforma citosólica é sintetizada a partir do segundo AUG (Figura 5, setas 4 e 5), não possuindo a extensão N-terminal, enquanto a isoforma longa, que carrega a seqüência de endereçamento mitocondrial, é resultado da tradução do primeiro AUG (Figura 5, seta 3; Porras *et al.*, 2006). A isoforma contendo a extensão N-terminal tem dois destinos: uma parte é importada para a mitocôndria e tem a cauda N-terminal cortada por uma peptidase, gerando a isoforma de 11.9 kDa que permance na matriz (Figura 5, seta 6), enquanto outra parte não é processada e permanece ligada à porção externa da membrana mitocondrial externa (Figura 5, seta 7).

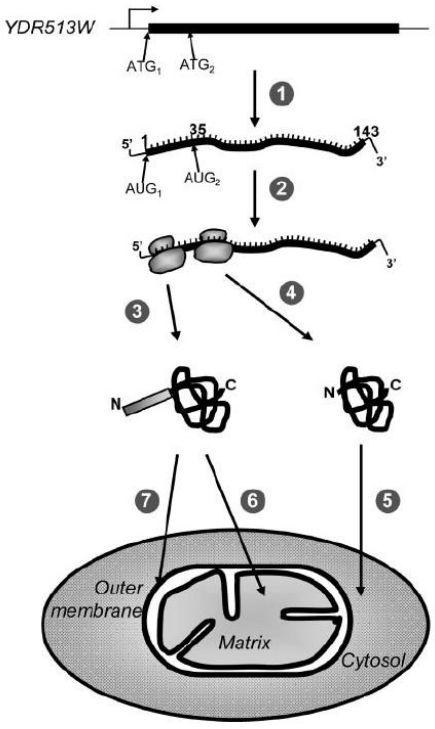

Figura 5: Esquema retirado de Porras *et al*., 2006, mostrando a origem das duas isoformas de Grx2 encontradas em *S. cerevisiae*.

A glutationa redutase (Glr1) de *S. cerevisiae* se localiza tanto no citosol quanto na mitocôndria (Outten & Culotta, 2004), permitindo a redução de GSSG, produzida no ciclo catalítico de Grxs, em ambos os compartimentos celulares. Como para *Sc*Grx2, as isoformas de Glr1 são sintetizadas a partir de dois códons de iniciação de transcrição localizados *in-frame*. A isoforma citosólica é sintetizada a partir do segundo AUG e a isoforma mitocondrial é resultado da tradução do primeiro AUG (Outten & Culotta, 2004).

A resistência de células de levedura ao estresse oxidativo induzido por peróxidos é afetada por deleções nos genes de Grxs ditiólicas. O duplo mutante Δ*grx1grx2* é muito sensível ao estresse oxidativo, e o mutante Δ*grx1* é bastante sensível ao ânion superóxido. Ambos os mutantes Δ*grx1* e Δ*grx2* são sensíveis a H_2O_2, sendo que o mutante Δ*grx2* é mais sensível a este oxidante que o mutante Δ*grx1*. A super expressão de *grx1* e *grx2* aumenta a resistência a H_2O_2 e a peróxidos orgânicos, como *tert*-butil hidroperóxido (*t*-BOOH) e hidroperóxido de cumeno (Luikenhuis *et al.*, 1998).

*Sc*Grx8 é uma isoforma ditiólica que foi descrita e caracterizada recentemente (Mesecke *et al.*, 2008a; Eckers *et al*., 2009). *Sc*Grx8 se localiza no citosol e é pouco abundante na célula. Além disso, *Sc*Grx8 apresenta atividade bastante baixa no ensaio de redução do dissulfeto misto formado entre HED e glutationa (β-ME-SG), sendo que sua atividade só é detectada neste ensaio quando usadas concentrações de enzima da ordem de micromolar, enquanto *Sc*Grx1 e *Sc*Grx2 são ativas em concentrações da ordem de nanomolar (Eckers *et al*., 2009; Discola *et al.*, 2009 - anexo 1). Aparentemente, a baixa atividade de *Sc*Grx8 no ensaio de redução do dissulfeto β-ME-SG se deve ao fato de sua redução por GSH ser lenta (Eckers *et al*., 2009).

*Sc*Grx8 apresenta baixa identidade com *Sc*Grx1 (30% identidade e 47% similaridade) e *Sc*Grx2 (24% identidade e 49% similaridade) e, diferentemente das Grxs ditiólicas clássicas, apresenta a seqüência CPDC em seu sítio ativo e não possui os resíduos do motivo de ligação à glutationa conservados (Figura 4). Além disso, *Sc*Grx8 apresenta um resíduo de triptofano anterior ao sítio ativo que não é encontrado em Grxs, mas é conservado em Trxs (Eckers *et al*., 2009).

Por ter sido descrita recentemente e por apresentar propriedades bioquímicas e estruturais distintas das outras duas Grxs ditiólicas de levedura, *Sc*Grx8 não foi objeto de estudo deste trabalho.

I.3.2 - Grxs monotiólicas de *S. cerevisiae*

Apesar de apresentarem a seqüência CGFS conservada em seus sítios ativos e 29% de identidade entre si (que aumenta para 71% quando consideradas apenas *Sc*Grx3 e *Sc*Grx4; Figura 4), *Sc*Grx3-5 parecem ter funções celulares diferentes. A perda das enzimas *Sc*Grx3, *Sc*Grx4 e *Sc*Grx5 resulta em um decréscimo na atividade de oxidorredutase em extratos celulares, e o mutante triplo é inviável (Rodríguez-Manzaneque *et al.*, 1999).

*Sc*Grx3 e *Sc*Grx4 são nucleares (Lopreiato *et al.*, 2004; Molina *et al.*, 2004) e possuem um domínio Trx N-terminal, fusionado ao domínio Grx (Bellí *et al.*, 2002; Vilella *et al.*, 2004), que é necessário para o endereçamento destas Grxs ao núcleo (Molina *et al.*,

2004). *Sc*Grx3 e *Sc*Grx4 estão envolvidas na inibição, dependente de ferro, do fator de transcrição Aft1, o qual regula a transcrição de genes envolvidos na homeostase de ferro (Ojeda *et al*., 2006; Pujol-Carrion *et al.*, 2006). Este fator de transcrição induz a expressão de genes do *regulon* do ferro em células de levedura deficientes em ferro, mas é inativado em condições nas quais a concentração de ferro intracelular é suficiente. Células sem *Sc*Grx3 e *Sc*Grx4 apresentam expressão constitutiva dos genes do *regulon* do ferro (Ojeda *et al*., 2006; Pujol-Carrion *et al.*, 2006).

A ausência de *Sc*Grx5 induz uma série de defeitos de crescimento. Mutantes sem *Sc*Grx5 não são capazes de crescer na presença de fontes não fermentáveis de carbono, acumulam ferro na mitocôndria e mostram uma diminuição nas atividades de enzimas que contém grupamentos ferro-enxofre (Rodríguez-Manzaneque *et al.*, 2002). Recentemente, foi demonstrado que *Sc*Grx5 é uma proteína mitocondrial envolvida na biogênese de proteínas ferro-enxofre (Rodríguez-Manzaneque *et al.*, 2002). A perda de *Sc*Grx5 também causa um aumento na sensibilidade a oxidantes e acúmulo de proteínas carboniladas na célula (Rodríguez-Manzaneque *et al.*, 1999).

O duplo mutante de levedura Δ*grx2grx5* é inviável (Rodríguez-Manzaneque *et al.*, 1999), indicando a necessidade de ao menos uma Grx na mitocôndria para a manutenção da atividade de oxidorredutase sobre substratos glutatiolados, uma vez que foi descrito que ambas, *Sc*Grx2 e *Sc*Grx5, são capazes de desglutatiolar substratos protéicos (Silva *et al.*, 2008 - anexo 2; Shenton *et al.*, 2002). Porém, *Sc*Grx2 parece não compensar a ausência de *Sc*Grx5 na biogênese de proteínas ferro-enxofre (Molina *et al.*, 2004).

As isoformas monotiólicas de levedura *Sc*Grx6 e *Sc*Grx7 foram identificadas recentemente e diferem das demais Grxs monotiólicas estudadas até o momento por apresentarem atividade como oxidorredutases no ensaio padrão para Grxs na redução do dissulfeto β-ME-SG (Meseck *et al.*, 2008b). Além disso, *Sc*Grx6 e *Sc*Grx7 apresentam resíduos diferentes em seus sítios ativos (CSYS e CPYS, respectivamente) em comparação às outras três Grxs monotiólicas de *S. cerevisiae* (Tabela 1).

Ambas, *Sc*Grx6 e *Sc*Grx7, contém um domínio transmembrana N-terminal que permite que elas se associem às membranas das vesículas do retículo endoplasmático e *cis*-Golgi na face do lúmen (Izquierdo *et al.*, 2008; Mesecke *et al*., 2008a). Os mutantes Δ*grx6*

e Δ*grx7* de levedura apresentam defeitos de crescimento e sensibilidade aumentada frente a agentes oxidantes como H_2O_2 e diamida (Mesecke *et al*., 2008a). Estes autores propõem que *Sc*Grx6 e *Sc*Grx7 não devem estar relacionadas ao enovelamento oxidativo de proteínas, mas devem se contrapor à oxidação de tióis específicos de substratos protéicos.

I.4 - Características gerais de Grxs

I.4.1 - Estrutura

Grxs possuem o conhecido enovelamento Tiorredoxina (Trx), que consiste em uma folha β central, geralmente composta por 4 fitas, rodeada por α-hélices (αβαβαββαα; Figura 6; Pan & Bardwell, 2006). Outras proteínas também apresentam este enovelamento básico, em alguns casos, com inserções ou extensões, como as próprias Trxs, as Proteínas Dissulfeto Isomerases (PDI; Kemmink *et al.,* 1997), Peroxirredoxinas (Prx; Wood *et al.,* 2003), Glutationa-Peroxidases (Epp *et al.*, 1983) Glutationa-S-Transferases (GST; Martin, 1995), DsbA (Martin *et al*., 1993), entre outras.

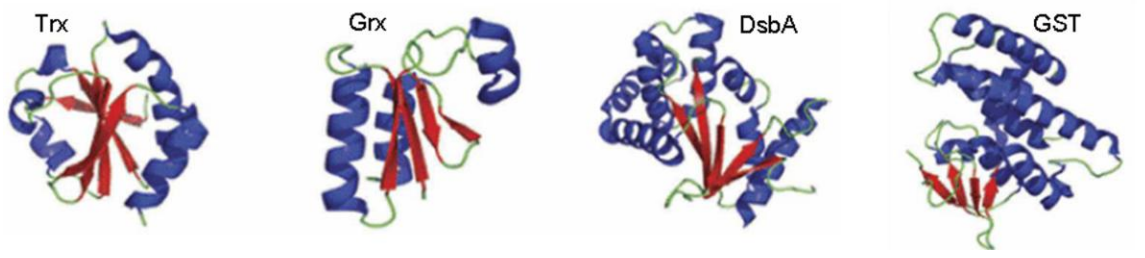

Figura 6: Enovelamento de proteínas da família Tiorredoxina, retirado de Pan & Bardwell, 2006. O enovelamento de proteínas da família Trx consiste em uma folha β central de quatro ou cinco fitas (em vermelho) rodeada por α-hélices (em azul).

Dentre as enzimas que apresentam o enovelamento Trx, algumas delas, como PDI, DsbA, Trx e Grxs, apresentam o motivo CXXC conservado em seus sítios ativos apesar de não compartilharem uma alta similaridade de seqüência. A localização do sítio ativo também é conservada entre estas proteínas, sendo que, em todas, a Cys N-terminal se encontra exposta na superfície da estrutura em um *loop* que conecta a segunda fita beta e uma hélice longa, enquanto a Cys C-terminal se encontra mais enterrada na estrutura

(Carvalho *et al*., 2006). E adjacente ao loop do sítio ativo, encontra-se outro loop que contém um resíduo de prolina conservado com configuração *cis,* o qual está relacionado ao reconhecimento de substratos nos membros da família Trx (Copley *et al*., 2004).

Todas estas enzimas estão envolvidas em reações de isomerização (PDI), formação (DsbA) ou quebra (Trx e Grx) de ligações dissulfeto (Carvalho *et al*., 2006). Os aminoácidos XX variam bastante entre elas (Trxs: Gly-Pro; PDIs: Gly-His; DsbA: Pro-His; Grxs: Pro-Tyr) e já foram descritos como os principais determinantes das diferentes propriedades redox destas enzimas (Huber-Wunderlich & Glockshuber, 1998; Mossner *et al*., 1998). Mais recentemente, foi descrito que o resíduo que ocupa a posição anterior à *cis*-prolina também modula as propriedades redox e a capacidade de interagir com substratos destas proteínas (Ren *et al.*, 2009).

I.4.2 - pK_a da cisteína reativa

A maioria das cisteínas (ligadas a uma cadeia polipeptídica ou livres) possui baixa reatividade, uma vez que suas sulfidrilas possuem um alto valor de pK_a (em torno de 8,5; Benesch & Benesch, 1955) e, portanto, encontram-se preferencialmente protonadas (SH) em pH fisiológico. Ao contrário, a forma desprotonada da sulfidrila, conhecida como tiolato (S^-), é mais nucleofílica e reage mais rapidamente com espécies oxidadas do que a forma protonada. O enovelamento protéico pode gerar um ambiente que promove a redução do pK_a das sulfidrilas, deixando-as preferencialmente desprotonadas em pH fisiológico, e ativos os resíduos de cisteína. Essa redução do pK_a de cisteínas ocorre, por exemplo, em proteínas da família Tiorredoxina, como as próprias Trxs, Grxs, Prxs, entre outras (Mossner *et al.,* 1998; Discola *et al.,* 2009; Ogusucu *et al.,* 2007).

Em Grxs, o pK_a da cisteína N-terminal de seus sítios ativos é geralmente bastante baixo, e este resíduo se encontra predominantemente na forma de tiolato em pH fisiológico, enquanto a cisteína C-terminal de seus sítios ativos se encontra geralmente protonada. A forma de tiolato da cisteína N-terminal de Grxs contribui para o ciclo catalítico como um bom nucleófilo na primeira reação (Figura 3, reações a, f) e, principalmente, como um bom grupo de saída (Figura 3, reações e, g).

Reações de intercâmbio tiol-dissulfeto catalisadas por Grxs são substituições nucleofílicas de um tiol ou tiolato (nucleófilo) em uma ligação dissulfeto que levam a oxidação do nucleófilo e a redução do grupo abandonador ou de saída (Jacob *et al.,* 2003). A velocidade destas reações depende do grupo abandonador. Bons grupos de saída são geralmente bases conjugadas de ácidos fortes, como o tiolato. É importante lembrar que quanto mais estabilizada é a densidade eletrônica negativa ou carga negativa do grupo de saída, menor é a energia do estado de transição, e mais rápida é a reação (Streitwieser & cols, 1998). Portanto, o pK_a baixo característico da cisteína N-terminal de Grxs está relacionado à estabilização da forma aniônica do enxofre na proteína.

Diversos fatores têm sido propostos para a estabilização do tiolato da cisteína N-terminal de Grxs e proteínas da família Trx:

- interações com resíduos de carga positiva próximos ao sítio ativo (Yang & Wells, 1991);
- potencial eletrostático positivo na região do sítio ativo, resultante da composição do ambiente local e não por interações de cargas diretas (Sun *et al.,* 1998);
- interação com o dipolo positivo da região N-terminal da α-hélice na qual se encontra o sítio ativo (Kortemme & Creigthon, 1995);
- ligação de hidrogênio entre o tiolato e o grupo amida da cisteína C-terminal (Eklund *et al*., 1992; Katti *et al*., 1995);
- ou ligação de hidrogênio entre o SH da cisteína C-terminal do sítio ativo e o tiolato da cisteína N-terminal (Jeng *et al.*, 1995; Jao *et al*., 2006).

II - OBJETIVOS

O objetivo geral deste projeto é a caracterização funcional e estrutural das glutarredoxinas ditiólicas Grx1 e Grx2 de S*accharomyces cerevisiae*, uma vez que, em comparação com outras vias de óxido-redução, pouco se sabe sobre mecanismo de ação e alvos biológicos desta classe de proteínas.

Para atingir esse objetivo, tentamos obter as estruturas de *Sc*Grx1 e *Sc*Grx2 em diferentes estados de oxidação, por meio da técnica de Cristalografia de proteínas, e analisar estas estruturas comparativamente. Hipóteses geradas podem ser testadas através de mutagênese sítio-dirigida, sendo possível a identificação de aminoácidos importantes para a catálise e/ou interação com substratos.

III - MATERIAIS E MÉTODOS

III.1 - Reagentes

Triptona, extrato de levedura, NaCl, ágar, etanol, isopropanol, ácido acético, ácido sulfúrico, glicose, H_2O_2, brometo de etídeo, acrilamida, bis-acrilamida, persulfato de amônia, TEMED, GSH, Glutationa redutase, BSA, Insulina de pâncreas bovino, diamida e azida de sódio, adquiridos junto a Sigma e Merck. Enzimas de restrição (*Bam*HI e *Nde*I), marcadores de massa molecular, agarose, Taq DNA polimerase, T4 DNA ligase, adquiridos junto a Invitrogen e Amersham. HED adquirido junto a Aldrich. EDTA e NADPH adquiridos junto a ICN Biomedicals Inc. IPTG, DTT, PMSF, imidazol, Hepes e SDS, adquiridos junto a USB. Monobromobimano, adquirido junto a Calbiochem. "QuickChange Site-Directed Mutagenesis Kit" da Stratagene. Kits de cristalização "Crystal Screen 1 e 2", "Index 1 e 2" e "Additive Screen 1, 2 e 3" da Hampton Research, "Wizard 1 e 2" da Emerald Biostructures. Oligonucleotídeos, adquiridos junto à IDT (Integrated DNA Technologies).

III.2 - Meio de Cultura para bactérias

LB: (1% triptona, 0,5% extrato de levedura e 1% NaCl).

*Os meios de cultura foram solidificados com a adição de ágar em uma concentração final de 2%.

III.3 - Linhagens e plasmídeo

Linhagens de *Escherichia coli*:

BL21(DE3); [F- , amp T, hsdSb (rB- mb-), gal, dcm (DE3) (Novagen).

DH5αF' :F' (Z80dlacZ_(lacZ)M15)_(lacZYA-argF)U169 recA1 endA1 hsdR17 (r_k^-,m_k^+).

XL1-Blue: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)].

Plasmídeo

Expressão: pET-15b (Novagen). A proteína recombinante resultante da expressão desse vetor apresenta uma cauda de histidina na região N-terminal. Outras características estão representadas abaixo.

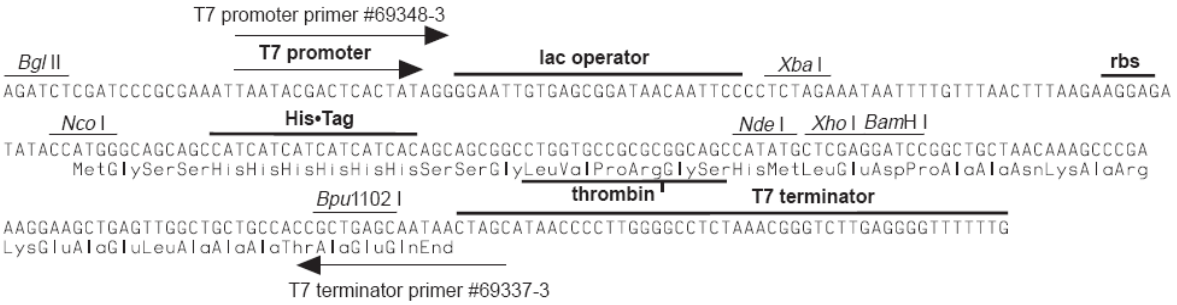

Figura 7: Seqüência de bases de nucleotídeos da região de clonagem e expressão do vetor pET-15b.

III.4 - Oligonucleotídeos

Os oligonucleotídeos (Tabela 2) foram desenhados de acordo com as seqüências dos genes presentes no banco de dados específico para *Saccharomyces cerevisiae* (*Saccharomyces* Genome Database: http://genome-www4.stanford.edu/SGD) e suas propriedades foram analisadas no website da "Integrated DNA Technologies" (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Os oligonucleotídeos utilizados para mutagênese sítio-dirigida, com exceção daqueles para a mutação *Scgrx2-C30S*, foram desenhados de acordo com as instruções do "QuickChange Site-Directed Mutagenesis Kit" da Stratagene. As enzimas de restrição a serem utilizadas em cada construção foram escolhidas após verificarmos se os genes não possuíam sítios de clivagem para as mesmas, utilizando a ferramenta disponível no website: www.restrictionmapper.org.

Tabela 2: Oligonucleotídeos utilizados. Os sítios de restrição estão sublinhados e os códons mutados estão destacados em negrito e itálico.

	Restrição	**Seqüência**
Scgrx1/YCL035C	*Nde*I e *Bam*HI	F: 5' CGCGATCCATATG ATG GTATCTCAAGAAAC 3' R: 5' CGCAAGCTTGGATCCTTAATTTGCAAGAATAGG 3'
*Scgrx1-S23A**	F: 5' GAG ATC TTC GTC GCA ***GCA*** AAA ACG TAC TGT CC 3' R: 5' GG ACA GTA CGT TTT ***TGC*** TGC GAC GAA GAT CTC 3'	
*Scgrx1-C30S**	F: 5' CG TAC TGT CCA TAC ***TCT*** CAT GCA GCC CTA AAC 3' R: 5' GTT TAG GGC TGC ATG ***AGA*** GTA TGG ACA GTA CG 3'	
*Scgrx1-Q52E**	F: 5' GTT CTG GTT TTG ***GAG*** TTG AAT GAC ATG AAG 3' R: 5' CTT CAT GTC ATT CAA ***CTC*** CAA AAC CAG AAC 3'	
*Scgrx1-D89S**	F: 5' CAT ATT GGA GGC AAC ***TCT*** GAC TTG CAG GAA TTG 3' R: 5' CAA TTC CTG CAA GTC ***AGA*** GTT GCC TCC AAT ATG 3'	
*Scgrx2-A23S**	F: 5' GAA GTG TTT GTT GCA ***TCC*** AAG ACA TAC TGC CC 3' R: 5' GG GCA GTA TGT CTT ***GGA*** TGC AAC AAA CAC TTC 3'	
Scgrx2-C30S	*Nde*I e *Bam*HI	F: 5' CGCGATCCATATG ATG GTA TCC CAG GAA ACA GTT GCT CAC GTA AAG GAT CTG ATT GGC CAA AAG GAA GTG TTT GTT GCA GCA AAG ACA TAC TGC CCT TAC ***AGC*** AAA GCT ACT TTG 3' R: 5' CGCAAGCTTGGATCCCTATTGAAATACCGGCTTC 3'
*Scgrx2-E52Q**	F: 5' G GCC CTT GTG TTG ***CAG*** TTA GAT GAA ATG AGC 3' R: 5' GCT CAT TTC ATC TAA ***CTG*** CAA CAC AAG GGC C 3'	
*Scgrx2-S89D**	F: 5' CAC ATT GGT GGT AAC ***GAT*** GAT TTG GAA ACT TTG 3' R: 5' CAA AGT TTC CAA ATC ***ATC*** GTT ACC ACC AAT GTG 3'	

* Oligonucleotídeos que foram usados para mutagênese sítio-dirigida utilizando o "QuickChange Site-Directed Mutagenesis Kit" da Stratagene.

III.5 - Preparação de bactérias eletrocompetentes

As bactérias foram crescidas em 1L de meio LB até atingirem OD_{600} entre 0,6 e 0,8. Então o frasco contendo as bactérias foi mantido em gelo por 30 minutos. Depois as células foram centrifugadas (15min/4°C/5000rpm) e o sobrenadante foi descartado. O *pellet* resultante foi ressuspendido em 1L de água gelada, centrifugado, e o sobrenadante removido. Novamente, o *pellet* foi ressuspendido em 500 mL de água gelada, centrifugado e ressuspendido em 20 mL de glicerol 10% gelado. Posteriormente, foi feita uma nova

centrifugação, o sobrenadante foi removido e o *pellet* remanescente foi ressuspendido em 2 mL de glicerol 10% gelado. Frações de 40 µL foram aliquotadas em gelo seco e estocadas em freezer -80˚C. Todo o processo de manipulação foi realizado em ambiente estéril. Este procedimento foi realizado seguindo o protocolo descrito por Ausebel & cols, 1987.

III.6 - Mini preparação plasmidial

Para obter o plasmídeo das colônias desejadas foi utilizada a mini preparação plasmidial (mini-prep), seguindo o método da hidrólise alcalina, de acordo com Sambrook & cols, 1989. O *pellet* de uma cultura de 1,5 mL, crescida a 37°C *overnight*, foi ressuspendido em 100 µL de solução GTE (Tris-HCl 25 mM, glicose 50 mM, EDTA 10 mM / pH 8,0) e deixado por 5 minutos no gelo. Então foram adicionados 200 µL de solução NaOH 0,2 M/SDS 1%, misturou-se por inversão, e a mistura foi deixada por 5 minutos no gelo. Após este intervalo, foram adicionados 150 µL de acetato de potássio 5 M pH 4,8, misturou-se por inversão, e a mistura foi deixada por 5 minutos no gelo. O material foi centrifugado (10min/4°C/15000rpm), e o sobrenadante contendo o DNA plasmidial foi transferido para outro tubo. Então foram adicionados 800 µL de isopropanol resfriado e misturou-se por inversão. O material foi novamente centrifugado, e o sobrenadante foi removido. O DNA plasmidial precipitado foi lavado com etanol 70% resfriado, centrifugado (10min/4°C/15000rpm) e seco sob vácuo. Finalmente, o DNA plasmidial foi ressuspendido em água e tratado com ribonuclease A (0,3 µg/µL), por 15 minutos a 37˚C, para digestão de RNA de alta massa molecular.

III.7 - Transformação bacteriana por eletroporação

Para transformar as bactérias, adicionamos cerca de 30 ng do plasmídeo em 40 µL de células eletrocompetentes (em fluxo laminar), previamente preparadas como descrito na seção III.5. Então as bactérias foram colocadas em uma cubeta (Bio-rad, 0,2 cm), a qual foi introduzida em um eletroporador (Gene pulser II - Bio-rad), e sobre a mesma foi aplicado um pulso (1,5 kV, 25 µF). Após este pulso, as células foram recuperadas em 1 mL de meio LB estéril e incubadas a 37˚C por 1 hora sob agitação. Finalmente, o meio LB com as células foi plaqueado, em meio sólido contendo 100µg/mL de Ampicilina, e incubado

overnight a 37˚C. Este procedimento foi realizado seguindo o protocolo descrito por Ausebel & cols, 1987.

III.8 - Clonagem gênica e mutagênese sítio-dirigida

Os genes de interesse (*Scgrx1/YCL035C, Scgrx2-C30S*) foram amplificados por PCR a partir do DNA genômico da linhagem W303 de *Saccharomyces cerevisiae*, usando os oligonucleotídeos específicos mostrados na Tabela 2. Os produtos amplificados de cada gene e o vetor de expressão pET-15b foram digeridos com as enzimas de restrição *Nde*I e *Bam*HI. Os fragmentos gerados, dos genes e do vetor, foram ligados, utilizando a enzima T4 DNA Ligase. Os plasmídeos resultantes (pET15b-*grx1*, pET15b-*grx2-C30S*) foram usados para transformar a bactéria eletrocompetente de *Escherichia coli DH5α* para a obtenção de um maior número de cópias dos mesmos.

Para determinar quais transformantes possuíam o inserto, foram feitas reações de PCR com algumas das colônias obtidas. As colônias que continham o inserto desejado foram utilizadas para a mini preparação plasmidial. Os plasmídeos obtidos foram sequenciados para verificar se as construções estavam corretas, e em caso positivo, usados para transformar, por eletroporação, a linhagem de expressão *BL21 (DE3)* de *E. coli*.

A construção pET15b-*grx2* nos foi enviada pelo Dr. José Antonio Bárcena (Departamento de Bioquímica y Biología Molecular, Universidade de Córdoba, Espanha) e transformada em *BL21 (DE3)* de *E. coli.*

As construções pET15b-*grx1-C30S,* pET15b-*grx1-S23A,* pET15b-*grx1-Q52E,* pET15b-*grx1-D89S,* pET15b-*grx2-A23S,* pET15b-*grx2-E52Q* e pET15b-*grx2-S89D* foram obtidas utilizando o "QuickChange Site-Directed Mutagenesis Kit" da Stratagene, os oligonucleotídeos mostrados na Tabela 2 e os plasmídeos pET15b-*grx1* e pET15b-*grx2,* os quais contém os genes que codificam as isoformas selvagens de *Sc*Grx1 e *Sc*Grx2. Os duplos mutantes pET15b-*grx1-S23A-Q52E* e pET15b-*grx2-A23S-E52Q* foram construídos utilizando o mesmo kit, porém foram usados como molde os plasmídeos pET15b-*grx1-S23A* e pET15b-*grx2-A23S* e os oligonucleotídeos para as mutações *Scgrx1-Q52E* e *Scgrx2-E52Q,* respectivamente. Com este kit de mutagênese os mutantes desejados puderam ser obtidos em poucas etapas, como esquematizado na Figura 8.

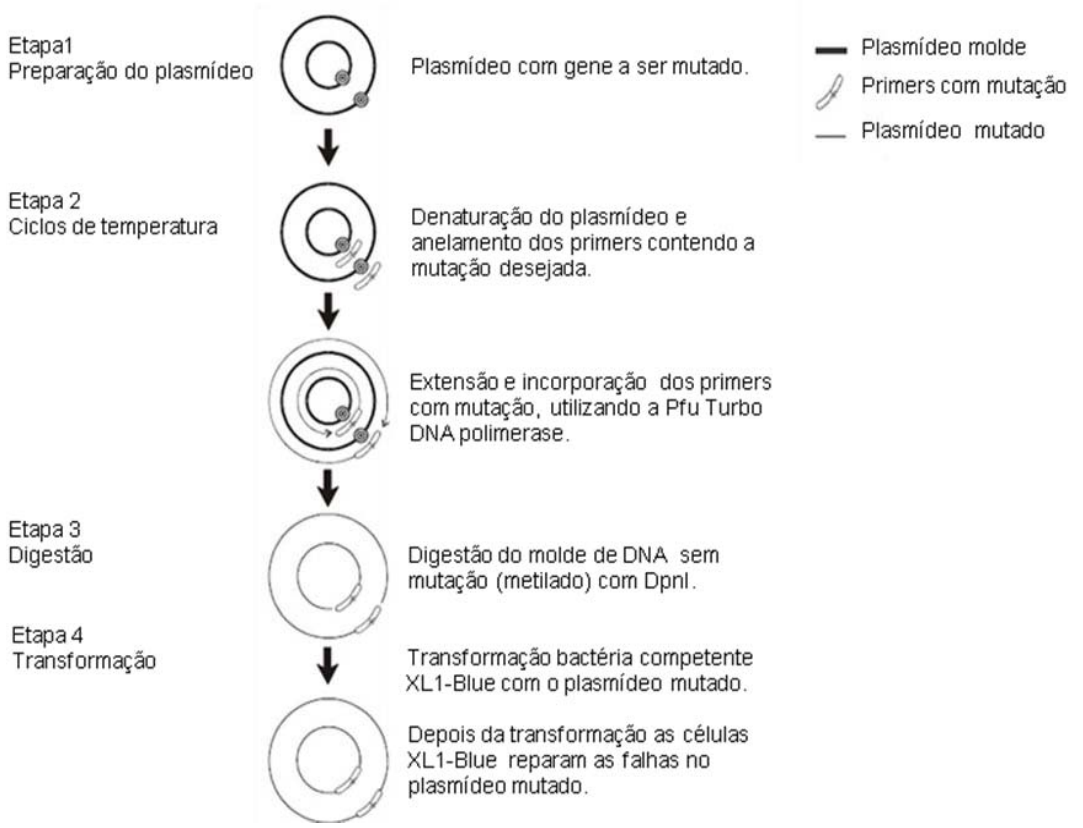

Figura 8: Etapas necessárias para a obtenção da mutação utilizando o "QuickChange Site-Directed Mutagenesis Kit" da Stratagene.

Após realizar as reações descritas acima, os plasmídeos dos transformantes, obtidos por mini-prep, foram seqüenciados, e aqueles que continham a mutação desejada foram usados para transformar a linhagem de expressão *BL21 (DE3)* de *E. coli*.

Os sequenciamentos dos plasmídeos foram realizados utilizando o sistema de análise de DNA MegaBACE 1000 (GE Healthcare) do Centro de Estudos do Genoma Humano da Universidade de São Paulo. As reações de sequenciamento foram realizadas utilizando o "DYEnamic ET Dye Terminator Kit" (com Thermo Sequenase™ II DNA Polimerase). As sequências foram analisadas pelo software MegAlign, dentro do pacote DNAstar.

III.9 - Expressão

As células foram inoculadas em meio LB com 100µg/mL de Ampicilina e cultivadas por 16 horas a 37°C em agitador orbital. Após este período, as culturas foram diluídas para OD_{600} = 0,2 em 1L de meio LB com 100µg/mL de Ampicilina e cultivadas até OD_{600} = 0,8. Então foi adicionado IPTG para induzir o promotor viral T7, que ativa a expressão gênica no vetor construído. A expressão de *Sc*Grx1, *Sc*Grx2 e de seus mutantes foi feita com 1 mM IPTG, por 3 horas a 37°C em agitador orbital. Após o período de indução, as células foram decantadas por meio de centrifugação 20min/4°C/5000rpm. O *pellet* de células obtido foi lavado com água MilliQ e novamente centrifugado (20min/4°C/5000rpm). O *pellet* final foi armazenado a -20°C até a purificação das proteínas.

III.10 - Purificação

Para a purificação das proteínas, o *pellet* de células foi ressuspendido em tampão Fosfato de Sódio 20mM pH 7,4/ 500 mM NaCl/ 20 mM imidazol pH 7,4. O rompimento das células foi feito por meio de sonicação, após a adição de 1 mM do inibidor de proteases PMSF. Depois o extrato celular foi tratado por 20 minutos, em gelo, com 1% de sulfato de estreptomicina (com agitação) e centrifugado por 45min/4°C/15000rpm. O precipitado contendo os restos celulares foi descartado, e o sobrenadante foi filtrado em membrana de 0,45 µm (Millipore).

Uma vez que todas as proteínas recombinantes construídas possuem uma cauda de histidina N-terminal, as proteínas foram purificadas em coluna de afinidade a níquel (HiTrap Chelating HP - Amersham Biosciences), ou afinidade a cobalto (Talon - BD Biosciences Clontech), com gradiente de imidazol, seguindo as orientações dos fabricantes.

III.10.1 - Purificação em coluna de afinidade a níquel

As proteínas *Sc*Grx1, *Sc*Grx2 e todos os seus mutantes foram purificados em coluna de afinidade a níquel com volume de 5 mL (HiTrap Chelating HP - Amersham

Biosciences), seguindo o protocolo descrito a seguir. Foi utilizada uma bomba peristáltica (Amersham Pharmacia) para a passagem das soluções pela coluna, na seguinte ordem:

1) 50 mL água MilliQ;
2) 2,5 mL $NiSO_4$ 0,1M;
3) 50 mL água MilliQ;
4) 50 mL Fosfato de sódio 20mM pH 7,4/ 500 mM NaCl/ 20 mM imidazol pH 7,4;
5) Extrato (preparado como descrito anteriormente);
6) 15 mL Fosfato de sódio 20mM pH 7,4/ 500 mM NaCl/ 50 mM imidazol pH 7,4;
7) 10 mL Fosfato de sódio 20mM pH 7,4/ 500 mM NaCl/ 100 mM imidazol pH 7,4;
8) 5 mL Fosfato de sódio 20mM pH 7,4/ 500 mM NaCl/ 150 mM imidazol pH 7,4;
9) 15 mL Fosfato de sódio 20mM pH 7,4/ 500 mM NaCl/ 500 mM imidazol pH 7,4;
10) 50 mL Fosfato de sódio 20mM pH 7,4/ 500 mM NaCl/ 50 mM EDTA;
11) 50 mL água MilliQ.

A coluna foi carregada com níquel nas etapas 2 e 3. Depois de carregada, a coluna foi equilibrada com o mesmo tampão no qual o extrato foi preparado (etapa 4), então o extrato foi passado para a ligação das proteínas à coluna (etapa 5). As lavagens das etapas 6, 7 e 8 foram necessárias para a remoção de proteínas contaminantes. A proteína de interesse foi eluída com 500 mM de imidazol (etapa 9) e coletada em tubos eppendorf. O níquel foi retirado da coluna com uma solução contendo o quelante EDTA (etapa 10), e a coluna foi limpa com água.

Para verificar quais frações eluídas da coluna continham proteína, utilizamos o método de detecção por Bradford, adicionando 5 µL da fração coletada em 500 µL da solução de Bradford (Bollag *et al.*, 1996). A solução de Bradford possui coloração cinza e a reação com proteínas provoca uma alteração da coloração para o azul. As frações que continham proteína foram aplicadas em Gel SDS-Page com o intuito de verificar se a massa molecular da proteína estava correto e se a mesma se encontrava pura.

III.10.2 - Purificação em coluna de afinidade a cobalto

*Sc*Grx1 também foi purificada em coluna de afinidade a cobalto de 4 mL (Talon - BD Biosciences Clontech). O extrato foi preparado como descrito anteriormente, porém,

neste caso, o *pellet* foi ressuspendido no tampão Tris-HCl 50 mM pH 7,5/ 100 mM NaCl. Utilizamos uma bomba peristáltica para a passagem das soluções pela coluna, na seguinte ordem:

1) 40 mL água MilliQ;
2) 40 mL Tris-HCl 50 mM pH 7,5/ 100 mM NaCl;
3) Extrato (preparado como descrito anteriormente);
4) 40 mL imidazol 20 mM pH 7,4/ Tris-HCl 50 mM pH 7,5/ 100 mM NaCl;
5) 4 mL imidazol 100 mM pH 7,4/ Tris-HCl 50 mM pH 7,5/ 100 mM NaCl;
6) 12 mL imidazol 200 mM pH 7,4/ Tris-HCl 50 mM pH 7,5/ 100 mM NaCl;
7) 40 mL EDTA 200 mM;
8) 40 mL água MilliQ;
9) 40 mL $CoCl_2$ 50 mM;
10) 28 mL água MilliQ;
11) 12 mL NaCl 0,3 M;
12) água MilliQ;
13) 40 mL Etanol 20%.

A coluna Talon - BD Biosciences Clontech foi armazenada em geladeira com etanol 20%, já carregada com cobalto. Então retiramos o etanol da coluna (etapa 1) e equilibramos a coluna com o tampão no qual o extrato foi preparado (etapa 2). Depois passamos o extrato na coluna (etapa 3) para a ligação das proteínas na mesma. As etapas 4 e 5 foram necessárias para eliminar proteínas contaminantes, e, na etapa 6, eluímos a proteína de interesse com 200 mM de imidazol. Então retiramos o metal da coluna com o quelante EDTA (etapa 7) e lavamos a coluna com água (etapa 8). As etapas 9-13 foram realizadas para carregar a coluna com cobalto e deixá-la com etanol para armazenamento.

Novamente, foi utilizado o método de Bradford para identificar as frações eluídas que continham proteína, e aplicamos estas frações em gel SDS-Page.

III.11 - Clivagem da cauda de histidina com Trombina

A cauda de histidina fusionada a *Sc*Grx1 e *Sc*Grx2 foi clivada utilizando o "Thrombin Clean Cleave Kit" da Sigma, seguindo as orientações do fabricante. Após 16

horas de clivagem, a trombina foi removida e as proteínas foram passadas novamente na coluna de níquel para retirar possíveis proteínas que não tiveram a cauda clivada. Os representantes que ainda continham cauda ficaram ligados na coluna, enquanto aqueles, cujas caudas foram clivadas, passaram direto pela coluna e foram coletados.

III.12 - Gel desnaturante de poliacrilamida (SDS-PAGE)

Os géis desnaturantes de poliacrilamida, utilizados para verificar pureza e tamanho das proteínas, foram feitos seguindo o protocolo (Bollag *et al.*, 1996):

Separating gel (14%): Misturou-se 2,5 mL da solução 30% Acrilamida/ 0,8% Bis-Acrilamida e 2,8 mL Tris 1M pH 8,8. E antes de despejar a mistura entre as placas, foram adicionados 50 µl de persulfato de amônio 10% e 10 µl TEMED para que a polimerização ocorresse.

Stacking gel (5%): Misturou-se 0,5mL da solução 30% Acrilamida/ 0,8% Bis-Acrilamida, 0,4mL de Tris 1M pH 6,8 e 2,1 mL de água destilada. Para que houvesse polimerização, antes de despejar a mistura entre as placas, foram adicionados 50 µl de persulfato de amônio 10% e 10 µl TEMED.

A amostra foi colocada no tampão 60 mM Tris-HCl pH 6,8/ 25% glicerol/ 2% SDS/ 0,1% bromofenol *blue*/ 30 mM DTT, e fervida por 15 minutos antes de ser aplicada no gel. O tampão de corrida utilizado foi 45 mM MES/ 50 mM Tris-HCl pH 7,3/ 0,1% SDS/ 0,8 mM EDTA. O padrão de massa molecular utilizado foi o *Bench Mark Protein Ladder* (Invitrogen). As corridas foram realizadas no sistema BioRad (*Mini Protean II*), e os géis foram corados com *Coomassie Blue* depois da eletroforese.

III.13 - Determinação da concentração das proteínas

As frações que continham as proteínas puras foram concentradas por ultracentrifugação em Microcon YM-10. Com o intuito de eliminar o imidazol, que interfere nas medidas espectrofotométricas a 280 nm, trocamos os tampões das proteínas por gel-filtração para Tris ou Hepes 5 mM, através de duas passagens consecutivas da amostra por uma coluna *desalting* PD10 (GE-Healthcare). Então determinamos as concentrações das enzimas espectrofotometricamente a 280 nm, utilizando os coeficientes

de extinção molar (ε) mostrados na Tabela 3, de acordo com a Lei de Beer-Lambert. Os valores de ε e massa molecular das Grxs de levedura (considerando a cauda de histidina) foram calculados com a ferramenta ProtParam do "Expasy Proteomics Server" (www.expasy.org).

Tabela 3: Coeficientes de extinção molar a 280 nm e massa molecular (considerando a cauda de histidina) das Grxs de levedura.

Proteína	**$\varepsilon_{Cys\ reduzidas}$ ($M^{-1}.cm^{-1}$)**	**$\varepsilon_{Cys\ oxidadas}$ ($M^{-1}.cm^{-1}$)**	**Massa molecular (kDa)**
*Sc*Grx1	5960	6085	14,67
*Sc*Grx1-S23A			14,66
*Sc*Grx1-C30S		5960	14,66
*Sc*Grx1-Q52E		6085	14,67
*Sc*Grx1-S23A-Q52E			14,66
*Sc*Grx1-D89S			14,65
*Sc*Grx2	4470	4595	14,13
*Sc*Grx2-A23S			14,14
*Sc*Grx2-C30S		4470	14,11
*Sc*Grx2-E52Q		4595	14,13
*Sc*Grx2-A23S-E52Q			14,14
*Sc*Grx2-S89D			14,16

III.14 - Ensaio de redução do dissulfeto misto β-ME-SG

A atividade como oxidorredutase das Grxs de levedura foi determinada no ensaio de redução do dissulfeto misto β-ME-SG, formado entre GSH e β-hidroxietil dissulfeto (HED) (Holmgren & Åslund, 1995).

O volume final utilizado em cada reação foi de 1,0 mL, e foram necessários os seguintes reagentes nas concentrações finais de: 0,1 M Tris-HCl pH 7,4; 2mM EDTA; 0,1 mg/mL BSA; 1mM GSH; 6 μg/ml GR; 0,7 mM HED e 0,2 mM NADPH.

Todos os reagentes acima, exceto o NADPH, foram misturados e deixados em um banho a 30°C por 3 minutos para que se formasse o dissulfeto misto β-ME-SG. Passado o tempo de incubação, foi adicionado o NADPH. Em seguida, foi adicionada a Grx, iniciando a reação, a qual foi acompanhada pela queda na absorbância a 340 nm, devido à oxidação do NADPH. O esquema abaixo mostra a seqüência de transferência de elétrons no sistema:

NADPH → GR → GSH → Grx → β-ME-SG; Grx reduz o substrato β-ME-SG e se oxida nesta reação, sendo reduzida por duas moléculas de GSH e gerando GSSG, a qual é reduzida por GR à custa dos elétrons do NADPH

Ao branco foram adicionados os mesmos reagentes descritos acima, exceto o NADPH e a Grx purificada, e o controle foi feito sem a adição de Grx ao sistema reacional. Também foi feito outro controle adicionando Grx à reação sem o substrato β-ME-SG. As concentrações de Grxs, da ordem de nanomolar, foram variadas para a determinação de suas atividades específicas. Todas as reações foram realizadas a 30°C.

III.15 - Determinação dos parâmetros cinéticos

Para determinar os valores de K_M e k_{cat} para as Grxs de levedura, medimos a atividade destas enzimas variando a concentração de HED entre 0,03 e 2,2 mM em concentrações fixas de GSH, a 30ºC (Mesecke *et al.*, 2008b; Mieyal *et al.,* 1991). Os ensaios foram feitos como aqueles para a determinação da atividade específica, utilizando as mesmas concentrações, descritas anteriormente, dos demais reagentes necessários. A concentração de enzima utilizada nos ensaios está entre parênteses: *Sc*Grx1 (500 nM), *Sc*Grx1-S23A (150 nM), *Sc*Grx1-C30S (148 nM), *Sc*Grx1-S23A-Q52E (200 nM), *Sc*Grx2 (50 nM), *Sc*Grx2-A23S (100 nM), *Sc*Grx2-C30S (150 nM) e *Sc*Grx2-A23S-E52Q (200 nM). As atividades medidas foram corrigidas subtraindo a medida da reação controle sem enzima. As velocidades iniciais das reações foram tratadas de acordo com Michaelis-Menten e Lineweaver-Burk.

III.16 - Determinação do pk_a das cisteínas reativas

Os valores de pk_a da cisteína mais reativa (N-terminal, Cys27) dos sítios ativos de *Sc*Grx1, *Sc*Grx2 e seus mutantes foram determinados por dois métodos independentes: inativação por alquilação com iodoacetamida (IAM) (Jao *et al.*, 2006; Gan *et al.*, 1990; Mieyal *et al*., 1991) e alquilação com o reagente fluorescente mono-bromobimano (MBB) (Kosower *et al*., 1979).

III.16.1 - Inativação com IAM

Esse método se baseia no fato de que IAM reage preferencialmente com a forma desprotonada das cisteínas, conhecida como tiolato, e também no fato de que a alquilação da cisteína N-terminal das Grxs leva à inativação da enzima.

As proteínas foram reduzidas com 100 mM DTT por 2 horas a temperatura ambiente. Depois o excesso de DTT foi removido por gel-filtração, através de duas passagens consecutivas da amostra por uma coluna *desalting* PD10 (GE-Healthcare) com 5 mM Tris-HCl pH 8,5 (*Sc*Grx1) e 5 mM Tris-HCl pH 7,5 (*Sc*Grx2). A concentração das enzimas foi determinada espectrofotometricamente a 280 nm, como descrito anteriormente.

As enzimas pré-reduzidas (250 nM *Sc*Grx1 e 20 nM *Sc*Grx2) foram incubadas por 3 minutos com 300 μM IAM, em diferentes pHs, a temperatura ambiente. Depois as enzimas foram deixadas em banho de gelo por 1 min e diluídas 100 vezes na solução do ensaio de atividade para parar a reação com IAM. As atividades das Grxs foram determinadas no ensaio de redução do dissulfeto misto β-ME-SG, como descrito anteriormente. As atividades das Grxs também foram medidas depois da incubação nos diferentes pHs, mas sem a adição de IAM. Por fim, para determinar o pK_a das cisteínas das Grxs construímos gráficos de atividade remanescente versus pH. A atividade remanescente foi calculada considerando a atividade da Grx incubada no pH sem IAM igual a 100%:

% atividade remanescente = atividade Grx no pH com IAM x 100/ atividade Grx no pH.

A curva obtida é uma sigmóide, que pode ser descrita pela equação de Hill. Uma vez que o valor do pK_a de uma dada espécie é igual ao valor de pH no qual 50% da espécie se encontra protonada e 50% desprotonada, o pK_a neste gráfico corresponde ao pH no qual

temos 50% de atividade remanescente (ponto de inflexão da curva). Fizemos a regressão de todas as curvas utilizando um valor de coeficiente de Hill igual a 1,0, no programa GraphPad Prism 4.

Os seguintes tampões foram utilizados na concentração de 10 mM nas incubações das Grxs com e sem IAM: KCl-HCl, pH 1,0 e 1,5; citrato-fosfato, pH 2,0; 2,5; 3,0; 3,5, 4,0; 5,0 e 6,0. A força iônica das incubações foi ajustada com o acréscimo de 0,5 M NaCl.

III.16.2 - Alquilação com MBB

A alquilação com MBB também é específica para o tiolato e gera um produto fluorescente com $\lambda_{excitação}$ = 396 nm e $\lambda_{emissão}$ = 482 nm. Se acompanharmos a velocidade de formação deste produto fluorescente em diferentes pHs podemos determinar o pK_a da cisteína N-terminal das Grxs, uma vez que a velocidade da reação será proporcional à concentração de Grxs com suas cisteínas na forma desprotonada nos diferentes pHs.

Inicialmente, as Grxs foram pré-reduzidas como descrito anteriormente e tiveram suas concentrações determinadas. Então as Grxs (10 µM) foram incubadas com 2 µM de MBB em diferentes pHs, e as reações foram acompanhadas por 15 minutos a 37ºC em um fluorímetro SPECTRAmax Gemini XPS (Molecular Devices), no laboratório da Dra. Marilene Demasi do Instituto Butantan. Os seguintes tampões foram utilizados na concentração de 50 mM: KCl-HCl, pH 1,0 e 1,5; citrato-fosfato, pH 2,0; 2,5; 3,0; 3,5; 4,0; 4,5; 5,0; e a força iônica foi ajustada com a adição de 0,5 M NaCl. A velocidade da reação de alquilação com MBB foi obtida a partir da regressão linear da curva, que representava a formação do produto fluorescente com o tempo, no seu trecho de maior inclinação. Por fim, construímos um gráfico das velocidades de alquilação com MBB versus pH, e novamente o pK_a pôde ser obtido no ponto de inflexão da curva. Neste caso, a curva obtida é também uma sigmóide, descrita pela equação de Hill (coeficiente de Hill igual a 1,0).

III.17 - Determinação da atividade peroxidásica

A atividade peroxidásica de *Sc*Grx1 e *Sc*Grx2 foi medida com hidroperóxido de cumeno (CHP), *tert*-butil hidroperóxido (*t*-BOOH) e H_2O_2, espectrofotometricamente a 340

nm, devido ao consumo de NADPH: NADPH → GR → GSH → Grx → peróxido (Collinson *et al.*, 2002).

O volume final utilizado em cada reação foi de 1,0 mL e foram necessários os seguintes reagentes nas concentrações finais de: 0,1 M Tris-HCl pH 7,4; 1mM GSH; 6 μg/ml GR; e 0,2 mM NADPH.

Todos os reagentes acima, exceto o NADPH, foram misturados e deixados em um banho a 30°C por 3 minutos para termostatizar. Passado o tempo de incubação, foi adicionado o NADPH, depois o peróxido, e, por fim, foi adicionada a Grx purificada, iniciando a reação. Todas as reações foram realizadas a 30°C. Ao branco não foram adicionados NADPH e a Grx. Ao controle foram adicionados todos os reagentes, com exceção de Grx. Nas nossas primeiras tentativas utilizamos 20 nM de Grx e variamos a concentração dos peróxidos entre 0,1 e 2,5 mM para medir a atividade peroxidásica (Collinson *et al.*, 2002), como não conseguimos detectar atividade, utilizamos depois 50 μM de proteína e 0,1 mM de peróxido.

III.18 - Redução de *Sc*Grx1 e *Sc*Grx2 pelas *Sc*Trrs

Com base no ensaio de redução do dissulfeto misto β-ME-SG, resolvemos testar se *Sc*Grx1 e *Sc*Grx2 poderiam ser reduzidas por ao menos uma das Tiorredoxinas Redutases de levedura, substituindo o sistema redutor das Grxs (GSH, GR e NADPH) por *Sc*Trr1 ou *Sc*Trr2 e NAPDH, de modo que teríamos:

NADPH → *Sc*Trr1/*Sc*Trr2 → *Sc*Grx1/*Sc*Grx2 → β-ME-SG.

A reação foi acompanhada pelo decréscimo na absorbância a 340 nm. Foram utilizados os reagentes: 0,1 M Tris-HCl pH 7,4, 2mM EDTA, 0,7mM GSH, 0,7 mM HED e 0,2 mM NADPH. Aos brancos não foram adicionados as enzimas e o NADPH. A concentração utilizada de *Sc*Grx1 e *Sc*Grx2 foi de 2 μM, e de *Sc*Trr1 e *Sc*Trr2 foi 0,5 μM. As reações foram realizadas a 30°C e o volume reacional foi de 1,0 mL.

III.19 - Redução do dissulfeto da Insulina

Para verificar se *Sc*Grx1 e *Sc*Grx2 são capazes de reduzir as pontes dissulfeto da insulina de pâncreas bovino, a insulina foi solubilizada em 20 mM de tampão citrato-fosfato pH 3,0, e o ensaio foi realizado como aquele descrito para o dissulfeto misto β-ME-SG, substituindo o HED por 30 μM de insulina (Vlamis-Gardikas *et al.*, 1997): NADPH → GR → GSH → Grx → insulina.

Ao branco foram adicionados todos os reagentes, exceto o NADPH e a Grx purificada, e o controle foi feito sem a adição de Grx ao sistema reacional. Foram utilizadas concentrações de *Sc*Grx1 e *Sc*Grx2 entre 10 nM e 10 μM. A reação foi acompanhada pela queda da absorbância a 340 nm, devido à oxidação de NADPH.

III.20 - Preparação do mutante *Sc*Grx2-C30S ligado à glutationa

Construímos a proteína mutante *Sc*Grx2-C30S com o intuito de obter a estrutura desta proteína ligada à glutationa, já que a cisteína C-terminal (Cys30) foi substituída por uma serina, impossibilitando o rompimento do dissulfeto misto entre a cisteína N-terminal (Cys27) de *Sc*Grx2-C30S e glutationa na ausência de um redutor externo.

Para obter o dissulfeto misto entre *Sc*Grx2-C30S e glutationa, a proteína mutante foi tratada inicialmente com 50 mM DTT a temperatura ambiente por 1hora para reduzir completamente toda a amostra. Depois o excesso de DTT foi removido por gel-filtração, através de duas passagens consecutivas da amostra por uma coluna *desalting* PD10 (GE-Healthcare). Em seguida, tratamos a proteína reduzida com 25 mM GSSG por 1 hora a temperatura ambiente. O excesso de GSSG também foi removido por gel filtração, passando a amostra duas vezes na coluna PD10. Seguindo esta seqüência de reações, esquematizada na Figura 9, conseguimos obter o dissulfeto misto *Sc*Grx2-SG na forma solúvel.

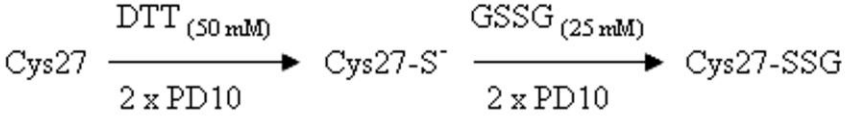

Figura 9: Esquema do procedimento realizado para a obtenção do dissulfeto misto entre *Sc*Grx2-C30S e glutationa.

III.21 - Cristalização e refinamentos

Foram realizados os ensaios iniciais de crescimento de cristais com *Sc*Grx1, *Sc*Grx2 e *Sc*Grx2-SG utilizando os kits "Crystal Screen 1 e 2" e "Index 1 e 2" da Hampton Research e "Wizard 1 e 2" da Emerald Biostructures, seguindo as orientações dos fabricantes.

A concentração da proteína utilizada nos ensaios de cristalização foi 10 mg/mL em tampão Tris-HCl 5mM pH 8,5 para *Sc*Grx1, 10 mg/mL em Tris-HCl 5 mM pH 7,4 para *Sc*Grx2 e 13 mg/ml para *Sc*Grx2-SG em tampão Hepes 5 mM pH 7,4. Foram realizados ensaios de cristalização com *Sc*Grx1 e *Sc*Grx2 sem tratamento e tratadas com 10mM *t*-BOOH, 1mM H_2O_2, 10mM DTT, 3mM Diamida, 10 mM GSH, e com *Sc*Grx2-C30S ligada à glutationa (como descrito anteriormente) e também reduzida com 10 mM DTT e 10 mM GSH. Antes de adicionar os reagentes oxidantes e redutores as proteínas foram centrifugadas por 5 minutos a 10000 rpm/4°C. As proteínas, depois de tratadas, foram mantidas a temperatura ambiente por uma hora. Exceto durante os tratamentos, as proteínas foram mantidas em banho de gelo até o término dos experimentos de cristalização. A temperatura de cristalização foi de 20°C. Com o intuito de obter a estrutura de *Sc*Grx2 na forma reduzida adicionamos nas gotas com cristais de *Sc*Grx2 já formados 50 mM DTT ou 10 mM GSH algumas horas ou dias antes das coletas de dados de difração.

Os ensaios foram realizados com o método da gota pendurada. O volume final das gotas foi 4µl (2µl da solução de proteína e 2µl da solução de cristalização), e o volume utilizado da solução de cristalização no poço foi de 300µl; nos refinamentos das condições de cristalização foram feitas gotas com um volume final de até 10 µl com o intuito de obter cristais maiores. Após os experimentos de cristalização, todas as placas foram observadas periodicamente com o auxílio de uma lupa.

Quando encontrados cristais em uma determinada condição de cristalização, foram feitos refinamentos desta condição, variando o pH, e a concentração de sal, proteína e precipitante, com o intuito de obter cristais adequados aos experimentos de difração.

Para tentar obter cristais melhores de *Sc*Grx1 e *Sc*Grx2 também foram usados os kits de aditivos "Aditive Screen 1, 2 e 3" da Hampton Research. Neste caso, a gota com um volume total de 10 µl era composta por 4,5 µl da solução protéica, 4,5 µl da solução de

cristalização (a mesma na qual haviam crescido os melhores cristais obtidos até o momento) e 1,0 µl da solução de aditivo do kit.

III.21.1 - *Microseeding*

A técnica de *microseeding* também foi utilizada na tentativa de melhorar os cristais de *Sc*Grx1 e *Sc*Grx2. Nesta técnica, cristais previamente nucleados são triturados, e as sementes microscópicas geradas são introduzidas em gotas novas, equilibradas até baixos níveis de supersaturação, de modo que o crescimento de cristais seja possível, mas não seja possível a formação de novos núcleos (Bergfors, 2003).

Cristais da proteína foram colocados em 100 µl da solução de cristalização e vortexados por tempo suficiente para que não se observasse mais pedaços de cristais na solução (~10 minutos). As soluções estoque com os núcleos dos cristais das Grxs foram diluídas 10, 100, 1000 e 10.000 vezes. Gotas contendo 4,5 µl da solução de cristalização (idêntica àquela que foram retirados os cristais para fazer a solução com as sementes) e 4,5 µl da proteína foram feitas e deixadas equilibrando por 8 horas; foram feitas também gotas com concentrações menores, em relação à condição inicial, de proteína e de precipitante. Depois deste tempo, 1,0 µl das soluções com sementes, nas diferentes diluições, foi adicionado às gotas.

III.22 - Coleta e processamento de dados de difração de raios-X

Com a obtenção de cristais apropriados, foram realizados os experimentos de difração de raios-X, utilizando a radiação gerada pela Luz Síncrotron. Todos os procedimentos de cristalização, coleta e análise dos dados de difrações de raios-X foram realizados no Laboratório Nacional de Luz Síncrotron (LNLS), com auxílio dos pesquisadores: Dra. Beatriz Gomes Guimarães e Dr. Marcos Antonio de Oliveira.

Os cristais foram mergulhados em soluções crio-protetoras antes dos experimentos de difração com raios-X. As soluções crio-protetoras foram feitas com base na composição da solução de cristalização, com acréscimo de 20, 25 e 30% de glicerol.

Todos os experimentos de difração de raios-X foram realizados na linha D03B-MX1 do LNLS. As medidas foram feitas em temperatura criogênica (110 K) através do

fluxo de nitrogênio líquido sobre a amostra. O comprimento de onda utilizado foi 1.427 Å, e os dados de difração de raios-X foram coletados em detector MARCCD.

Os dados foram processados utilizando os programas MOSFLM (Leslie, 1992) e SCALA (Kabush, 1998; Blessing, 1995) do pacote CCP4. O número de moléculas por unidade assimétrica foi determinado através do cálculo do coeficiente de Matthews (Matthews, 1968).

III.23 - Resolução e refinamento das estruturas de *Sc*Grx2

A estrutura de *Sc*Grx2 na forma oxidada (obtida com o tratamento de 10 mM *t*-BOOH) foi resolvida por substituição molecular com o auxílio do programa AmoRe (Navaza, 1994), utilizando um modelo teórico construído pelo programa Modeller (Claude *et al.*, 2004), a partir das coordenadas atômicas da Grx de *Sus scrofa* (código PDB: 1KTE; Katti *et al.*, 1995) e a seqüência de aminoácidos de *Sc*Grx2. As demais estruturas de *Sc*Grx2 e do mutante *Sc*Grx2-C30S ligado à glutationa foram resolvidas por substituição molecular com o auxílio do programa Phaser (Read, 2001), utilizando as coordenadas atômicas da estrutura refinada de *Sc*Grx2 oxidada.

Para os refinamentos das estruturas foi utilizado o programa Refmac 5 (Murshudov *et al.*, 1997). Utilizamos também modelos gerados por análises TLSMD *(translation/libration/screw/motion/determination*; http://skuld.bmsc.washington.edu/~tlsmd) para tentar melhorar os valores de R_{factor} e R_{free} nos últimos ciclos de refinamento da estrutura do mutante *Sc*Grx2-C30S (Winn *et al.*, 2001). Os programas O (Jones *et al.,* 1991) e Coot (Emsley & Cowtan, 2004) foram utilizados para visualização, substituição de aminoácidos e ordenação da molécula no interior do mapa de densidade eletrônica. A análise estereoquímica dos modelos refinados foi realizada utilizando o programa PROCHECK (Laskowski *et al.*, 1993). As representações gráficas foram criadas com o programa PyMOL (Delano, 2002). Os alinhamentos das estruturas de *Sc*Grx2 foram realizados utilizando o programa Coot (Emsley & Cowtan, 2004).

IV - RESULTADOS E DISCUSSÃO

IV.1 - Expressão e purificação de *Sc*Grx1, *Sc*Grx2 e *Sc*Grx2-C30S

Todos os ensaios enzimáticos e de cristalização com *Sc*Grx1, *Sc*Grx2 e com o mutante *Sc*Grx2-C30S foram realizados com as proteínas recombinantes, purificadas como descrito em "Materiais e Métodos". A Figura 10 mostra o gel SDS-Page contendo as Grxs purificadas. A expressão destas proteínas apresenta bom rendimento, sendo obtidos em média 50 mg/L de cultura de *Sc*Grx1, e cerca de 25 mg/L de cultura de *Sc*Grx2 e *Sc*Grx2-C30S.

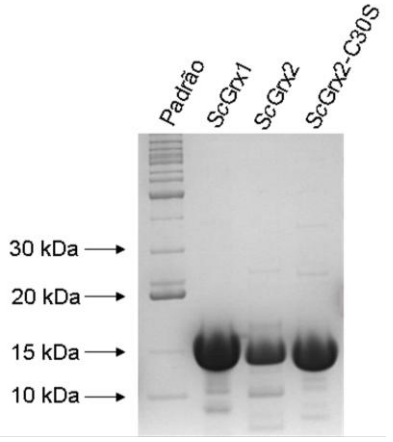

Figura 10: Gel SDS-Page mostrando as Grxs ditiólicas de levedura e o mutante *Sc*Grx2-C30S purificadas em coluna de afinidade a Niquel.

Obtivemos *Sc*Grx1, *Sc*Grx2 e *Sc*Grx2-C30S puras através de um gradiente de imidazol feito em coluna de afinidade a níquel, como descrito em "Materiais e Métodos". Para determinar a concentração das proteínas e trabalhar com elas em ensaios de atividade e cristalização, procuramos um tampão sem imidazol (para evitar interferência na medida de concentração a 280 nm) e pouco sal (para evitar que se formassem cristais de sal nos ensaios de cristalização), no qual as Grxs permanecessem solúveis na maior concentração possível. O tampão escolhido para *Sc*Grx1 foi Tris-HCl 5 mM pH 8,5, para *Sc*Grx2, Tris-HCl 5 mM pH 7,4, e para o mutante *Sc*Grx2-C30S, Hepes 5 mM pH 7,4.

IV.2 - Atividades específicas de *Sc*Grx1 e *Sc*Grx2

O ensaio de redução do dissulfeto misto formado entre HED e GSH (β-ME-SG) foi utilizado para a determinação das atividades específicas das Grxs de levedura como oxidorredutases. Com este intuito, foram feitas medidas de velocidade inicial em concentrações crescentes de Grx. As reações foram acompanhadas espectrofotometricamente a 340 nm, devido à oxidação do NADPH. A Figura 11 mostra a variação da absorbância a 340 nm em função do tempo para uma reação controle, sem Grx, e para uma reação na qual foi adicionada *Sc*Grx2. As velocidades das reações foram determinadas a partir da regressão linear das curvas de absorbância versus tempo nos trechos de maior inclinação, utilizando o programa Origin 6.1. A velocidade da reação controle foi subtraída das demais medidas feitas na presença de Grx, em triplicata.

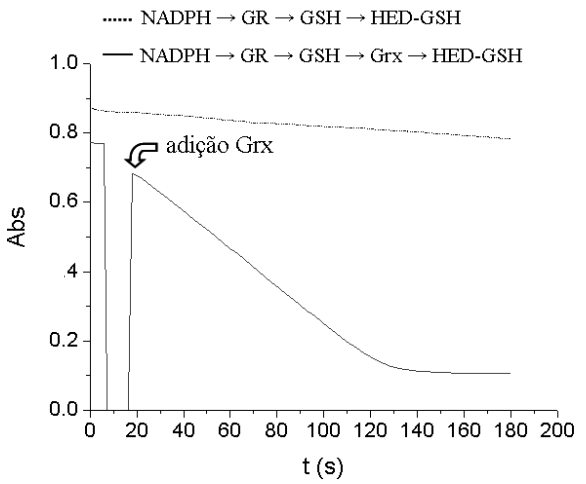

Figura 11: Gráfico de absorbância (λ = 340 nm) versus tempo da reação de redução do dissulfeto misto β-ME-SG sem Grx (linha pontilhada) e com a adição de *Sc*Grx2 (linha contínua).

Para calcular a velocidade inicial (V_0) da reação em número de mols de NADPH consumidos por minuto, utilizamos a expressão:

V_0 = dAbs/dt x V/ε, onde dAbs/dt é a variação da absorbância com o tempo, V é o volume final da reação (1,0 mL) e ε é o coeficiente de extinção molar do NADPH a 340 nm (6290 $M^{-1}.cm^{-1}$).

Para obter a atividade específica, foi construído um gráfico de velocidade (nmol/min) versus a quantidade de Grx (pmol) adicionada. O coeficiente angular da reta obtida corresponde à atividade específica da enzima. Os gráficos foram construídos utilizando o programa Origin 6.1, e os coeficientes angulares foram obtidos através da regressão linear dos dados feita pelo mesmo programa (Figura 12).

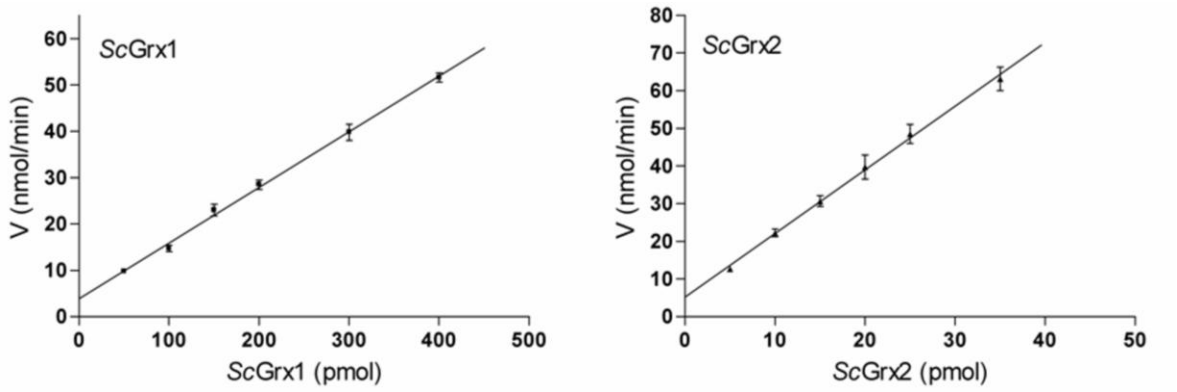

Figura 12: Gráficos de velocidade de consumo de NADPH (nmol/min) versus quantidade de Grx (pmol) adicionada, usados para a determinação das atividades específicas de *Sc*Grx1 e *Sc*Grx2.

*Sc*Grx2 apresenta uma atividade específica como oxidorredutase de 125 ± 7 $\mu mol.min^{-1}.mg^{-1}$, enquanto *Sc*Grx1 apresenta uma atividade específica de 8,2 ± 0,3 $\mu mol.min^{-1}.mg^{-1}$. Portanto, *Sc*Grx2 é cerca de quinze vezes mais ativa neste ensaio do que *Sc*Grx1 (Tabela 4). Apesar de ter sido sugerido que *Sc*Grx2 poderia ser mais ativa como oxidorredutase do que *Sc*Grx1, através de observações obtidas em experimentos com extratos de levedura (Luikenhuis *et al.*, 1998), essa grande diferença de atividade não era esperada, uma vez estas proteínas compartilham 64 % de identidade e 85% de similaridade de seqüência de aminoácidos.

Para verificar se as caudas de histidina N-terminais poderiam interferir na atividade de *Sc*Grx1 e *Sc*Grx2, estando relacionadas à grande diferença observada, medimos a atividade de *Sc*Grx1 e *Sc*Grx2 sem as caudas de histidina, clivando as mesmas com Trombina. A Figura 13 mostra as formas de *Sc*Grx1 e *Sc*Grx2 com e sem cauda de histidina; as formas cujas caudas foram clivadas (*Sc*Grx1c e *Sc*Grx2c) apresentam tamanho menor. Como pode ser observado na Tabela 4, as enzimas sem cauda são um pouco mais

ativas que suas formas fusionadas, porém *Sc*Grx2c continua sendo muito mais ativa que *Sc*Grx1c.

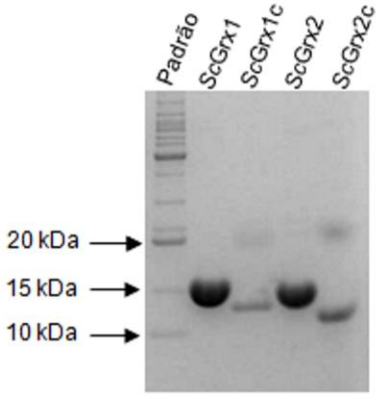

Figura 13: Gel SDS-Page com as amostras de *Sc*Grx1 e *Sc*Grx2 com cauda e sem a cauda de histidina (*Sc*Grx1c e *Sc*Grx2c).

Tabela 4: Atividades específicas de *Sc*Grx1, *Sc*Grx2 com cauda e sem cauda (*Sc*Grx1c e *Sc*Grx2c) no ensaio de redução do dissulfeto misto β-ME-SG, e a relação entre as atividades de *Sc*Grx2 e *Sc*Grx1 das formas com cauda e sem cauda.

	Atividade específica ($\mu mol.min^{-1}.mg^{-1}$)	atividade *Sc*Grx2/ atividade *Sc*Grx1
*Sc*Grx1	8,2 ± 0,3	15,2
*Sc*Grx2	125 ± 7	
*Sc*Grx1c	10,0 ± 0,2	17,0
*Sc*Grx2c	170 ± 10	

Considerando que uma possível contaminação de *Sc*Grx1 com metal (níquel) durante o processo de purificação poderia interferir na atividade de *Sc*Grx1, testamos a atividade de *Sc*Grx1 purificada na coluna de cobalto. O cobalto se liga mais fortemente à coluna do que o níquel, de modo que não haveria desprendimento de Co da coluna e contaminação da proteína durante o processo de purificação. Porém atividade de *Sc*Grx1 é a mesma, sendo purificada em coluna de níquel ou cobalto (Tabela 5).

Testamos também se o tampão no qual deixamos *Sc*Grx1 (Tris 5 mM pH 8,5) poderia não mantê-la estável, levando à desnaturação e, conseqüentemente, à uma diminuição de atividade. É importante lembrar que o tampão utilizado no ensaio com HED foi Tris-HCl 100 mM pH 7,4, em todos as medidas. Como mostrado na Tabela 5, a variação

de pH entre 7,4 e 8,5 no tampão em que *Sc*Grx1 é mantida não altera sua atividade. *Sc*Grx1 não é estável em pHs inferiores a 7,0, sofrendo precipitação.

Tabela 5: Atividade de *Sc*Grx1 purificada em coluna de níquel (Ni) ou cobalto (Co) mantida em diferentes tampões.

Tampão/coluna	V (Δabs/min) 10 nM *Sc*Grx1	V (Δabs/min) 150 nM *Sc*Grx1
Fosfato K 5 mM pH 7,0/ Co	0,000243	0,00169
Fosfato Na 5 mM pH 7,4/ Co	0,000322	0,00232
Tris 5 mM pH 8,0/ Co	0,000317	0,00234
Tris 5 mM pH 8,5/ Co	0,000327	0,00237
Tris 5 mM pH 8,5/ Ni	-	0,00228

Após estes testes, concluímos que *Sc*Grx2 realmente é mais ativa do que *Sc*Grx1, já que a cauda de histidina e o tampão não alteram significativamente a atividade de *Sc*Grx1 e que não há interferência no ensaio de atividade devido à contaminação com metal.

IV.3 - Cinética Bi-substrato

Para caracterizar melhor as atividades de *Sc*Grx1 e *Sc*Grx2 como oxidorredutases no ensaio de redução do dissulfeto misto β-ME-SG e entender se a diferença de atividade observada entre estas enzimas se deve a uma diferença de afinidade pelo substrato (diferença no K_M) ou a uma diferença no *turnover* (k_{cat}), resolvemos fazer experimentos de cinética bi-substrato e determinar seus parâmetros cinéticos.

Como temos dois substratos para Grx no ensaio de redução do dissulfeto misto β-ME-SG (β-ME-SG e GSH), é necessário realizar a variação da concentração de um dos substratos, em diferentes concentrações fixas do outro substrato, para determinar os valores de K_M para ambos os substratos e a velocidade máxima real (V_{max}) da reação. O esquema geral da reação deste ensaio: NADPH → GR → GSH → Grx → β-ME-SG,

mostra que Grx reage primeiro com o dissulfeto misto β-ME-SG e que depois é reduzida por GSH. Então podemos variar a concentração de HED em concentrações fixas de GSH (Figura 14), determinar as constantes aparentes da reação (Tabela 6) e o K_M^{HED} real, e depois, com base nestas constantes, construir um gráfico secundário (Figura 16) e determinar o K_M^{GSH} e a V_{max} real da reação (Tabela 7), como descrito por Segel, 1975.

Cada curva mostrada para *Sc*Grx1 e *Sc*Grx2 nas Figuras 14 e 15 corresponde à média de três experimentos. Para todas as medidas de velocidade em diferentes concentrações de substrato foi subtraída a velocidade da reação controle que não continha Grx. A partir da regressão não-linear das curvas de Michaelis-Menten (Figura 14), determinamos as constantes aparentes para o substrato HED nas diferentes concentrações de GSH (Tabela 6), utilizando o programa GraphPad Prism4. Anteriormente, foram feitos testes para verificar que a concentração de GR usada não era limitante.

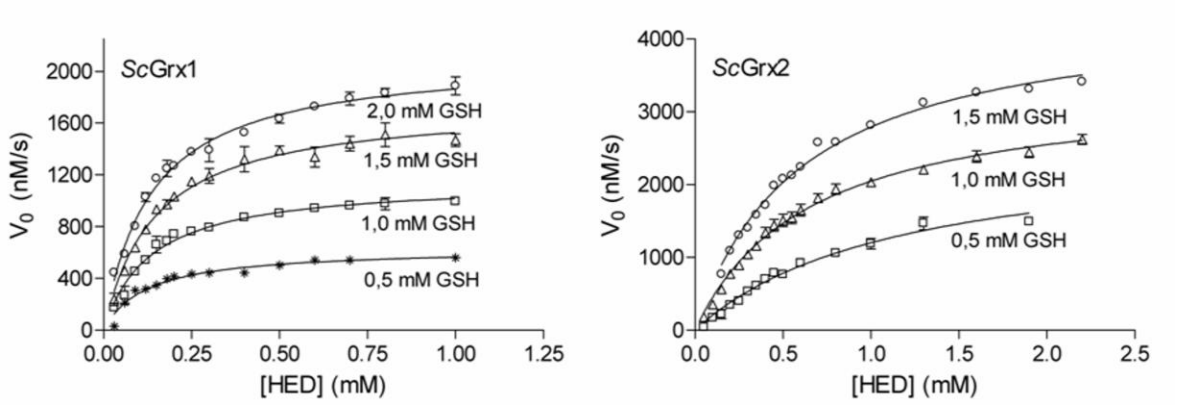

Figura 14: Gráficos de velocidade inicial versus a concentração de HED para *Sc*Grx1 e *Sc*Grx2 em concentrações diferentes de GSH. A concentrações de *Sc*Grx1 e *Sc*Grx2 utilizadas foram de 500 e 50 nM, respectivamente.

Tabela 6: Constantes cinéticas aparentes para a redução do dissulfeto misto β-ME-SG por *Sc*Grx1 e *Sc*Grx2, determinadas através da regressão não-linear de gráficos de Michaelis-Menten.

*Sc*Grx1			*Sc*Grx2		
[GSH] (mM)	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	[GSH] (mM)	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})
0,5	0,12 ± 0,02	1,3 ± 0,1	0,5	0,9 ± 0,1	47 ± 4
1,0	0,12 ± 0,01	2,2 ± 0,1	1,0	0,7 ± 0,1	66 ± 2
1,5	0,14 ± 0,01	3,5 ± 0,1	1,5	0,6 ± 0,1	85 ± 2
2,0	0,13 ± 0,01	4,2 ± 0,1			

As constantes aparentes para o HED também podem ser obtidas a partir dos gráficos de duplo recíproco, segundo o tratamento de Lineweaver-Burk (Figura 15). Esse tratamento de dados produziu valores de constantes muito semelhantes aos mostrados na Tabela 6. Além disso, o K_M real para o HED pode ser calculado a partir do ponto no qual as três retas se cruzam, neste ponto temos que $x = -1/K_M^{HED}$. O K_M^{HED} calculado para *Sc*Grx1 é 0,2 mM e para *Sc*Grx2 é 1,1 mM, indicando que *Sc*Grx1 tem maior afinidade pelo primeiro substrato da reação (Tabela 7). Por outro lado, os valores de k_{cat}^{app} mostram que *Sc*Grx2 cicla muito mais rapidamente do que *Sc*Grx1.

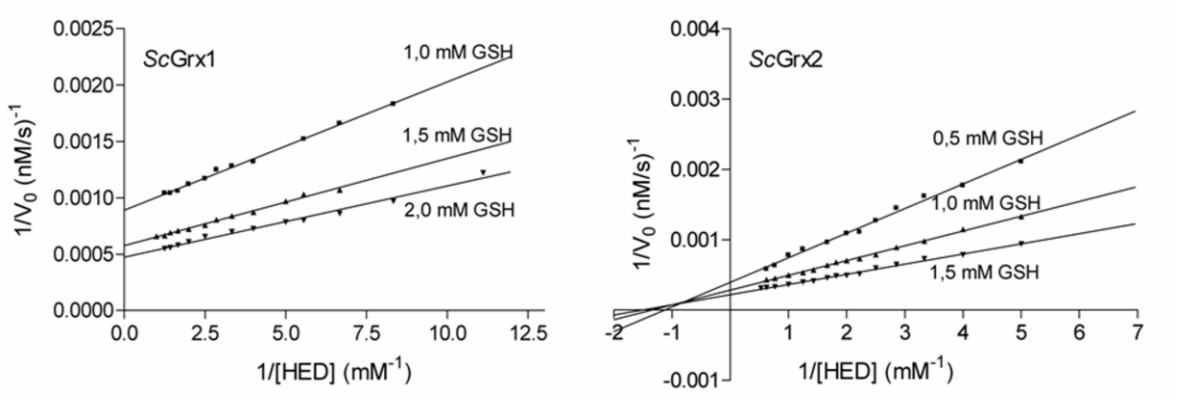

Figura 15: Gráficos representando os duplos recíprocos (Lineweaver-Burk): 1/V versus 1/[HED] para três concentrações diferentes de GSH.

Considerando que o mecanismo desta reação é seqüencial ordenado (veja discussão a seguir), podemos, a partir dos parâmetros aparentes (V_{max}^{app}), fazer um segundo gráfico de 1/ V_{max}^{app} versus 1/[GSH], e determinar o K_M^{GSH} real e a V_{max} real da reação (Segel, 1975). Nestes gráficos secundários temos: quando y = 0, x = -1/K_M^{GSH}, e quando x = 0, y = 1/ V_{max}; os valores obtidos estão mostrados na Tabela 7.

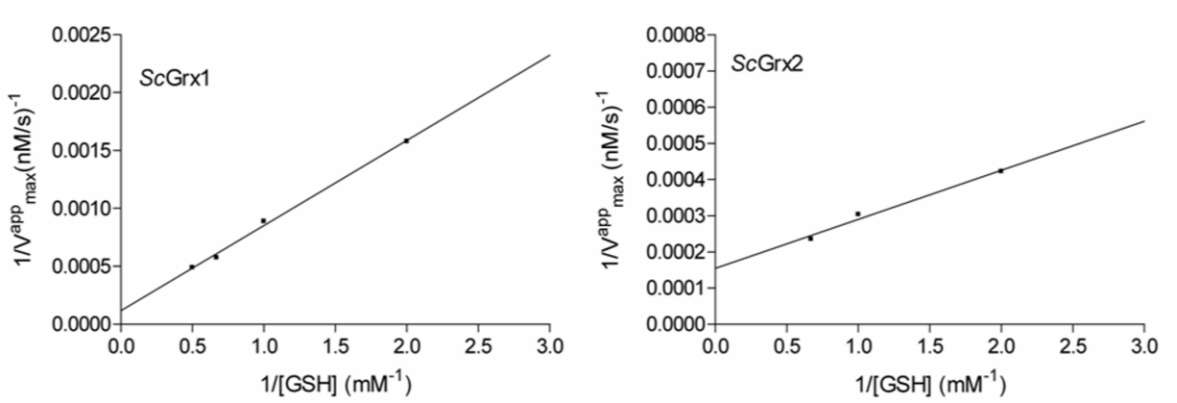

Figura 16: Gráficos secundários de 1/ V_{max}^{app} versus 1/[GSH] para *Sc*Grx1 e *Sc*Grx2.

Tabela 7: Constantes cinéticas reais para a redução do dissulfeto misto β-ME-SG por *Sc*Grx1 e *Sc*Grx2, obtidas através de gráficos secundários de 1/V_{max}^{app} versus 1/[GSH].

Enzima	K_M^{HED} (mM)	K_M^{GSH} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M $(M.s)^{-1}$
*Sc*Grx1	0,2 ± 0,1	6,2 ± 2,5	17 ± 7	(2,7 ± 1,5) x 10^3
*Sc*Grx2	1,1 ± 0,4	0,9 ± 0,2	129 ± 20	(1,5 ± 0,4) x 10^5

Os dados da Tabela 7 mostram que a afinidade de *Sc*Grx1 por GSH é muito menor que a afinidade de *Sc*Grx2, o que pode ser observado nos seus respectivos valores de K_M^{GSH}. Considerando que a etapa limitante de velocidade da reação global é a de redução do dissulfeto misto Grx-SG por GSH (Srinivasan *et al*., 1997), o fato de *Sc*Grx1 possuir uma afinidade maior pelo primeiro substrato da reação é sobrepujado pela maior afinidade de *Sc*Grx2 por GSH. Além disso, *Sc*Grx2 apresenta um maior *turnover* (maior k_{cat}) que *Sc*Grx1 na reação, de modo que a eficiência catalítica de *Sc*Grx2 (k_{cat}/K_M) é cerca de duas ordens de grandeza superior a de *Sc*Grx1. Corroborando o alto K_M^{GSH} encontrado para *Sc*Grx1, resultados recentemente publicados mostram que *Sc*Grx1 apresenta maior

especificidade pelo tripeptídeo ECG, análogo à GSH que contém apenas ligações α, do que por GSH na etapa limitante de velocidade de redução do dissulfeto Grx-SG (Saaranen *et al.*, 2009).

É importante salientar aqui que estamos sempre nos referindo à afinidade de *Sc*Grx1 ou *Sc*Grx2 glutatioladas (Grx-SG) por GSH, em relação à reação na qual o dissulfeto misto Grx-SG é reduzido por uma molécula de GSH, e não à afinidade destas Grxs reduzidas por GSH.

Outro interesse em se obter os gráficos de duplo recíproco (Figura 15) era analisar o perfil das curvas nas diferentes concentrações de GSH, o que possibilitaria fazer inferências a respeito do mecanismo no qual a reação se processa. Reações catalisadas que envolvem dois substratos (reações bi-substrato) são geralmente classificadas em dois grandes grupos, de acordo com o mecanismo pelo qual se processam (Voet & Voet, 2004):

Reações Seqüenciais: reações nas quais todos os substratos se combinam com a enzima, formando um complexo ternário, antes de a reação ocorrer e os produtos serem liberados. Reações que se processam segundo este mecanismo podem ser reconhecidas através dos gráficos de duplo recíproco no quais as retas (correspondentes a concentrações diferentes de um dos substratos, no nosso caso, as retas referentes a diferentes concentrações de GSH) se cruzam a esquerda do eixo 1/V.

Reações Ping Pong: reações nas quais um ou mais produtos são liberados antes que todos os substratos tenham sido combinados à enzima. Reações que se processam segundo este mecanismo podem ser reconhecidas através dos gráficos de duplo recíproco no quais as retas são paralelas.

Como pode ser observado na Figura 15, para *Sc*Grx2 é muito claro que as curvas se interceptam a esquerda do eixo 1/V, o que indica que a reação deve ter mecanismo Seqüencial. As retas para *Sc*Grx1 também se interceptam a esquerda de 1/V, porém como o K_M^{HED} é bastante pequeno neste caso, as retas se interceptam longe deste eixo.

A princípio, era esperado que o mecanismo para Grxs neste ensaio fosse o Ping-Pong. Como mostrado na Figura 3, no mecanismo proposto, primeiro Grx atacaria o dissulfeto misto β-ME-SG, liberando β-mercaptoetanol e permanecendo ligada a GS, e depois outra molécula de GSH reduziria o dissulfeto misto Grx-SG. Portanto, um produto

seria liberado antes que todos os substratos tivessem se combinado à enzima, o que caracteriza o mecanismo do tipo Ping-Pong. Ensaios com outros substratos glutatiolados, como por exemplo, Cys-SG (dissulfeto misto entre Cys e glutationa), mostram que Grxs apresentam mecanismo do tipo Ping-Pong nestas reações (Gallogly *et al.,* 2008; Gravina & Mieyal, 1993). O motivo desta diferença de comportamento pode estar relacionado ao fato de que o dissulfeto misto β-ME-SG é formado em uma pré-incubação. Quando a reação é iniciada com a adição de Grx, não se sabe ao certo quanto do dissulfeto misto β-ME-SG realmente foi formado. No caso do substrato Cys-SG, que pode ser obtido comercialmente, o dissulfeto misto já está previamente formado. É importante ressaltar que o HED é o substrato clássico usado na literatura para medir a atividade de Grxs, e como o principal interesse de nosso estudo é a comparação entre *Sc*Grx1 e *Sc*Grx2 e todos os ensaios são feitos em condições idênticas para ambas, estas ressalvas a respeito do HED não devem comprometer nossas análises.

IV.4 - pK_a das cisteínas reativas de *Sc*Grx1 e *Sc*Grx2

Grxs de outros organismos, como porco (Gan & Wells, 1987) e humano (Jao *et al.,* 2006; Gallogly *et al.,* 2008), tiveram os valores de pK_a das cisteínas reativas (N-terminal) determinados. Em todos os casos, o valor de pK_a destas cisteínas é bastante inferior ao de cisteínas livres (8,4) e situa-se entre 3,5 e 4,6. Devido a este baixo valor de pK_a, as cisteínas N-terminais de Grxs estão predominantemente desprotonadas em pH fisiológico, na forma de tiolato, possuindo, portanto, uma reatividade maior que cisteínas livres.

Resolvemos determinar os valores de pK_a da Cys27 de *Sc*Grx1 e *Sc*Grx2, considerando que uma diferença significativa de pK_a entre elas implicaria em uma diferente reatividade, explicando a diferença de atividade observada para estas enzimas. No nosso caso, esperaríamos que o pK_a da Cys27 de *Sc*Grx1 fosse significativamente maior do que de *Sc*Grx2.

O método experimental freqüentemente utilizado para a determinação do pK_a de cisteínas de Grxs consiste na inativação destas proteínas por alquilação da cisteína N-terminal com iodoacetamida (IAM). Este método já foi utilizado para *Hs*Grx1 (Jao *et al.*,

2006; Mieyal *et al.,* 1991) e *Hs*Grx2 (Gallogly *et al.,* 2008) e *Sc*Grx2 (Gan *et al.*, 1990) e, portanto, permite comparações. Porém, existem críticas sobre este método, uma vez que é necessário medir a atividade da enzima incubada em diferentes pHs e sabe-se da influência do pH sobre a atividade enzimática. A princípio, essa influência é eliminada, pois o pK_a é determinado a partir da atividade remanescente, que é calculada comparando-se as atividades da enzima alquilada e não alquilada, medidas no mesmo pH. De qualquer forma, achamos mais confiável determinar o pK_a também por outro método, sugerido pelo pesquisador Dr. Gerardo Ferrer-Sueta da Universidad de la República (Uruguai), no qual acompanhamos a velocidade de alquilação da cisteína com o reagente fluorescente monobromobimano (MBB; Kosower *et al.,* 1979).

Os valores obtidos com o método de inativação com IAM são muito similares àqueles determinados previamente para *Sc*Grx2 (Gan *et al.*, 1990) e *Hs*Grx1 (Jao *et al.*, 2006), usando o mesmo método. Os resultados obtidos com o método de alquilação com MBB são mais baixos (Tabela 8), porém para nossas análises o ponto importante destes resultados é que os valores de pK_a da Cys27 de *Sc*Grx1 e *Sc*Grx2 são bastante baixos, como os descritos para outras Grxs, e próximos entre si.

Tabela 8: Valores de pK_a da cisteína N-terminal (Cys27) dos sítios ativos de *Sc*Grx1 e *Sc*Grx2, determinados pelos métodos de inativação com IAM e alquilação com MBB.

	pK_a Cys27 (MBB)	pK_a Cys27 (IAM)
*Sc*Grx1	3,2 ± 0,2	4,0 ± 0,2
*Sc*Grx2	3,1 ± 0,2	3,5 ± 0,2

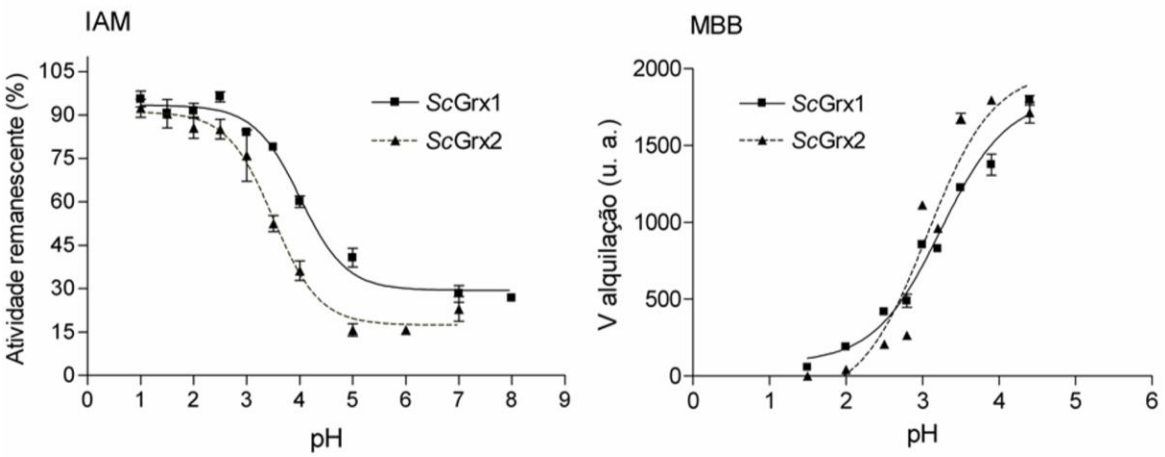

Figura 17: Gráficos utilizados para a determinação do pK_a de *Sc*Grx1 e *Sc*Grx2 pelos métodos de inativação com IAM (à esquerda) e de alquilação com MBB (à direita).

Reações de intercâmbio tiol-dissulfeto são do tipo Sn^2 (substituição nucleofílica binuclear) e suas velocidades dependem, portanto, da basicidade (pK_a) do nucleófilo e do grupo de saída (Gilbert, 1990; Szajewski & Whitesides, 1980). Para estas reações, quanto menor o pK_a do enxofre, o qual é o grupo de saída, mais rápida é a velocidade da reação (Srinivasan *et al.*, 1997). Para reações de intercâmbio tiol-dissulfeto de um mesmo tipo, a constante de segunda ordem da reação aumenta por um fator de quatro com o decréscimo de uma unidade no pK_a do grupo de saída ($k_A/k_B = 4^{(pKaB - pKaA)}$) (Gilbert, 1990; Szajewski & Whitesides, 1980). Assumindo que a redução do intermediário Grx-SG por uma molécula de GSH é a etapa limitante da reação no ensaio com HED, de modo que o pK_a do tiolato da Cys27 é determinante na velocidade da reação, a diferença nos valores de pK_a da Cys27 de *Sc*Grx1 (B) e *Sc*Grx2 (A) resultaria em uma constante de segunda ordem 2 vezes maior para a reação catalisada por *Sc*Grx2 em comparação à catalisada por *Sc*Grx1, considerando os valores obtidos com IAM, e 1,15 vezes maior, considerando os resultados com MBB (Tabela 8). Entretanto, a razão entre as constantes de segunda ordem observadas para *Sc*Grx1 e *Sc*Grx2 (Tabela 7) é muito maior do que a prevista devido a pequena diferença do pK_a da Cys27 destas enzimas, sugerindo que outros fatores são responsáveis pela diferença de atividade entre *Sc*Grx1 e *Sc*Grx2.

Embora a equação de Brønsted se aplique muito bem a tióis de baixa massa molecular (Szajewski & Whitesides, 1980), alguns desvios podem ocorrer quando são

consideradas moléculas complexas como proteínas (Trujillo *et al.*, 2007), indicando que características estruturais podem ser mais importantes do que o pK_a das cisteínas reativas no controle da velocidade de reações de troca tiol-dissulfeto (Jao *et al.*, 2006).

É importante mencionar que, neste trabalho, foi assumido que a etapa limitante da reação no ensaio com HED para Grxs é a redução do dissulfeto misto Grx-SG por uma molécula de GSH, como descrito para a *Hs*Grx1 (Srinivasan *et al*., 1997). Isto por que as atividades de *Sc*Grx2 e *Sc*Grx1 apresentam uma dependência em relação ao pH (Figura 18) similar àquela apresentada por *Hs*Grx1, com um pH ótimo próximo a 9,0. E também, os resíduos de Cys27 de *Sc*Grx1 e *Sc*Grx2 apresentam valores de pK_a similares ao de *Hs*Grx1, sugerindo que o pK_a do segundo substrato (GSH; pK_aCys_{GS} = 8,5) está envolvido na etapa limitante da reação (Srinivasan *et al*., 1997; Gallogly *et al.*, 2008). Considerando todas estas informações, pode-se concluir que os valores de pK_a para a Cys27 de *Sc*Grx1 e *Sc*Grx2 não são um fator determinante na diferença de atividade observada entre estas enzimas.

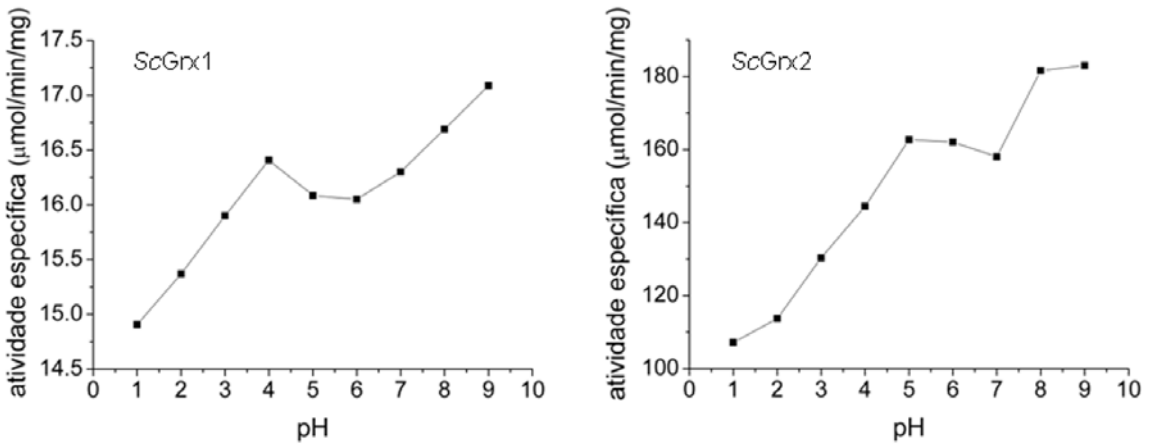

Figura 18: Atividade de *Sc*Grx1 e *Sc*Grx2 em função do pH.

Se considerássemos que a etapa limitante da reação fosse a primeira (Figura 3, reação f), na qual o tiolato da Cys27 ataca a ligação dissulfeto de β-ME-SG, a velocidade da reação seria principalmente dependente da porcentagem de cisteínas de *Sc*Grx1 e *Sc*Grx2 que estariam na forma desprotonada no pH do ensaio de atividade (7,4). Com base na equação de Henderson-Hasselbalch: $pH = pK_a + \log [S^-]/[SH]$ (Po & Senozan, 2001) e

os valores de pK_a das Cys27 de *Sc*Grx1 e *Sc*Grx2, podemos calcular a porcentagem das cisteínas que estariam na forma de tiolato em pH 7,4 para *Sc*Grx1 e *Sc*Grx2. Considerando os valores obtidos com MBB, temos que 99,99% das Cys27 de *Sc*Grx1 e *Sc*Grx2 estão desprotonadas neste pH, e considerando os valores obtidos com IAM temos 99,96% das Cys27 de *Sc*Grx1 e 99,98% das Cys27 de *Sc*Grx2 estão na forma de tiolato em pH 7,4. Mesmo considerando a primeira etapa da reação como limitante de velocidade, não haveria uma diferença significativa de desprotonação entre as Cys27 de *Sc*Grx1 e *Sc*Grx2 no pH usado nos ensaios de atividade, e conseqüentemente, não deveria haver significativa diferença de reatividade entre as mesmas. Portanto, os valores de pK_a da Cys27 de *Sc*Grx1 e *Sc*Grx2 não explicam a grande diferença de atividade entre estas duas enzimas.

IV.5 - Atividade peroxidásica

A próxima etapa foi avaliar se *Sc*Grx1 e *Sc*Grx2 também apresentavam diferenças para outras atividades enzimáticas. Uma vez que essas enzimas apresentam valores de pK_a bastante baixos e esse fator tem sido associado a reatividade com peróxidos (Ogosucu *et al.*, 2007; Trujillo *et al.*, 2007), decidimos analisar se essas enzimas apresentavam atividade peroxidásica.

A capacidade de *Sc*Grx2 catalisar a redução direta de peróxidos foi acompanhada através da queda na absorbância a 340 nm, devido à oxidação do NADPH (Collinson *et al.,* 2002). Porém observamos que *Sc*Grx2 não é eficiente na degradação de peróxidos. A velocidade da reação catalisada por *Sc*Grx2 é a mesma que a da redução dos peróxidos no sistema reacional sem a enzima, pois GSH reduz peróxidos. A Figura 19 mostra os gráficos de velocidade versus concentração de peróxido para H_2O_2 e hidroperóxido de cumeno (CHP).

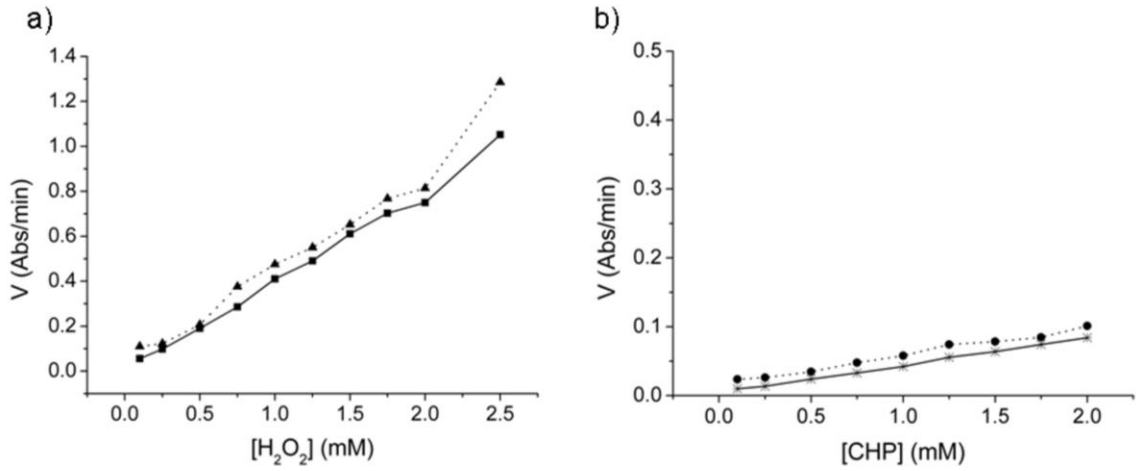

Figura 19: (a) Gráfico de velocidade de redução de H_2O_2 versus concentração de H_2O_2; os quadrados representam o controle sem *Sc*Grx2, e os triângulos, às reações com 20 nM de *Sc*Grx2. (b) Gráfico de velocidade de redução de CHP versus concentração de CHP; as cruzes representam o controle sem *Sc*Grx2, e os círculos, às reações com 20 nM de *Sc*Grx2.

As peroxirredoxinas (Prxs) de levedura catalisam a redução de hidroperóxidos quando presentes em concentrações da ordem de micromolar (Jeong *et al.*, 1999; Munhoz & Netto, 2004). Nestes ensaios utilizamos *Sc*Grx2 em concentrações da ordem de nanomolar, como usado no ensaio de redução do dissulfeto misto β-ME-SG. Talvez não tenhamos detectado a atividade peroxidásica de *Sc*Grx2 devido à baixa concentração de enzima utilizada. Então resolvemos tentar medir a atividade peroxidásica e *Sc*Grx1 e *Sc*Grx2 usando concentrações de 50 µM de proteína e 0,1 mM de peróxido (H_2O_2, *t*-BOOH e CHP). As velocidades das reações de redução de peróxidos por *Sc*Grx1 e *Sc*Grx2 estão mostradas na Tabela 9. As velocidades foram determinadas a partir da porção do gráfico de absorbância versus tempo que apresentava inclinação máxima.

Tabela 9: Velocidade da redução de peróxidos por *Sc*Grx1 e *Sc*Grx2.

Reação	v (dA/min) H_2O_2	v (dA/min) *t*-BOOH	v (dA/min) CHP
***Sc*Grx1**			
A) NADPH → GR → GSH → peróxido	0,056	0,036	0,013
B) NADPH → GR → GSH → *Sc*Grx1	0,230	0,227	0,233
C) NADPH→ GR→ GSH→ *Sc*Grx1 → peróxido	0,224	0,223	0,230
***Sc*Grx2**			
A) NADPH → GR → GSH → peróxido	0,053	0,040	0,015
B) NADPH → GR → GSH → *Sc*Grx2	0,157	0,158	0,153
C) NADPH→ GR→ GSH→ *Sc*Grx2 → peróxido	0,155	0,150	0,150

Os resultados mostram que *Sc*Grx1 e *Sc*Grx2 não são eficientes como peroxidases, nem mesmo quando a concentração destas enzimas nos ensaios é da ordem de micromolar. Na verdade, o consumo de NADPH observado deve-se ao fato de que parte das moléculas de *Sc*Grx1/*Sc*Grx2 em solução devem estar oxidadas e parte da GSH é consumida na redução destas moléculas oxidadas, pois as reações B e C apresentam velocidades muito próximas. Se Grxs fossem capazes de reduzir os peróxidos teríamos observado um aumento na velocidade da reação C em relação à reação B. Quando utilizamos concentrações de Grxs da ordem de nanomolar, como no ensaio com HED, não observamos consumo de NADPH devido à redução de moléculas de Grx oxidadas, uma vez que a concentração de Grx é muito menor.

É importante mencionar que foi descrito na literatura que *Sc*Grx1 e *Sc*Grx2 possuem atividade peroxidásica mesmo quando utilizadas em concentrações da ordem de nanomolar (Collinson *et al.*, 2002). Os parâmetros cinéticos de *Sc*Grx1 e *Sc*Grx2 frente a peróxidos determinados por estes autores podem ser obtidos se não forem consideradas as reações controle, nas quais observamos a redução de peróxidos por GSH.

A alta reatividade de Prxs na redução de peróxidos é atribuída parcialmente ao baixo pK_a da Cys peroxidásica de seus sítios ativos (Trujillo *et al.*, 2007). Considerando este fator, teoricamente, Grxs poderiam ter atividade peroxidásica, pois possuem um resíduo de Cys com pK_a até um pouco menor em seus sítios ativos (Discola *et al.*, 2009 -

anexo 1), em comparação às Prxs (Trujillo *et al.*, 2007). Porém, nossos resultados e resultados de Pedrajas *et al.*, 2002 mostram que Grxs não apresentam atividade peroxidásica.

A quebra da ligação O-O (1,49 Å) de peróxidos, catalisada por Prxs, é mais difícil do que a de ligações dissulfeto S-S (2,05 Å), catalisadas por Trxs e Grxs, uma vez que a primeira ligação é mais curta e mais forte do que a segunda (Lazzeretti & Zanasi, 1997). Além do baixo pK_a da Cys reativa, outras características estruturais parecem estar relacionadas a alta reatividade das Prxs frente a peróxidos. Foi proposto que a substituição da Cys N-terminal do motivo CXXC por uma Thr e a utilização da Cys C-terminal como nucleófilo em Prxs, confere a estas proteínas um maior controle sobre a orientação do substrato no sítio ativo, favorecendo a clivagem da ligação O-O (Copley *et al.*, 2004).

IV.6 - Interação entre as *Sc*Grxs e *Sc*Trrs

Foi descrito na literatura que *Ec*Grx4 e *Hs*Grx2 são reduzidas pelas Tiorredoxinas Redutases (Trr) dos respectivos organismos (Fernandes *et al.*, 2005; Johansson *et al.*, 2004). Uma vez que em nosso laboratório são expressas *Sc*Trr1 (citosólia; Chae *et al.*, 1994) e *Sc*Trr2 (mitocondrial; Pedrajas *et al.*, 1999), resolvemos testar se estas enzimas seriam capazes de reduzir *Sc*Grx1 e *Sc*Grx2. Para isso, repetimos o ensaio de redução do dissulfeto misto β-ME-SG, substituindo o sistema redutor de Grxs (NADPH, GR e GSH) pelas *Sc*Trrs e NADPH.

Os resultados mostraram que *Sc*Trr1 e *Sc*Trr2 não reduzem *Sc*Grx1 e *Sc*Grx2 (Figura 20). Utilizamos as Trrs na concentração de 0,5 μM, sendo que estas enzimas catalisam a redução de seus substratos, Trxs, em concentrações 20 vezes menores (25 nM). E utilizamos *Sc*Grx1 e *Sc*Grx2 na concentração de 2 μM, sendo que estas enzimas catalisam a reação de redução de β-ME-SG quando presentes em concentrações da ordem de nanomolar (Figura 11). Mesmo utilizando concentrações bastante altas de Grxs e Trrs, não observamos a redução de *Sc*Grxs pelas *Sc*Trrs.

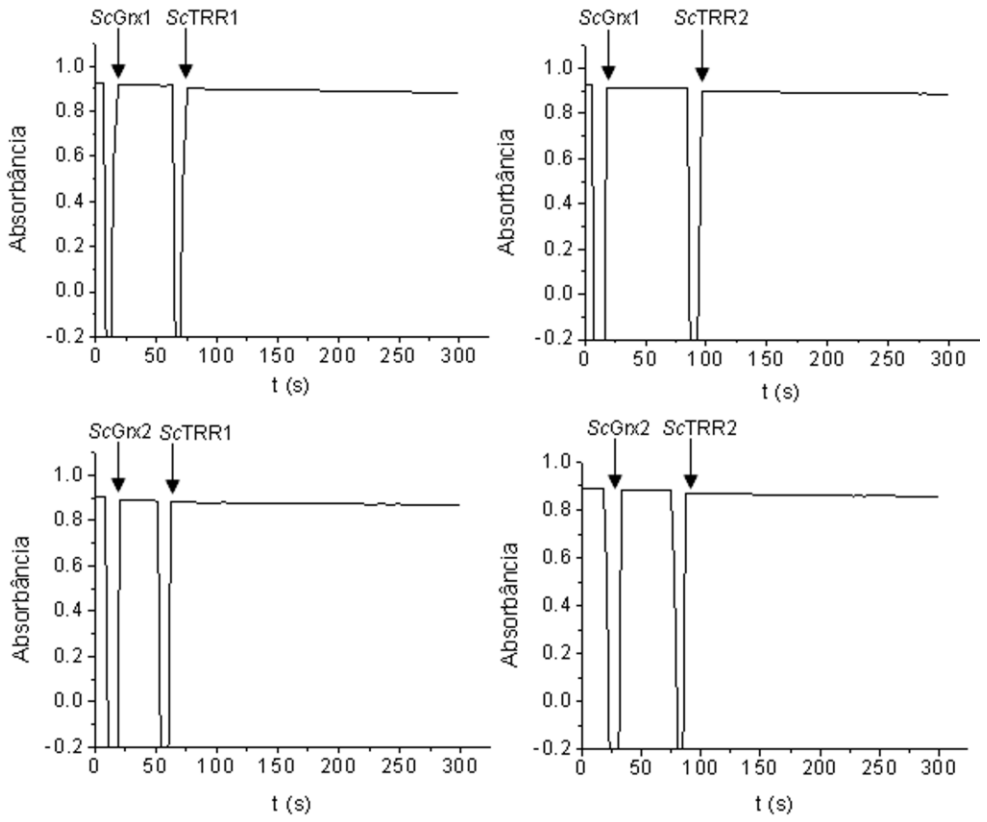

Figura 20: Ensaio teste da redução de *Sc*Grx1 e *Sc*Grx2 pelas Trrs (*Sc*Trr1 e *Sc*Trr2) de levedura.

IV.7 - Redução do dissulfeto da Insulina

A redução de pontes dissulfeto em proteínas é realizada pelas Grxs através do mecanismo ditiólico, envolvendo, portanto, as duas cisteínas dos sítios ativos das Grxs. Ao contrário, a redução de dissulfetos mistos entre GSH e proteínas ou outros compostos de baixa massa molecular, como o HED, é realizado por Grxs através do mecanismo monotiólico, o qual envolve apenas a Cys N-terminal do sítio ativo. Como discutido anteriormente, *Sc*Grx1 é muito menos ativa que *Sc*Grx2 na redução do dissulfeto misto β-ME-SG, porém não sabemos se estas enzimas também apresentam diferença de atividade em reações que se processam pelo mecanismo ditiólico. Para verificar isso, seria importante medir as atividades de *Sc*Grx1 e *Sc*Grx2 na redução de um dissulfeto protéico.

Até o momento, nenhum substrato passível de redução pelo mecanismo ditiólico foi descrito para *Sc*Grx1 e *Sc*Grx2. De modo geral, é atribuída às Grxs e Trxs a redução do

dissulfeto da Ribonucleotídeo Redutase (RR) durante a síntese de desoxirribonucleotídeos, porém foi descrito para *S. cerevisiae,* que os principais redutores da RR são as Trxs (Camier *et al.*, 2007). Além disso, a RR de levedura não é disponível comercialmente, o que torna difícil a realização de ensaios *in vitro* para determinar se *Sc*Grx1 e *Sc*Grx2 são capazes de reduzí-la através do mecanismo ditiólico. Uma vez que não temos RR, e que foi descrito que *Ec*Grx1 é capaz de reduzir pontes dissulfeto da insulina (Holmgren & Åslund, 1995), resolvemos testar se as Grxs de levedura também reduziriam insulina. Para isso foram realizados ensaios como aquele descrito para a redução do dissulfeto misto β-ME-SG, substituindo apenas o HED por 30 µM de insulina de pâncreas bovino (Vlamis-Gardikas *et al.*, 1997).

Ao branco foram adicionados todos os reagentes, exceto o NADPH e a Grx purificada, e o controle foi feito sem a adição de Grx ao sistema reacional. Foram utilizadas concentrações de *Sc*Grx1 e *Sc*Grx2 entre 10 nM e 10 µM. No ensaio de redução do dissulfeto misto β-ME-SG, é possível medir a atividade de *Sc*Grx1 e *Sc*Grx2 usando estas enzimas em concentrações da ordem de nanomolar. Como nenhuma atividade foi observada no ensaio com insulina usando concentrações de *Sc*Grx1 e *Sc*Grx2 desta ordem, testamos também concentrações maiores (ordem de micromolar). Como pode ser observado na Figura 21, *Sc*Grx1 e *Sc*Grx2 não foram capazes de reduzir as pontes dissulfeto da insulina mesmo quando usadas em altas concentrações na reação.

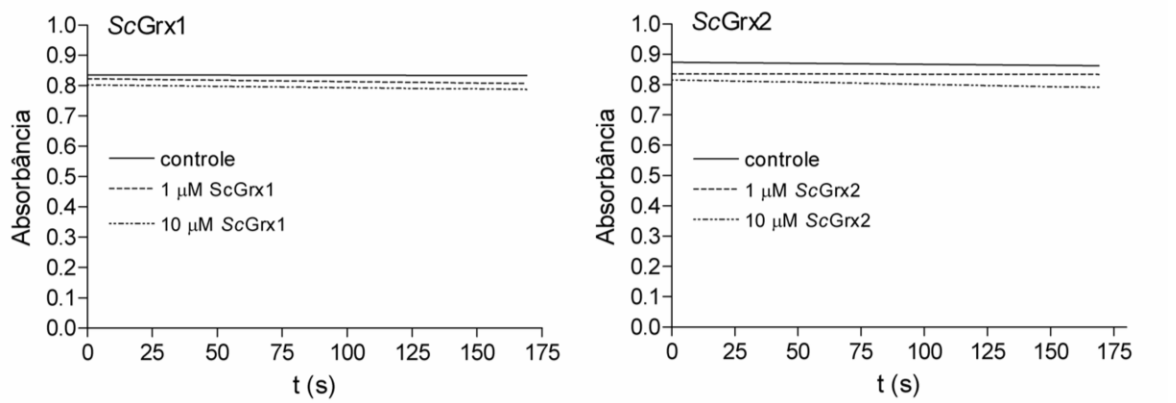

Figura 21: Gráficos de absorbância (λ = 340 nm) versus tempo (s) da reação de redução das pontes dissulfeto da insulina por *Sc*Grx1 e *Sc*Grx2.

Até o momento, não encontramos um substrato para testar a atividade de *Sc*Grx1 e *Sc*Grx2 no mecanismo ditiólico. É importante lembrar que todas as Grxs ditiólicas estudadas até o momento catalisam reações que se processam através do mecanismo monotiólico, mas nem todas catalisam reações pelo mecanismo ditiólico (Lillig *et al.*, 2008). Um aspecto importante a ser considerado nesse sentido seria interações proteína-proteína de Grxs com alvos biológicos, as quais devem envolver aspectos estruturais.

IV.8 - Cristalização de *Sc*Grx1

Foram feitos ensaios de crescimento de cristais com *Sc*Grx1 sem tratamento e com a proteína tratada com diamida, *t*-BOOH, H_2O_2 e DTT, usando os kits "Crystal Screen 1 e 2" e "Wizard 1 e 2". Cresceram cristais apenas da proteína tratada com diamida na condição 3 do kit Wizard 2 (WII-3: 20% PEG 8000/ 0,1 M Tris-HCl pH 8,5/ 0,2 M $MgCl_2$), e de *Sc*Grx1 sem tratamento na condição 31 do kit Wizard 1 (WI-31: 20% PEG 8000/ 0,1 M Fosfato-Citrato pH 4,2/ 0,2 M NaCl); Figuras 22A e 22B, respectivamente. Como pode ser observado na Figura 22A, conseguimos apenas microcristais de *Sc*Grx1 na condição WII-3, e na condição WI-31 conseguimos cristais aparentemente mais promissores (Figura 22B).

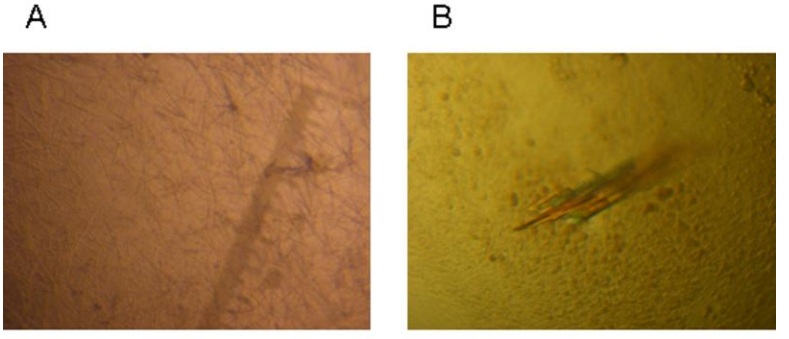

Figura 22: Cristais de *Sc*Grx1 obtidos. A) Microcristais de *Sc*Grx1 tratada com diamida, obtidos na condição WII-3. B) Cristais de *Sc*Grx1 sem tratamento, obtidos na condição WI-31.

Foram feitos refinamentos de ambas as condições de cristalização de *Sc*Grx1, variando o pH (entre 4,0 e 9,5), e as concentrações de proteína (5, 10 e 15 mg/mL), precipitante (5 a 20% PEG 8000) e sal (0,05 a 0,2 M). Tentamos também a técnica de

microseeding e aditivos com os kits "Aditive Screen 1, 2 e 3", porém não conseguimos obter cristais maiores e bem formados para os experimentos de difração de raios-X. Enquanto tentávamos melhorar os cristais de *Sc*Grx1, foi publicado um artigo (Håkansson & Winther, 2007), descrevendo as estruturas cristalográficas de *Sc*Grx1 nas formas reduzida e ligada à glutationa. Dessa forma, concentramos nossos estudos na resolução de estrutura de *Sc*Grx2.

IV.9 - Estruturas de *Sc*Grx2

IV.9.1 - Determinação da estrutura de *Sc*Grx2 na forma oxidada

Cristalização e difração

Inicialmente, foram feitos ensaios de crescimento de cristais de *Sc*Grx2 com os kits "Crystal Screen 1 e 2". Foram obtidos diversos cristais de *Sc*Grx2 tratada com *t*-BOOH, H_2O_2, diamida e com a proteína sem tratamento, todos, na solução de cristalização: 0,2 M de acetato de amônio/ 30% PEG 4000/ 0,1M acetato de sódio pH 4,6 (solução 10 do kit Crystal Screen 1: CS1-10).

Foram feitos refinamentos, variando-se o pH (entre 4,0 e 9,5), a concentração de PEG 4000 (entre 12 e 30%) e a concentração de acetato de amônio (entre 0,05 e 0,2M) com o intuito de obter cristais melhores para a coleta de dados de difração. Observamos cristais entre pH 4 e pH 4,6, em concentrações de acetato de amônio entre 0,1 e 0,2 M, e entre 25 e 30% de PEG 4000, porém os cristais eram iguais nestas faixas de variação (semelhantes ao da Figura 23). Cristalizamos também a proteína a 4°C, mas novamente os cristais obtidos eram muito semelhantes aos obtidos a 20°C. Outras tentativas foram feitas para obter cristais melhores: utilizamos os kits "Additive Screen 1, 2 e 3" e também a técnica de *microseeding*, porém não conseguimos melhorar os cristais.

Cristais de *Sc*Grx2 tratada com *t*-BOOH e com diamida e também de *Sc*Grx2 sem tratamento difrataram até resoluções de 2,05 Å, 2,2 Å e 2,15 Å, respectivamente. Estes cristais foram imersos em uma solução crio-protetora com 20% glicerol antes do início da coleta dos dados de difração de raios-X. O detector foi mantido a uma distância de 90 mm, e foram coletadas 180 imagens com $\Delta\phi$=1°.

Figura 23: Cristal de *Sc*Grx2 sem tratamento. Todos os cristais de *Sc*Grx2 obtidos eram agulhas semelhantes à mostrada acima.

Como não conseguimos obter cristais da proteína reduzida previamente com DTT ou GSH, novas placas de cristalização foram feitas com a proteína sem tratamento na condição CS1-10, e nas gotas nas quais cresceram cristais adicionamos DTT (50 mM) ou GSH (10 mM). Os cristais permaneceram intactos após a adição destes agentes redutores. Coletamos conjuntos de dados, com 180 imagens cada ($\Delta\phi$=1°), de diferentes cristais de *Sc*Grx2 tratados com DTT (que difrataram até resoluções de 2,12 Å, 2,3 Å, e 2,5 Å), e conjuntos de dados de cristais de *Sc*Grx2 tratados com GSH (que difrataram a 2,65 Å, 2,8 Å e 3,1 Å). As estatísticas de alguns destes conjuntos de dados estão mostradas na Tabela 10.

Foram feitos também outros ensaios de crescimento de cristais com o kits "Index 1 e 2" e "Wizard 1 e 2" com a proteína tratada por 1 hora a temperatura ambiente com 10 mM DTT e com 10 mM GSH. As placas de cristalização foram observadas periodicamente, porém apenas um cristal da proteína tratada com DTT foi observado na condição 8 do kit Wizard 1 (2,0M sulfato de amônio/0,1M citrato pH 5,5). Este cristal difratou até 2,5 Å de resolução; como para os demais conjuntos de dados, foram coletadas 180 imagens com $\Delta\phi$=1°.

Processamento dos dados e substituição molecular

Todos os conjuntos de dados foram processados com o auxílio dos programas MOSFLM (Leslie, 1992) e SCALA (Kabush, 1998; Blessing, 1995) do pacote CCP4. O número de moléculas por unidade assimétrica foi determinado calculando-se o coeficiente de Matthews (Matthews, 1968), cujo valor encontrado foi 2,3 $Å^3$/Da para todos os

conjuntos de dados coletados. A porcentagem de solvente nos cristais variou entre 45,5 e 45,8%, e, em todos os casos, havia apenas uma molécula na unidade assimétrica. As estatísticas dos dados de difração de raios-X coletados estão mostradas na Tabela 10. As estatísticas dos dados de difração de raios-X coletados para os primeiros cristais de *Sc*Grx2 (proteína sem tratamento e tratada com *t*-BOOH) constam no trabalho publicado na seção F da revista *Acta Crystallographica* (Discola *et al.,* 2005 - anexo 5).

Tabela 10: Estatísticas dos dados de difração coletados. Os valores entre parênteses correspondem aos dados da última camada de resolução.

	t-BOOH	Sem tratamento	Diamida	DTT1	DTT2	DTT3 *	GSH
Grupo espacial	$P4_12_12$	$P4_12_12$	$P4_12_12$	$P4_12_12$	$P4_12_12$	$P4_12_12$	$P4_12_12$
Parâmetros de cela unitária (Å)	a = b = 47,6341 c=94,5919	a = b = 47,6595 c=95,0028	a = b = 47,7749 c=94,6508	a = b = 47,6998 c =95,1715	a = b = 47,3719 c=94,8469	a = b = 47,2429 c=94,9426	a = b = 48,3563 c=95,5466
Resolução (Å)	2,05	2,15	2,20	2,12	2,30	2,50	3,10
Número de reflexões totais	90136	68434	87346	184740	166187	141962	43787
Número de reflexões únicas	7295	6439	6046	6703	5332	5201	2316
Completeza (%)	99,8 (99,8)	100 (100)	100 (100)	96,6 (96,6)	98,6 (98,6)	100 (100)	99,2 (99,2)
Multiplicidade (%)	12,3 (11,2)	10,6 (10,7)	14,4 (14,9)	13,1 (12,0)	14,5 (13,5)	7,8 (8,1)	6,4 (6,7)
R_{sym}	0,080 (0,294)	0,103 (0,328)	0,100 (0,322)	0,064 (0,293)	0,058 (0,272)	0,085 (0,387)	0,089 (0,351)
$\langle I/\sigma(I)\rangle$	6,3 (2,1)	5,5 (2,2)	6,5 (2,2)	9,1 (2,6)	9,9 (2,7)	7,9 (2,0)	7,3 (2,4)

* Cristal de *ScGrx*2 tratada com DTT na condição 8 do kit Wizard 1. Os demais conjuntos de dados foram coletados a partir de cristais obtidos na condição 10 do kit Crystal Screen 1.

Inicialmente, foi feita uma tentativa de substituição molecular com as coordenadas da Grx de *Sus scrofa* (código PDB: 1KTE; Katti *et al.,* 1995), utilizando o programa

AmoRe (Navaza, 1994). No momento, esta era a proteína com estrutura determinada que apresentava maior identidade seqüencial com *Sc*Grx2 (36% de identidade e 58% de similaridade). Porém, não foi encontrada nenhuma solução para a proteína sem tratamento e nem para a proteína tratada com *t*-BOOH. A segunda tentativa de substituição molecular foi realizada com um modelo teórico criado pelo programa Modeller (Claude *et al.,* 2004), a partir das coordenadas atômicas de 1KTE e da seqüência de aminoácidos de *Sc*Grx2. Neste caso, o programa AmoRe indicou uma solução para ambos cristais.

A estrutura da *Sc*Grx2 tratada com *t*-BOOH foi refinada com o auxílio do programa Refmac5 (Murshudov *et al.,* 1997). Quando terminado o refinamento desta estrutura, utilizamos suas coordenadas como modelo para substituição molecular para os demais conjuntos de dados coletados de cristais de *Sc*Grx2 com outros tratamentos.

Refinamento das estruturas

A primeira estrutura resolvida por substituição molecular usando o modelo teórico foi a de *Sc*Grx2 tratada com *t*-BOOH. O primeiro ciclo de refinamento do modelo gerou R_{factor} = 0,496 e R_{free} = 0,600; ao final do refinamento do modelo obtivemos R_{factor} = 0,194 e R_{free} = 0,227. Nesta estrutura, as cisteínas 27 e 30 do sítio ativo de *Sc*Grx2 se encontram na forma oxidada, formando uma ligação dissulfeto. Este modelo foi usado para resolver as fases dos demais conjuntos de dados coletados de *Sc*Grx2, por substituição molecular. Os primeiros ciclos de refinamento das demais estruturas de *Sc*Grx2 geraram valores mais baixos de R_{factor} e R_{free} (aproximadamente 0,2 e 0,3, respectivamente) em comparação aos obtidos para a primeira estrutura resolvida com o modelo teórico; as cadeias estavam bem posicionadas dentro dos mapas de densidade eletrônica e já era possível verificar o estado de oxidação das cisteínas. Como já havíamos determinado a estrutura de *Sc*Grx2 na forma oxidada, nosso objetivo era obter a estrutura de *Sc*Grx2 na forma reduzida. Porém, as demais estruturas também apresentavam as cisteínas oxidadas e não foram observadas mudanças ao final dos refinamentos. Então, as últimas estruturas determinadas (DTT3 e GSH) passaram apenas pelo primeiro ciclo de refinamento e, por esta razão, os parâmetros estatísticos destas duas moléculas não constam na tabela 11.

Os modelos foram refinados (com exceção dos mencionadas acima), e foi utilizado o programa PROCHECK (Laskowski *et al.,* 1993) para analisar a estereoquímica dos mesmos. As estatísticas dos refinamentos e a análise da estereoquímica são mostradas na Tabela 11. A Figura 24 mostra o diagrama de Ramachandran da estrutura de *Sc*Grx2 tratada com *t*-BOOH; os diagramas das demais estruturas não são mostrados por serem semelhantes. Nenhum aminoácido de *Sc*Grx2 foi localizado em região proibida.

Tabela 11: Estatísticas dos refinamentos das estruturas de *Sc*Grx2 e análise estereoquímica.

	t-BOOH	Sem tratamento	Diamida	DTT1	DTT2
R_{factor}	0,194	0,193	0,191	0,214	0,209
R_{free}	0,227	0,255	0,248	0,254	0,251
$Rms_{ligação}$	0,012	0,014	0,012	0,011	0,011
$Rms_{ângulo}$	1,332	1,441	1,340	1,33	1,33
B factor médio (Å^2)					
Cadeia principal	18,716	19,507	20,583	29,9332	25,733
Cadeias laterais	22,240	23,597	23,971	30,850	27,128
Análises Ramachandran (%)					
Regiões favorecidas	97,9	97,9	96,9	96,8	95,8
Regiões adicionalmente permitidas	2,1	2,1	3,1	3,2	4,2
Regiões não permitidas	0	0	0	0	0

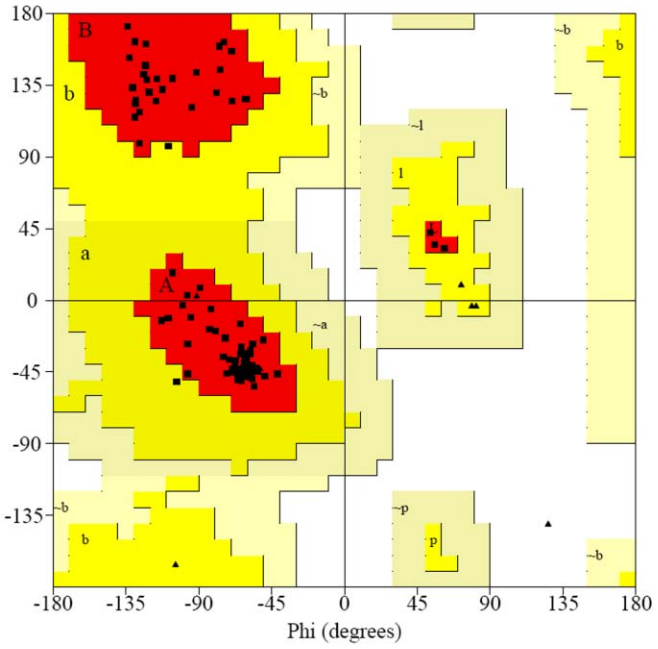

Figura 24: Diagrama de Ramachandran da estrutura de *Sc*Grx2 tratada com *t*-BOOH.

IV.9.2 - Determinação da estrutura de *Sc*Grx2-C30S ligada à glutationa

Cristalização e difração

Construímos a proteína mutante *Sc*Grx2-C30S, na qual a Cys30 (C-terminal) foi substituída por uma serina, com o objetivo de determinar sua estrutura na forma ligada à glutationa (*Sc*Grx2-SG) e na forma reduzida. Uma serina no lugar da Cys30 C-terminal permitiria a formação de um dissulfeto misto estável entre a Cys27 N-terminal e a molécula de glutationa, uma vez que a Cys27 não seria atacada pela Cys30 para liberar a glutationa e formar o dissulfeto intramolecular (Figura 3, reação d). O dissulfeto misto com glutationa, no mutante *Sc*Grx2-C30S, pode ser reduzido apenas por um agente redutor externo. Além disso, neste mutante não ocorre a formação do dissulfeto intramolecular no sítio ativo, sendo possível, então, obter em princípio a estrutura de *Sc*Grx2 na forma reduzida.

O dissulfeto misto entre o mutante *Sc*Grx2-C30S e glutationa (*Sc*Grx2-SG) foi obtido com o tratamento da proteína (pré-reduzida) com GSSG, como descrito em "Materiais e Métodos". Realizamos ensaios de crescimento de cristais de *Sc*Grx2-SG e de

*Sc*Grx2-C30S tratada com 10 mM DTT ou 10 mM GSH com os kits "Crystal Screen 1 e 2" e "Wizard 1 e 2". Obtivemos os cristais de *Sc*Grx2-SG, mostrados na figura 25A, na condição 22 do kit "Crystal Screen 1" (CS1-22: 30% PEG 4000/ 0,1 M Tris-HCl pH 8,5/ 0,2 M acetato de sódio). Um dos cristais desta gota foi submetido a experimentos de difração de raios-X e difratou a 2,9 Å. Foi coletado um conjunto de dados de difração deste cristal com 220 imagens com $\Delta\phi$=1°, usando solução crio-protetora com 20% glicerol. Este conjunto de dados foi processado, porém não apresentava boa qualidade (estatísticas mostradas na Tabela 12), então resolvemos tentar melhorar os cristais para realizar novos experimentos de difração.

Foram feitos refinamentos desta condição de cristalização (CS1-22), variando o pH (entre 4,0 e 9,5), as concentrações de sal (0,05 a 0,2 M acetato de sódio) e precipitante (15 a 30% PEG 4000). Conseguimos obter cristais de *Sc*Grx2-SG em quase todas as condições testadas nos refinamentos e submetemos os melhores a experimentos de difração de raios-X. Coletamos um conjunto de dados a 1,9 Å de resolução de um cristal obtido na condição 30% PEG 4000/ 0,1 M acetato de sódio pH 5,4/ 0,2 M acetato de sódio (Figura 22B). Foram coletadas 240 imagens com $\Delta\phi$=1°, usando solução crio-protetora com 20% glicerol.

Não conseguimos obter cristais do mutante *Sc*Grx2-C30S tratado com DTT ou GSH.

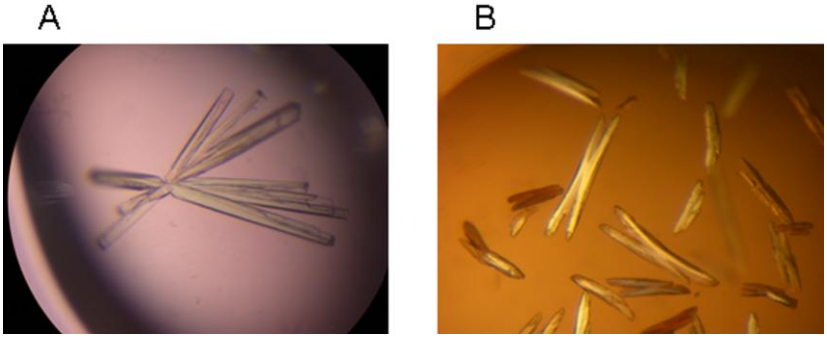

Figura 25: Cristais de *Sc*Grx2-SG. A) Cristais obtidos no *screening* inicial, na condição CS1-22. B) Cristais obtidos após o refinamento da condição CS1-22; a diferença desta condição em relação à inicial é o tampão, neste caso foi usado 0,1 M acetato de sódio pH 5,4, e não Tris pH 8,5.

Processamento dos dados e substituição molecular

Os conjuntos de dados foram processados com o auxílio dos programas MOSFLM (Leslie, 1992) e SCALA (Kabush, 1998; Blessing, 1995) do pacote CCP4. O número de moléculas na unidade assimétrica foi determinado através do cálculo do coeficiente de Matthews (Matthews, 1998). As estatísticas dos conjuntos de dados coletados a 2,9 Å e a 1,91 Å de resolução são mostradas na Tabela 12.

Tabela 12: Estatísticas dos dados de difração dos cristais de *Sc*Grx2-SG.

	*Sc*Grx2-SG (figura 25A)	*Sc*Grx2-SG (figura 25B)
Grupo espacial	$P4_12_12$	$P2_12_12_1$
Parâmetros de cela unitária (Å)	a=b= 44,2581, c= 105,4259	a= 37,0076, b= 45,2051, c= 105,0348
Resolução (Å)	2,90	1,91
λ (Å)	1,427	1,427
Número de reflexões totais	231652	247826
Número de reflexões únicas	6277	14326
Completeza (%)	100 (100)	99,7 (99,7)
Multiplicidade (%)	9,8 (9,7)	8,6 (8,2)
R_{sym}	0,096 (0,501)	0,070 (0,389)
$\langle I/\sigma(I)\rangle$	25,9 (4,6)	23,6 (4,5)

Como pode ser observado na Tabela 12, as estatísticas do primeiro conjunto de dados coletado a 2,9 Å de resolução não estão muito boas. Tentamos fazer a substituição molecular com estes dados, usando como modelo as coordenadas da estrutura de *Sc*Grx2 oxidada, porém não encontramos uma solução para o problema das fases.

O processamento do segundo conjunto de dados, coletado a 1,91 Å de resolução, mostrou que a qualidade do mesmo era superior a do primeiro conjunto coletado (Tabela 12). Foram encontradas duas moléculas na unidade assimétrica, a porcentagem de solvente no cristal era de 33%, e o coeficiente de Matthews igual a 1,83 $Å^3$/Da. Utilizando o

programa Phaser (Read, 2001) e as coordenadas da estrutura de *Sc*Grx2 oxidada (resolvida e refinada anteriormente a 2,05 Å de resolução) para a substituição molecular, encontramos uma solução para as fases de *Sc*Grx2-SG.

Refinamento da estrutura de *Sc*Grx2-SG

O refinamento do modelo da *Sc*Grx2-SG foi feito com o programa Refmac5 (Murshudov *et al.,* 1997). Utilizamos também modelos gerados por análises TLSMD *(translation /libration/ screw/ motion/ determination*; http://skuld.bmsc.washington.edu/~tlsmd) para tentar melhorar os valores de R_{factor} e R_{free} nos últimos ciclos de refinamento. TLSMD analisa os movimentos entre domínios da estrutura cristalina, fragmentando a cadeia e gerando múltiplos segmentos que são modelados como corpos rígidos sofrendo movimentação vibracional TLS *(translation/libration/screw*) (Winn *et al.*, 2001). O programa PROCHECK (Laskowski *et al.,* 1993) foi usado para analisar a estereoquímica do modelo.

O primeiro ciclo de refinamento do modelo gerou R_{factor} = 0,399 e R_{free} = 0,431, ao final do refinamento chegamos a R_{factor} = 0,196 e R_{free} = 0,262. Idealmente, o valor de R_{free} alcançado ao final do refinamento deveria ser mais baixo, em torno de 0,22-0,2. Porém não conseguimos baixá-lo além de 0,26, mesmo utilizando fragmentos gerados por análises com TLSMD ou rodando ciclos de refinamento no programa CNS (Brunger *et al.*, 1987; Brunger *et al.*, 1990), o qual simula o aquecimento das moléculas, de modo a possibilitar a saída de um mínimo local de energia para o mínimo global. As estatísticas do refinamento da estrutura de *Sc*Grx2-SG estão mostradas na Tabela 13. Logo nos primeiros ciclos de refinamento, foi possível distinguir as densidades eletrônicas das duas moléculas de glutationa ligadas, uma a cada molécula de proteína.

Tabela 13: Estatísticas do refinamento da estrutura de *Sc*Grx2-SG e análise estereoquímica.

R_{factor}	0,196
R_{free}	0,262
$Rms_{ligação}$	0,012
$Rms_{ângulo}$	1,392
B fator médio (Å^2)	
Cadeia principal	13,084
Cadeias laterais	16,943
Análises Ramachandran (%)	
Regiões favorecidas	95,8
Regiões adicionalmente permitidas	4,2
Regiões não permitidas	0

A Figura 26 mostra o diagrama de Ramachandran para o modelo da estrutura de *Sc*Grx2-SG; 95,8% dos resíduos estão em regiões mais favorecidas e 4,2% dos resíduos estão em regiões adicionalmente permitidas; não há resíduos de aminoácidos em regiões não permitidas.

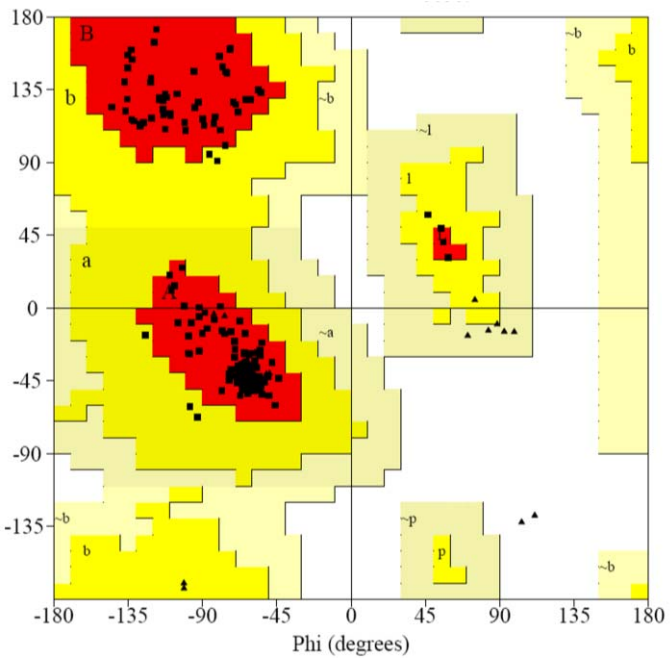

Figura 26: Diagrama de Ramachandran da estrutura de *Sc*Grx2-SG.

IV.9.3 - Análise das estruturas de *Sc*Grx2

Enovelamento e sítio ativo

Inicialmente esperávamos obter estruturas de *Sc*Grx2 em dois estados de oxidação diferentes: proteína tratada com DTT/GSH (cisteínas reduzidas) e proteína tratada com diamida/*t*-BOOH/H_2O_2 (cisteínas oxidadas, formando ligação dissulfeto). Porém, todas as estruturas de *Sc*Grx2 resolvidas, mesmo sob diferentes tratamentos, apresentavam as cisteínas do sítio ativo oxidadas, formando uma ligação dissulfeto intramolecular (Figura 28A). A sobreposição destas estruturas mostrou que todas eram idênticas, sendo observadas apenas ligeiras diferenças nas posições de algumas cadeias laterais mais móveis que se localizam na superfície da proteína, como lisinas e ácidos glutâmicos (Figura 27). Como obtivemos várias estruturas de *Sc*Grx2 na forma oxidada, resolvemos fazer nossas análises utilizando apenas a estrutura de *Sc*Grx2 obtida com o tratamento com *t*-BOOH, já que o modelo apresentava melhor qualidade. Mesmo com o mutante *Sc*Grx2-C30S, não conseguimos obter a estrutura de *Sc*Grx2 reduzida, porém obtivemos a estrutura de *Sc*Grx2 ligada à glutationa por um dissulfeto misto formado entre a Cys27 de *Sc*Grx2 e a cisteína da glutationa (Figura 28B).

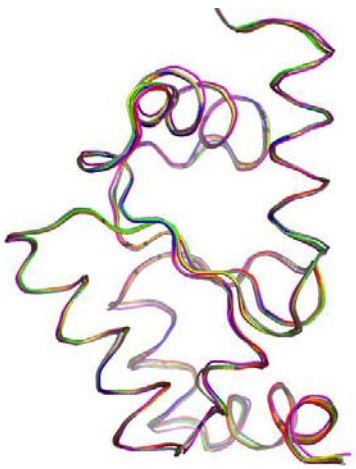

Figura 27: Alinhamentos entre as sete estruturas de *Sc*Grx2 oxidada (t-BOOH em azul, sem tratamento em vermelho, diamida em verde, DTT1 em amarelo, DTT2 em rosa, DTT3 em laranja e GSH em preto).

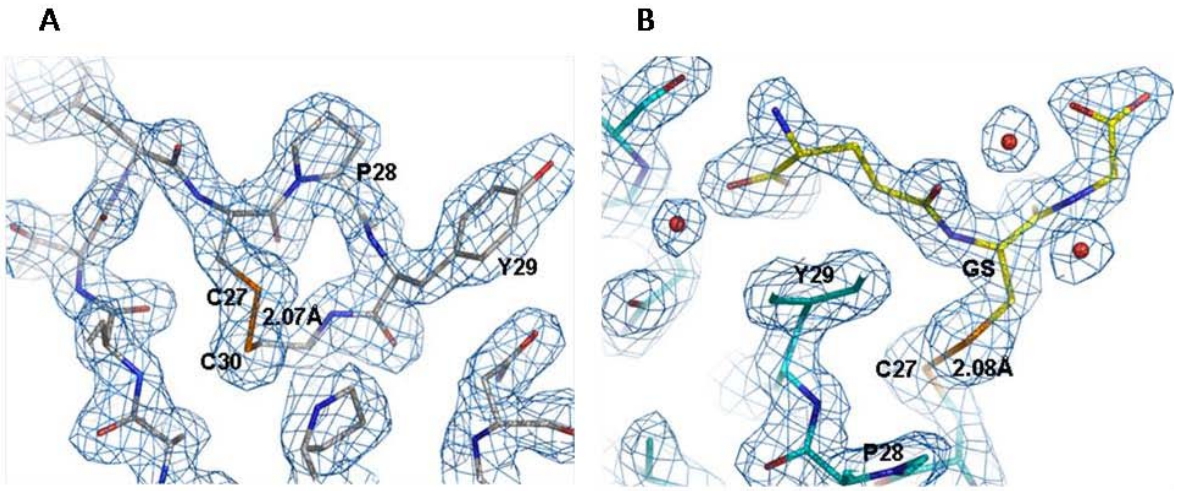

Figura 28: Mapas de densidade eletrônica (2Fo – Fc, contorno de 1.0 σ) dos sítios ativos de A) *Sc*Grx2 oxidada e B) *Sc*Grx2-SG. A continuidade da densidade eletrônica entre os átomos de enxofre da Cys27 e Cys30, e Cys27 e cisteína da glutationa mostram as ligações dissulfeto.

*Sc*Grx2 é uma proteína globular e possui o conhecido enovelamento Tiorredoxina, que consiste em uma folha-β no centro da estrutura, rodeada por α-hélices (Figura 29). A folha-β central possui quatro fitas: β1, β3 e β4 são antiparalelas entre si, enquanto a fita β2 é paralela à β1. As α-hélices α1 e α3 se localizam de um lado da folha-β, enquanto α2, α4 e α5 estão do outro lado da mesma. As hélices α4 e α5 são continuação uma da outra em seqüência, mas posicionam-se quase perpendicularmente uma em relação à outra. A Gly98 que se localiza entre as hélices α4 e α5 é a responsável pela mudança abrupta de direção (este resíduo de glicina é conservado em Grxs). Os elementos estruturais secundários estão ligados por alças e voltas. E mesmo estes segmentos de cadeia não regulares são firmemente posicionados devido a fortes interações, geralmente mediadas por átomos da cadeia principal.

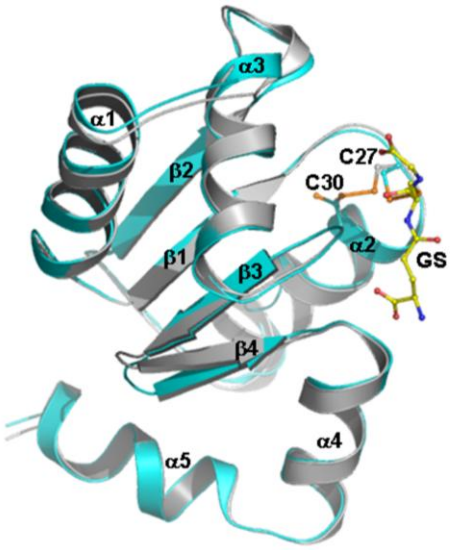

Figura 29: Enovelamento da *Sc*Grx2: folha-β com quatro fitas-β ao centro da estrutura, rodeada por α-hélices. *Sc*Grx2$_{ox}$ (cinza) e *Sc*Grx2-SG (azul claro); a molécula de glutationa é mostrada em amarelo.

A Cys27 localiza-se no final do *loop* que precede a segunda α-hélice, e a Cys30 localiza-se na primeira volta desta hélice. Deste modo, a Cys27 encontra-se mais exposta ao solvente na superfície da estrutura, enquanto que a Cys30 encontra-se mais enterrada no interior da molécula (Figura 29). A localização da Cys27 na superfície da molécula explica o fato de que nestas proteínas a cisteína N-terminal é a mais reativa, pois seu tiol fica acessível à glutationa e outros substratos. As posições das cisteínas são características bastante conservadas em oxidorredutases tiólicas.

Na vizinhança do sítio ativo de *Sc*Grx2, existem dois resíduos carregados positivamente, Lys24 e Lys31, os quais podem interagir e estabilizar o tiolato da Cys27, favorecendo a redução do pK_a do mesmo. Além disso, o sítio ativo de *Sc*Grx2 se localiza na porção N-terminal da segunda α-hélice, de modo que a interação do tiolato com o pólo positivo da região N-terminal da hélice pode também ser um fator relacionado ao baixo pK_a de *Sc*Grx2.

Estrutura de *Sc*Grx2-C30S ligada à glutationa

O cristal de *Sc*Grx2-SG apresentava duas moléculas na unidade assimétrica. As estruturas dos dois monômeros são ligeiramente diferentes devido ao diferente empacotamento das moléculas no cristal. O alinhamento de 106 resíduos dos monômeros A e B resulta em um rmsd de 0,5 Å. Além disso, a cadeia do monômero A está completa com

os 109 resíduos de aminoácidos, enquanto a cadeia do monômero B se inicia no segundo resíduo e apresenta densidade eletrônica um pouco fraca para os últimos dois resíduos. Há uma molécula de glutationa ligada a cada um dos monômeros.

A molécula de glutationa ligada ao monômero A está mais exposta ao solvente, enquanto a glutationa ligada ao monômero B está próxima a uma das faces do monômero A (Figura 30). Além disso, as moléculas de glutationa ligadas apresentam duas conformações diferentes: a glicina da glutationa (Gly_{GS}) tem diferentes orientações em cada um dos monômeros (Figura 31).

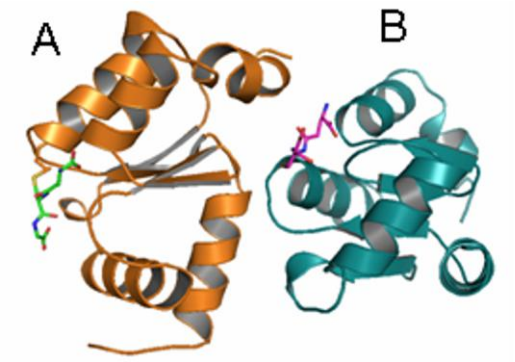

Figura 30: Arranjo das duas moléculas na unidade assimétrica do cristal de *Sc*Grx2-SG. O monômero A está representado em laranja, e a molécula de GSH a ele ligada, em verde. O monômero B está representado em azul claro, e a molécula de GSH a ele ligada, em rosa.

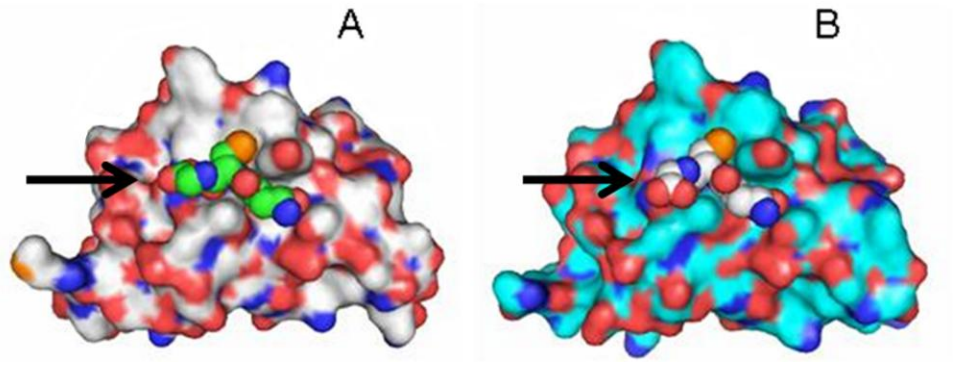

Figura 31: Superfície da estrutura de *Sc*Grx2-SG. A) Superfície do monômero A (átomos de C em branco) e sua molécula de GSH (átomos de C em verde). B) Superfície do monômero B (átomos de C em azul claro) e sua molécula de GSH (átomos de C em branco). As setas indicam as diferentes configurações adotas pela Gly_{GS} em cada um dos monômeros.

As interações entre os resíduos das moléculas de glutationa e os resíduos dos monômeros A e B são as mesmas, com exceção da Gly_{GS} ligada ao monômero B, que não estabelece interações com nenhum resíduo da cadeia polipeptídica. A Figura 32 mostra as interações observadas entre a molécula de glutationa e o monômero A da estrutura de *Sc*Grx2-SG. No monômero A, o grupo α-carboxilato do resíduo C-terminal da glutationa (Gly_{GS}) estabelece interação elestrostática com o grupo amino da cadeia lateral da Lys24 e faz uma ligação de hidrogênio com o grupo amino da cadeia lateral da Gln63. O grupo amino do γ-glutamato da glutationa (γ-Glu_{GS}) estabelece uma ligação de hidrogênio com a hidroxila da Ser89, enquanto seu grupo carboxilato estabelece ligações de hidrogênio com os grupos amino da cadeia principal da Ser89 e da Asn88. Em ambos monômeros, a Cys da glutationa (Cys_{GS}) está covalentemente ligada a Cys27, através de uma ligação dissulfeto, e seus grupos amino e carbonila estabelecem ligações de hidrogênio com os grupos carbonila e amino da Val75, respectivamente. O resíduo de Val75 precede a Pro76 com configuração *cis*, a qual deixa a carbonila da Val75 exposta na superfície da proteína.

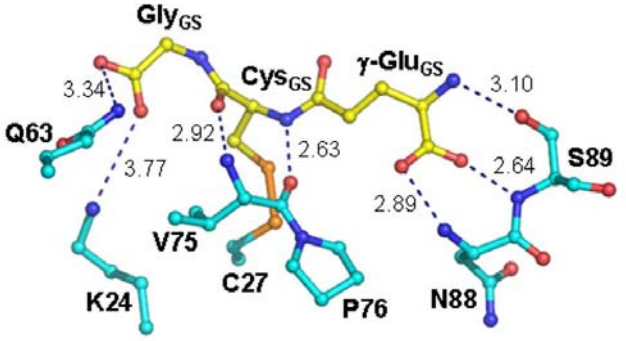

Figura 32: Resíduos de *Sc*Grx2 (azul claro) envolvidos em interações com a molécula de glutationa (amarelo) na estrutura de *Sc*Grx2-SG. As ligações de hidrogênio e as pontes salinas estão indicadas por linhas pontilhadas azuis, com as distâncias entre os átomos em angstrom.

Além das ligações de hidrogênio e das pontes salinas, interações de van der Waals também são importantes para a ligação da molécula de glutationa na superfície da glutarredoxina. Os resíduos de *Sc*Grx2 envolvidos em interações de van der Waals com glutationa são: Pro28, Tyr29, Ser30, Thr74, Val75 e Pro76 (Figura 33).

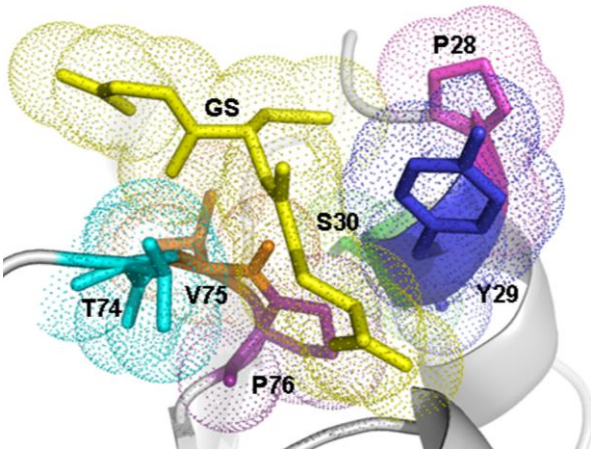

Figura 33: Representação dos resíduos Pro28 (rosa), Tyr29 (azul escuro), Ser30 (verde), Thr74 (azul claro), Val75 (laranja) e Pro76 (roxo) de *Sc*Grx2 que estabelecem interações de van der Waals com a molécula de glutationa (amarelo) na estrutura de *Sc*Grx2-SG.

Comparação entre *Sc*Grx2$_{ox}$ e *Sc*Grx2-SG

A sobreposição das estruturas de *Sc*Grx2$_{ox}$ e *Sc*Grx2-SG foi feita com o programa Coot (Emsley & Cowtan, 2004). As estruturas de *Sc*Grx2$_{ox}$ e *Sc*Grx2-SG são bastante similares (Figura 29); o alinhamento de *Sc*Grx2$_{ox}$ com o monômero A de *Sc*Grx2-SG resulta em um rmsd de 0.59 Å para todos os 109 Cα, enquanto o alinhamento de *Sc*Grx2$_{ox}$ com o monômero B resulta em um rmsd de 0.54 Å para 106 Cα. Apesar da grande semelhança estrutural, algumas mudanças conformacionais podem ser observadas entre *Sc*Grx2$_{ox}$ e *Sc*Grx2-SG, principalmente no loop que contém o sítio ativo, situado entre a fita-β1 e a hélice-α2 (Figura 34). Quando apenas os resíduos do loop do sítio ativo destas estruturas são alinhados, o rmsd aumenta para 1.69Å. Devido às interações entre a molécula de glutationa e o resíduo de Val75, o loop do sítio ativo na estrutura de *Sc*Grx2-SG está mais próximo ao loop que conecta α3 e β3 (onde Val75 está localizada), em comparação a sua posição na estrutura de *Sc*Grx2$_{ox}$.

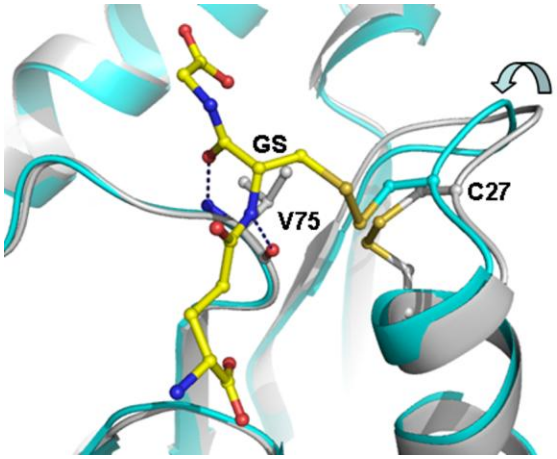

Figura 34: Mudança conformacional do loop do sítio ativo entre *Sc*Grx2$_{ox}$ (cinza) e *Sc*Grx2-SG (azul claro); a molécula de glutationa está representada em amarelo.

Além disso, podem ser observadas diferenças conformacionais entre os resíduos de cisteínas do sítio ativo de *Sc*Grx2$_{ox}$ e *Sc*Grx2-SG. A cadeia lateral da Cys27 está mais exposta ao solvente na estrutura glutatiolada do que na oxidada, e a cadeia lateral da Ser30, que substitui a Cys30 no mutante *Sc*Grx2-C30S, possui uma conformação desfavorável ao ataque sobre o dissulfeto misto entre a Cys27 e a glutationa na estrutura de *Sc*Grx2-SG. A hidroxila da Ser30 forma, em *Sc*Grx2-SG, uma rede de ligações de hidrogênio com o grupo carboxilato do Glu52, através de duas moléculas de água (Figura 35a). Esta rede de ligações de hidrogênio parece estar relacionada com a configuração mais enterrada da Ser30 em *Sc*Grx2-SG. Na estrutura de *Sc*Grx2$_{ox}$, o carboxilato do Glu52 forma uma ligação de hidrogênio com uma molécula de água, a qual forma uma ligação de hidrogênio com a carbonila da Lys24 (Figura 35a). Em *Sc*Grx2-SG, a carbonila da Lys24 faz uma ligação de hidrogênio com o grupo amino da Cys27 (Figura 35a). Esta última interação provavelmente é responsável por posicionar corretamente o resíduo de Lys24 e permitir, assim, a interação entre sua cadeia lateral e a molécula de glutationa (Figura 32). Na estrutura de *Sc*Grx2$_{ox}$ a cadeia lateral da Lys24 estabelece ligação de hidrogênio com a carbonila da cadeia lateral da Gln63.

Outro resíduo que muda sua conformação da estrutura de *Sc*Grx2$_{ox}$ para *Sc*Grx2-SG é a Tyr29. Na estrutura de *Sc*Grx2-SG, a Tyr29 está mais próxima da molécula de glutationa e a estabiliza por contatos de van der Waals (Figura 35b).

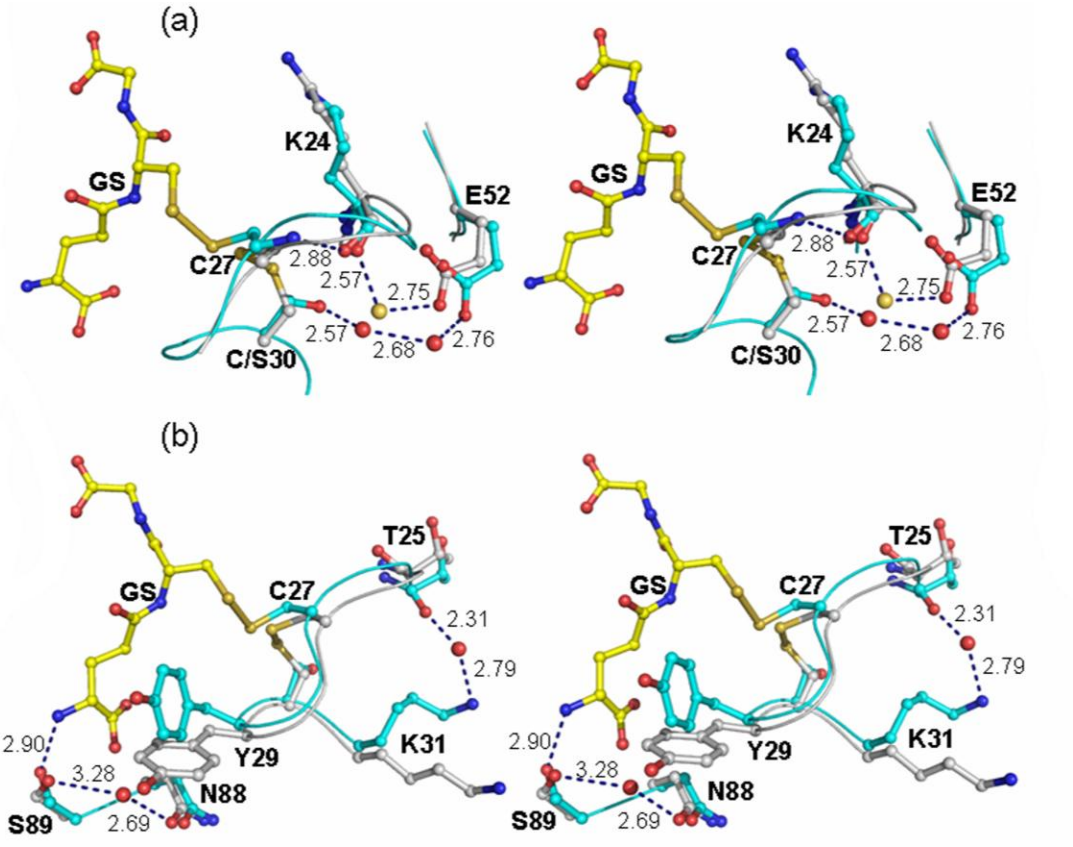

Figura 35: Visão estereoscópica representando as alterações conformacionais locais entre *Sc*Grx2$_{ox}$ (cinza) e *Sc*Grx2-SG (azul claro). A molécula de glutationa está representada em amarelo e as ligações de hidrogênio são indicadas por linhas azuis pontilhadas, com as distâncias atômicas em angstrom. As moléculas de água da estrutura de *Sc*Grx2$_{ox}$ são mostradas em amarelo, e as moléculas de água da estrutura de *Sc*Grx2-SG são mostradas em vermelho. (a) Alterações conformacionais entre os resíduos de Cys27, Cys/Ser30, Lys24, e a rede de ligações de hidrogênio entre Ser30 e Glu52 mediadas por moléculas de água. (b) Alterações conformacionais dos resíduos Thr25, Lys31, Tyr29 e Ser89.

Adicionalmente, a Ser89 também sofre alterações conformacionais entre as duas estruturas de *Sc*Grx2. Na estrutura de *Sc*Grx2-SG, a hidroxila da Ser89 estabelece uma ligação de hidrogênio com o grupo α-amino do γ-Glu$_{GS}$ (Figura 35b), enquanto na estrutura

oxidada, a hidroxila da Ser89 estabelece uma ligação de hidrogênio, mediada por uma molécula de água, com a carbonila da cadeia lateral da Asn88.

A carbonila da Thr25 sofre um "flip" quando comparadas suas configurações nas estruturas de *Sc*Grx2$_{ox}$ e *Sc*Grx2-SG, passando a estabelecer interação com a cadeia lateral da Lys31, mediada por uma molécula de água, em *Sc*Grx2-SG (Figura 35b). Na estrutura de *Sc*Grx2$_{ox}$, a cadeia lateral da Lys31 está mais distante da Thr25, de modo que não são observadas interações entre estes resíduos. Analisando outros rotâmeros possíveis para a Lys31 em *Sc*Grx2-SG, observamos que seria possível a interação direta da cadeia lateral da Lys31, em uma conformação diferente, com a carbonila da Thr25, por uma ligação de hidrogênio. Estas mudanças conformacionais entre as estruturas glutatiolada e oxidada, observadas com Thr25 e Lys31, parecem ser importantes para orientar apropriadamente o loop do sítio ativo e os resíduos envolvidos na ligação da molécula de glutationa durante o ciclo catalítico. Alterações similares com os resíduos que precedem a cisteína N-terminal do sítio ativo já foram observadas em outras estruturas de Grxs, quando comparados diferentes estados de oxidação (Bacik & Hazes, 2007). Isto sugere que estas mudanças conformacionais podem ser uma estratégia geral adotada por Grxs durante a catálise.

IV.9.4 - Comparação das estruturas de *Sc*Grx2 com outras Grxs

Outras estruturas de Grxs foram determinadas e depositadas no PDB em diferentes estados de oxidação. Para verificar quais Grxs possuíam maior semelhança estrutural com *Sc*Grx2, submetemos as coordenadas das estruturas de *Sc*Grx2 ao Dali Server (http://ekhidna.biocenter.helsinki.fi/dali_server/) para compará-las a estruturas disponíveis no PDB. Também realizamos o alinhamento da seqüência de aminoácidos de *Sc*Grx2 com as seqüências de proteínas cujas estruturas 3D já foram depositadas no PDB, utilizando a ferramenta BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi/). A Tabela 14 mostra as Grxs que apresentavam maior semelhança estrutural com *Sc*Grx2.

Tabela 14: Relação de estruturas de Grxs determinadas em diferentes estados de oxidação e que apresentam similaridade estrutural com *Sc*Grx2. As estruturas de Grxs na forma glutatiolada foram alinhadas com *Sc*Grx2-SG, e as estruturas nas formas reduzida e oxidada foram alinhadas com *Sc*Grx2$_{ox.}$ O valor de rmsd obtido está angstrom, e, entre parênteses, está o número de resíduos de *Sc*Grx2 alinhados (*Sc*Grx2 possui 109 resíduos). Para as estruturas que possuíam mais de um monômero, foi feito o alinhamento utilizando o monômero A.

Grx (identidade de seqüência com *Sc*Grx2)	Estado oxidação	Código PDB	rmsd (Å) (número resíduos alinhados)	Referência
*Sc*Grx1 (64% identidade)	reduzida	2JAD	0,7 (108)	Håkansson & Winther, 2007.
	glutatiolada	2JAC	0,7 (108)	
	oxidada	3C1R	0,7 (109)	Yu *et al.*, 2008.
	glutatiolada	3C1S	0,7 (106)	
***Sc*Grx2**	oxidada	3D4M	0 (109)	Discola *et al.*, 2009.
	glutatiolada	3D5J	0,6 (A, 109); 0,5 (B, 106) *	
*Hs*Grx1 (35% identidade)	reduzida	1JHB	1,5 (91)	Sun *et al.*, 1998.
	glutatiolada	1B4Q	1,7 (99)	Yang *et al.*, 1998.
*Hs*Grx2 (35% identidade)	glutatiolada	2FLS	1,2 (97)	Johansson *et al.*, 2007.
Grx *S.scrofa* (porco) (36% identidade)	oxidada	1KTE	1,4 (91)	Katti *et al.*, 1995.
*Ec*Grx1 (25% identidade)	oxidada	1EGO	1,8 (67)	Xia *et al.*, 1992.
	reduzida	1EGR	2,0 (72)	Sodano *et al.*, 1991.
	glutatiolada	1GRX	2,1 (64)	Bushweller *et al.*, 1994.
*Ec*Grx3 (34% identidade)	oxidada	1FOV	1,7 (83)	Nordstrand *et al.*, 2000.
	glutatiolada	3GRX	1,7 (79)	Nordstrand *et al.*, 1999.
Grx poxviral (27% identidade)	oxidada	2HZE	1,8 (94)	Bacik & Hazes, 2007.
	reduzida	2HZF	2,0 (93)	
Grx C1 *Populus Tremula X Tremuloides* (46% identidade)	holo	2E7P	1,3 (104)	Rouhier *et al.*, 2007.
	Reduzida	1Z7P	1,3 (103)	Feng *et al.*, 2006.

*Alinhamento entre a estruturas de *Sc*Grx2$_{ox}$ (3D4M) e os monômeros A e B de *Sc*Grx2-SG (3D5J).

A sobreposição das estruturas de *Sc*Grx2 com as estruturas das Grxs da Tabela 14 mostrou que a *Sc*Grx2 é mais similar estruturalmente a *Sc*Grx1 e às Grxs de mamífero, planta e poxviral do que às Grxs de *E. coli* (Figura 36). Podemos observar na Figura 36 que todas as Grxs analisadas, com exceção de *Ec*Grx1 e *Ec*Grx3, possuem os mesmos elementos estruturais secundários, apesar de haver diferenças no tamanho das α-hélices e fitas-β. *Ec*Grx1 e *Ec*Grx3 não possuem a primeira α-hélice presente nas demais Grxs, e *Ec*Grx1 não possui também a última α-hélice. Ainda não foi descrito o papel destas α-hélices em Grxs.

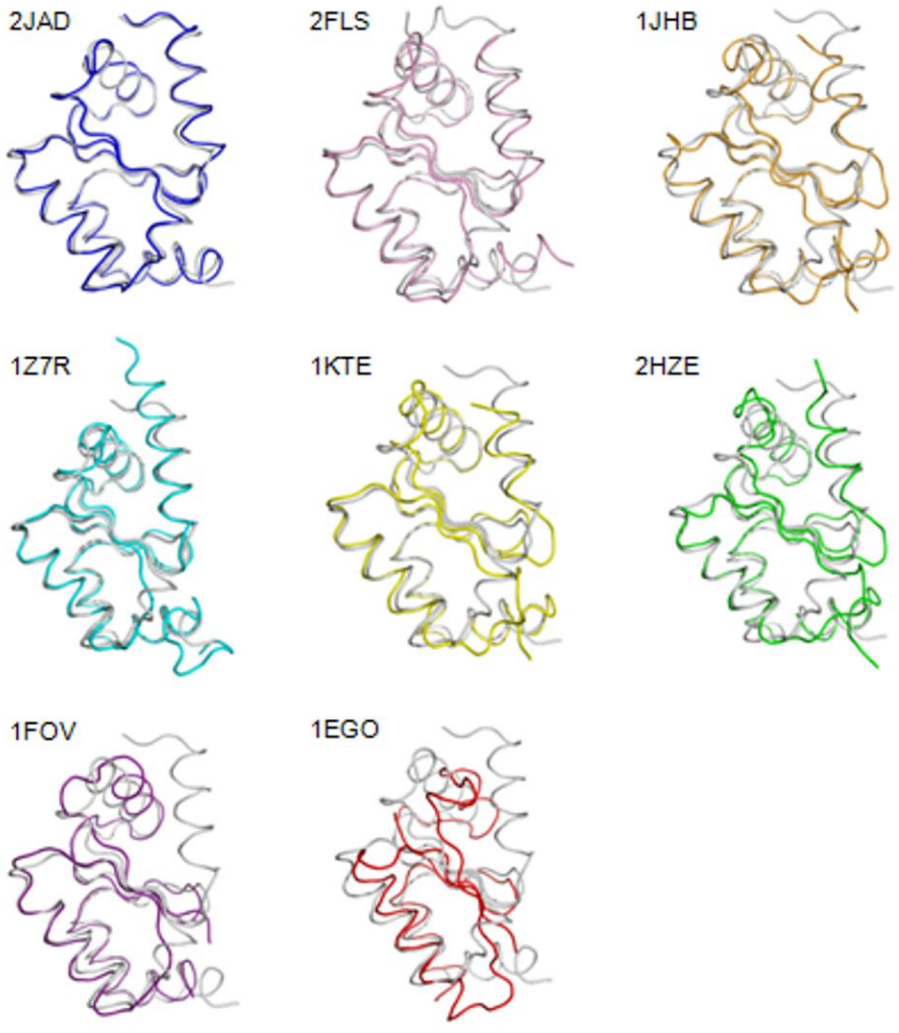

Figura 36: Alinhamentos das estruturas de *Sc*Grx2 (cinza) e as estruturas de outras Grxs. As formas reduzida e oxidada das Grxs foram alinhadas à *Sc*Grx2$_{ox}$, enquanto as formas glutatioladas foram alinhadas à *Sc*Grx2-SG.

Como pode ser observado na Tabela 14, outras Grxs tiveram suas estruturas determinadas na forma glutatiolada. A comparação destas estruturas mostra que as interações entre Grx e glutationa são conservadas (Figura 37). Entretanto, o sítio de ligação à glutationa de *Sc*Grx2 é mais similar ao de Grxs de mamíferos do que de Grxs de bactéria (Figura 37b), assim como o enovelamento de *Sc*Grx2 é mais similar ao de Grxs de mamíferos (Figura 36).

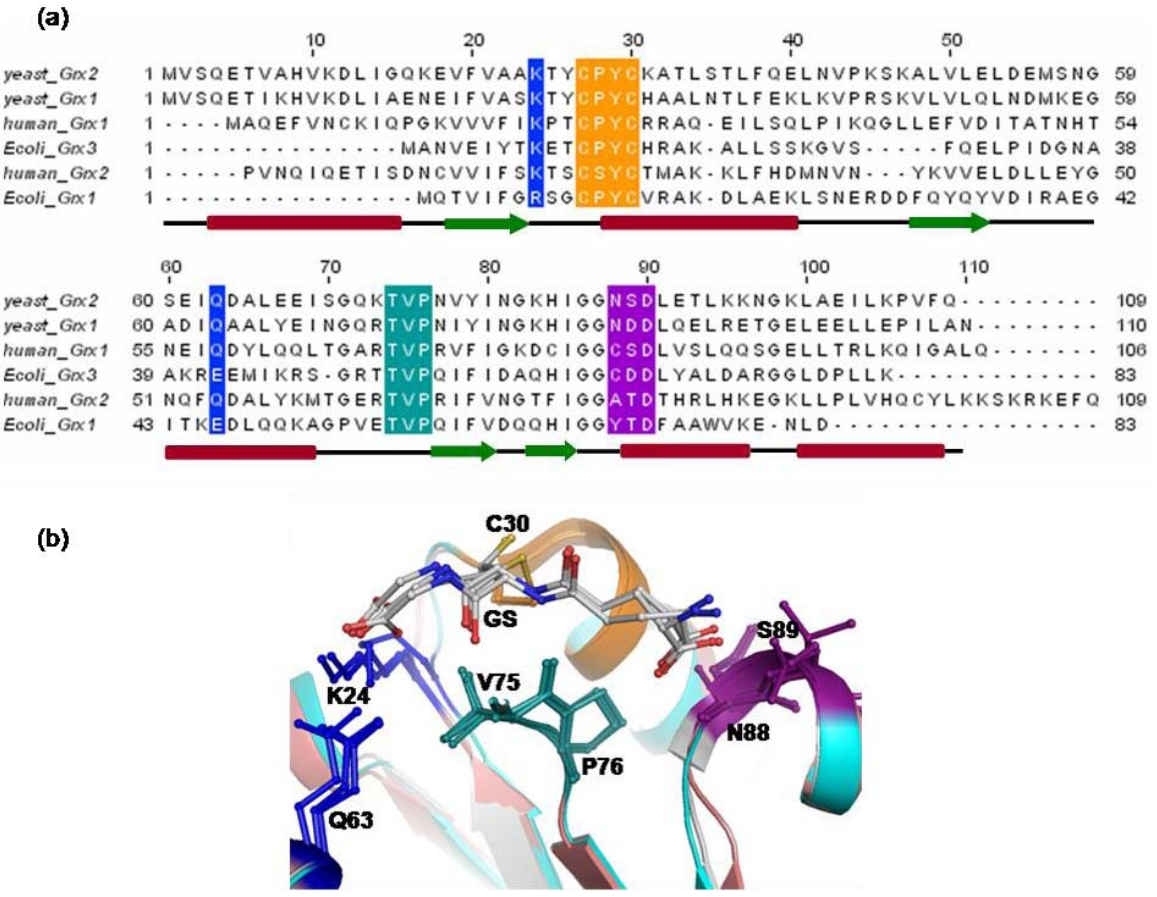

Figura 37: Alinhamento das Grxs que tiveram suas estruturas glutatioladas determinadas. (a) O alinhamento das seqüências foi feito com o programa ClustalW (Chenna *et al.,* 2003), e a representação, com o programa Jalview (Clamp *et al.,* 2004). Os resíduos do sítio ativo estão em laranja, e os resíduos em azul estão envolvidos em interações com Gly_{GS}. Em verde estão os resíduos do motivo TVP, que interagem com o resíduo de Cys_{GS}, e em roxo, os resíduos que interagem com γ-Glu_{GS}. Os elementos estruturais secundários de *Sc*Grx2 são mostrados na parte de baixo do alinhamento (setas indicam fitas-β, e os cilindros, α-hélices). (b) Alinhamento das estruturas glutatioladas de *Sc*Grx2 (azul claro), *Sc*Grx1 (cinza) e *Hs*Grx2 (rosa). Os resíduos envolvidos em interações com a molécula de glutationa (cinza) estão identificados com o número da seqüência de *Sc*Grx2 e com as mesmas cores usadas no alinhamento de seqüência.

Em todas as Grxs que tiveram suas estruturas glutatioladas determinadas, a cadeia principal do resíduo de Cys_{GS} estabelece pontes de hidrogênio com a cadeia principal de um resíduo de Val conservado no motivo TVP (Figura 37), como mostrado para a Val75 de *Sc*Grx2 (Figura 32). E em todas elas, a Pro com configuração *cis* deixa a carbonila do resíduo de Val exposta na superfície.

A Ser89 de *Sc*Grx2, responsável pelas ligações de hidrogênio com o grupo carboxilato de γ-Glu_{GS}, é conservada na *Hs*Grx1 (Ser84), mas é substituída por Asp89, Thr80, Thr73 e Asp66 em *Sc*Grx1, *Hs*Grx2 e *Ec*Grx1 e *Ec*Grx3, respectivamente. As cadeias laterais destes resíduos de Asp e Thr, que substituem a Ser89 de *Sc*Grx2, estabelecem as mesmas interações com o carboxilato do γ-$Glu_{GS,}$ uma vez que possuem um grupo OH (doador de hidrogênio).

Em relação aos resíduos que interagem com o grupo α-carboxilato da $Gly_{GS,}$ um resíduo com carga positiva (na maioria Lys) é conservado na posição que corresponde à Lys24 de *Sc*Grx2. A Gln63 é conservada nas Grxs de humano e levedura, sendo substituída por Glu em *Ec*Grx1 e *Ec*Grx3. Em todas as Grxs analisadas, o resíduo de carga positiva (Lys24 em *Sc*Grx2) interage com grupo α-carboxilato da Gly_{GS}, porém só são observadas interações de Gly_{GS} com a Gln nas Grxs de levedura e *Hs*Grx2. Em *Ec*Grx1 e *Ec*Grx3, esta segunda interação com a Gly_{GS} é feita com os resíduos de Lys45 e Arg41, respectivamente, os quais ocupam a posição anterior ao resíduo equivalente a Gln63 em *Sc*Grx2 (Figura 37a). Em *Hs*Grx1, a segunda interação com a Gly_{GS} é feita com o resíduo de Arg73, que ocupa a posição anterior à Thr do motivo TVP. Podemos observar que nas Grxs de levedura e humano um resíduo de carga positiva é conservado nesta posição, e possivelmente pode estar envolvido em interações com a glutationa durante o ciclo catalítico destas Grxs. Nas estruturas de *Sc*Grx2, não há densidade eletrônica para a cadeia lateral da Lys73, anterior ao motivo TVP, pois este resíduo está bastante exposto ao solvente e é, provavelmente, muito móvel.

IV.10 - Comparações estruturais entre *Sc*Grx1 e *Sc*Grx2

Recentemente, foram determinadas as estruturas cristalográficas do mutante *Sc*Grx1-C30S nas formas reduzida e glutatiolada (códigos PDB: 2JAD e 2JAC, respectivamente; Håkansson & Winther, 2007). Realizamos, então, a comparação das estruturas de *Sc*Grx1 e *Sc*Grx2 com o intuito de identificar possíveis características estruturais destas enzimas que pudessem estar relacionadas à grande diferença de atividade observada.

*Sc*Grx1 e *Sc*Grx2 possuem enovelamentos muito similares, o que pode ser evidenciado pelos valores de rmsd resultantes dos alinhamentos de suas estruturas, mostrados na Tabela 14. Porém, há uma diferença clara entre as conformações adotadas pela Ser30 nas estruturas de *Sc*Grx1-SG e *Sc*Grx2-SG. Na estrutura de *Sc*Grx1-SG, a cadeia lateral da Ser30 está voltada em direção a Cys27, enquanto na estrutura de *Sc*Grx2-SG, a cadeia lateral da Ser30 está voltada para o lado oposto (Figura 38). De fato, a distância entre o oxigênio do grupo hidroxila da Ser30 e o enxofre da Cys27 é de 3,47 Å em *Sc*Grx1-SG e de 5,14 Å em *Sc*Grx2-SG. Assumindo que um resíduo de cisteína na posição 30 teria configuração similar à serina nos mutantes C30S em ambas estruturas, a Cys30 em *Sc*Grx1-SG seria capaz de atacar a Cys27 e formar um dissulfeto intramolecular, enquanto a Cys30 em *Sc*Grx2-SG estaria em uma conformação desfavorável ao ataque sobre o dissulfeto misto entre a Cys27 e glutationa. Se a formação do dissulfeto intramolecular é desfavorável em *Sc*Grx2, a redução do dissulfeto misto *Sc*Grx2-SG por um nucleófilo externo, como GSH, é, então, favorecida (ver Figura 3). Conseqüentemente, o mecanismo monotiólico, pelo qual a reação se processa no ensaio com HED, é favorecido.

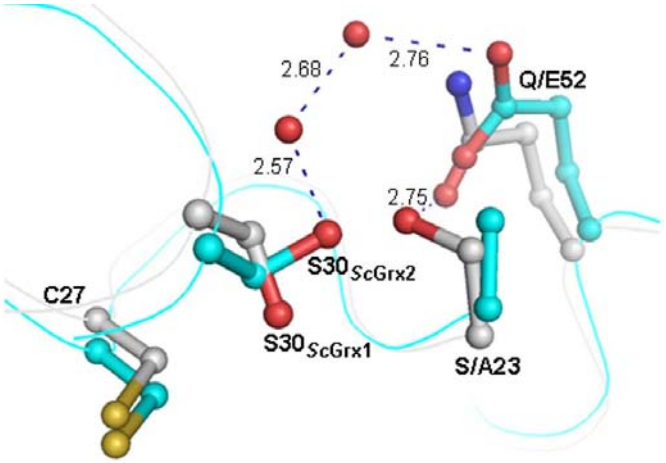

Figura 38: Conformações das cadeias laterais de Ser30 nas estruturas dos mutantes C30S de *Sc*Grx1 (branco) e *Sc*Grx2 (azul claro), ligados à glutationa.

Corroborando a nossa hipótese, na estrutura de *Ec*Grx3 na forma glutatiolada (código PDB: 3GRX; Nordstrand *et al*., 1999), a Ser14 (que substitui a cisteína C-terminal do sítio ativo no mutante *Ec*Grx3-C14S) apresenta uma conformação mais enterrada, similar a apresentada pela Ser30 de *Sc*Grx2. Em todas as demais estruturas de Grxs descritas na forma glutatiolada (todas obtidas com mutantes, nos quais a cisteína C-terminal do sítio ativo foi substituída por uma serina), incluindo *Ec*Grx1-SG (Bushweller *et al.*, 1994), a conformação desta serina é similar àquela encontrada na estrutura de *Sc*Grx1-SG. A conformação mais enterrada da Ser30 na estrutura de *Sc*Grx2-SG parece estar relacionada à interação com o grupo carboxilato do resíduo Glu52, mediada por duas moléculas de água. (Figura 38). A distância entre um dos átomos de O do grupo carboxilato do Glu52 e o grupo hidroxila da Ser30 é 5.02 Å na estrutura de *Sc*Grx2-SG. *Ec*Grx3 possui o resíduo Glu30, cujo grupo carboxilato tem um de seus átomos de O distante 5.74 Å da hidroxila da Ser14 na estrutura glutatiolada. O resíduo Glu30 de *Ec*Grx3 não ocupa a mesma posição que o Glu52 ocupa em *Sc*Grx2-SG, embora as distâncias entre seus grupos carboxilatos e as hidroxilas das Ser14 e Ser30, respectivamente, sejam próximas em ambas estruturas. Além disso, em ambas as estruturas, a Ser está voltada para o lado oposto em relação ao dissulfeto misto formado entre a cisteína N-terminal e glutationa, ao contrário do observado nas outras estruturas de Grxs glutatioladas. E, coincidentemente, *Ec*Grx3 possui atividade (no ensaio com HED) monotiólica maior que *Ec*Grx1, embora a diferença de

atividade entre *Ec*Grx1 e *Ec*Grx3 seja de apenas duas vezes, e não quinze como observado entre *Sc*Grx1 e *Sc*Grx2.

Outras Grxs, como a Grx de porco (Katti *et al.,* 1995) e *Hs*Grx1 (Yang *et al.,* 1998), possuem um resíduo de Asp na posição equivalente ao Glu52 de *Sc*Grx2. Entretanto, nestas Grxs há um resíduo de Ile, que pode bloquear o contato (direto ou indireto) entre o Asp e a Cys C-terminal do sítio ativo. Em *Sc*Grx2, na posição equivalente ocupada por esse resíduo de Ile das Grxs de mamíferos, existe o resíduo de Ala23 que, devido a sua cadeia lateral curta, não impede a interação entre a Ser30 e o Glu52 (Figura 38). *Ec*Grx3 também não possui um resíduo volumoso que poderia impedir interações entre Ser14 e Glu30. *Sc*Grx1 possui um resíduo de Gln52 substituindo o Glu52 de *Sc*Grx2, entretanto a Gln52 estabelece ligações de hidrogênio com a hidroxila da Ser23 (em *Sc*Grx2: Ala23) e não interage com a Ser30.

Em resumo, nossas análises sugerem que a conformação desfavorável da Cys30 de *Sc*Grx2 ao ataque sobre o dissulfeto misto Cys27-SG pode estar relacionada a interações estabelecidas com o resíduo de Glu52, as quais são possíveis devido a não interferência da cadeia lateral da Ala23. Em contraste, a conformação favorável da Cys30 de *Sc*Grx1 ao ataque sobre o dissulfeto misto Cys27-SG, possivelmente, se deve ao fato de que aquelas interações observadas em *Sc*Grx2 não são possíveis em *Sc*Grx1, devido à substituição da Ala23 de *Sc*Grx2 pela Ser23 em *Sc*Grx1, que forma uma ligação de hidrogênio com a Gln52, impedindo a interação entre Cys30 e Gln52.

Finalmente, uma diferença no sítio de ligação do γ-Glu_{GS} poderia também contribuir para a diferença de atividade entre *Sc*Grx1 e *Sc*Grx2. Em *Sc*Grx2-SG, o grupo hidroxila da Ser89 forma uma ligação de hidrogênio com o grupo α-amino do γ-Glu_{GS} (Figura 32), enquanto em *Sc*Grx1-SG, o carboxilato do Asp89 forma uma ligação de hidrogênio com o grupo α-amino do γ-Glu_{GS}. Então *Sc*Grx1 poderia apresentar uma atividade menor que *Sc*Grx2, devido a repulsão entre as cargas negativas do carboxilato do γ-Glu_{GS} e do carboxilato do Asp89, durante a aproximação do substrato β-ME-SG. De fato, foi descrito para a Grx do bacterifago T4, que a substituição de um Asp por uma Ser nessa posição leva a um aumento da atividade como oxidorredutase da enzima (Nikkola *et al.,* 1991).

IV.11 - Mutantes de *Sc*Grx1 e *Sc*Grx2

Com o intuito de testar nossas hipóteses, construímos os seguintes mutantes: *Sc*Grx1-S23A, *Sc*Grx1-Q52E, *Sc*Grx1-S23A-Q52E, *Sc*Grx1-C30S, *Sc*Grx1-D89S, *Sc*Grx2-A23S, *Sc*Grx2-E52Q, *Sc*Grx2-A23S-E52Q e *Sc*Grx2-S89D, utilizando o kit de mutagênese da Stratagene, conforme descrito em "Materiais e Métodos". Conseguimos expressar e purificar todos os mutantes (Figura 39) seguindo os mesmos protocolos já estabelecidos para *Sc*Grx1 e *Sc*Grx2 selvagens.

É possível observar na Figura 39 que os mutantes *Sc*Grx2-S89D e *Sc*Grx2-A23S-E52Q apresentam duas bandas, uma com o tamanho esperado (aproximadamente 15 kDa), e outra menor. Esta banda de tamanho menor corresponde à proteína sem a cauda de histidina N-terminal. O aparecimento desta segunda banda já foi observado anteriormente com *Sc*Grx1 e *Sc*Grx2; aparentemente, alguns dias após a purificação estas proteínas começam a perder suas caudas de histidina. Observamos que *Sc*Grx1 e *Sc*Grx2 não se ligam na coluna de Níquel quando são passadas novamente alguns dias depois de já terem sido purificadas. Como também já testamos as atividades destas proteínas com e sem a cauda de histidina e verificamos que, em ambos os casos, a atividade é semelhante, este comportamento não parece comprometer a utilização das enzimas. De qualquer forma, sempre testamos as atividades das proteínas logo após sua purificação.

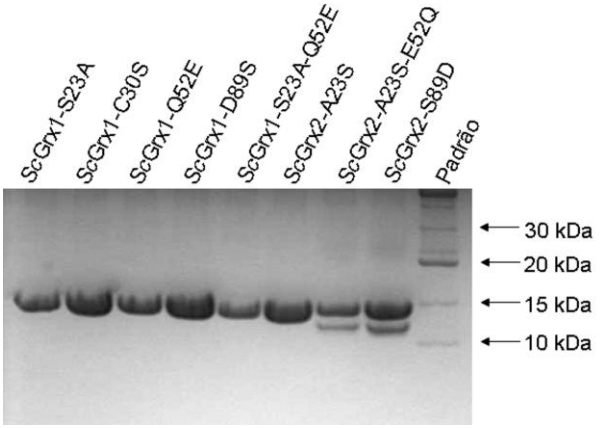

Figura 39: Gel SDS-Page mostrando os mutantes de *Sc*Grx1 e *Sc*Grx2.

O ensaio de redução do dissulfeto misto β-ME-SG foi utilizado para a determinação das atividades específicas dos mutantes de *Sc*Grx1 e *Sc*Grx2 (Figuras 40 e 41), assim como descrito para as proteínas selvagens. Os resultados são mostrados na Tabela 15.

Tabela 15: Atividades específicas de *Sc*Grx1, *Sc*Grx2 e seus mutantes no ensaio de redução do dissulfeto misto β-ME-SG.

Atividade específica ($\mu mol.min^{-1}.mg^{-1}$)			
*Sc*Grx1	8,2 ± 0,3	*Sc*Grx2	125 ± 7
*Sc*Grx1-S23A	28,2 ± 0,3	*Sc*Grx2-A23S	67 ± 1
*Sc*Grx1-C30S	39 ± 1	*Sc*Grx2-C30S	38 ± 2
*Sc*Grx1-Q52E	12,2 ± 0,2	*Sc*Grx2-E52Q	132 ± 9
*Sc*Grx1-S23A-Q52E	24,5 ± 0,4	*Sc*Grx2-A23S-E52Q	54 ± 2
*Sc*Grx1-D89S	16,6 ± 0,4	*Sc*Grx2-S89D	115 ± 5

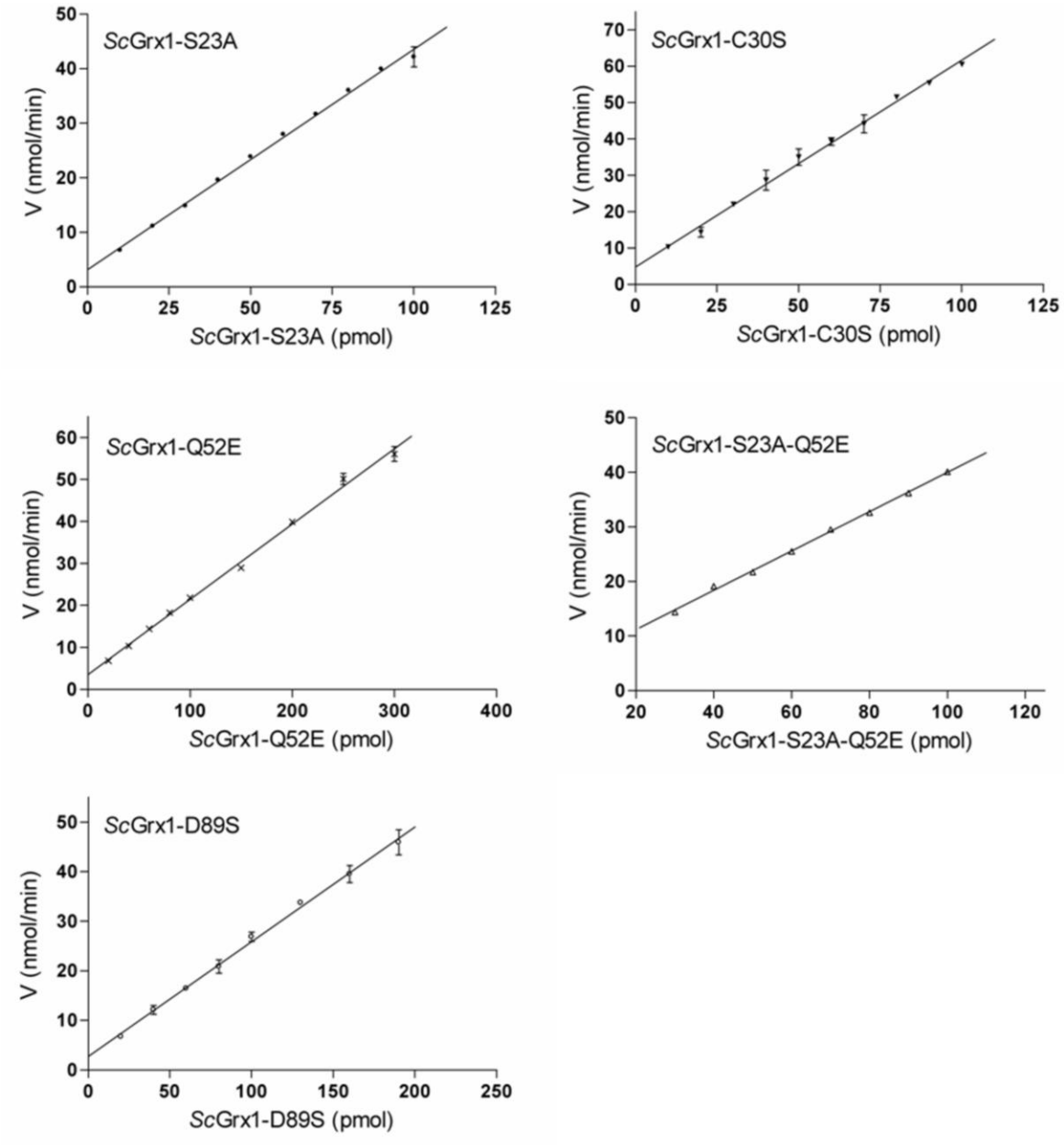

Figura 40: Gráficos de velocidade de consumo de NADPH (nmol/min) versus quantidade de Grx (pmol) adicionada, usados para a determinação das atividades específicas dos mutantes de *Sc*Grx1.

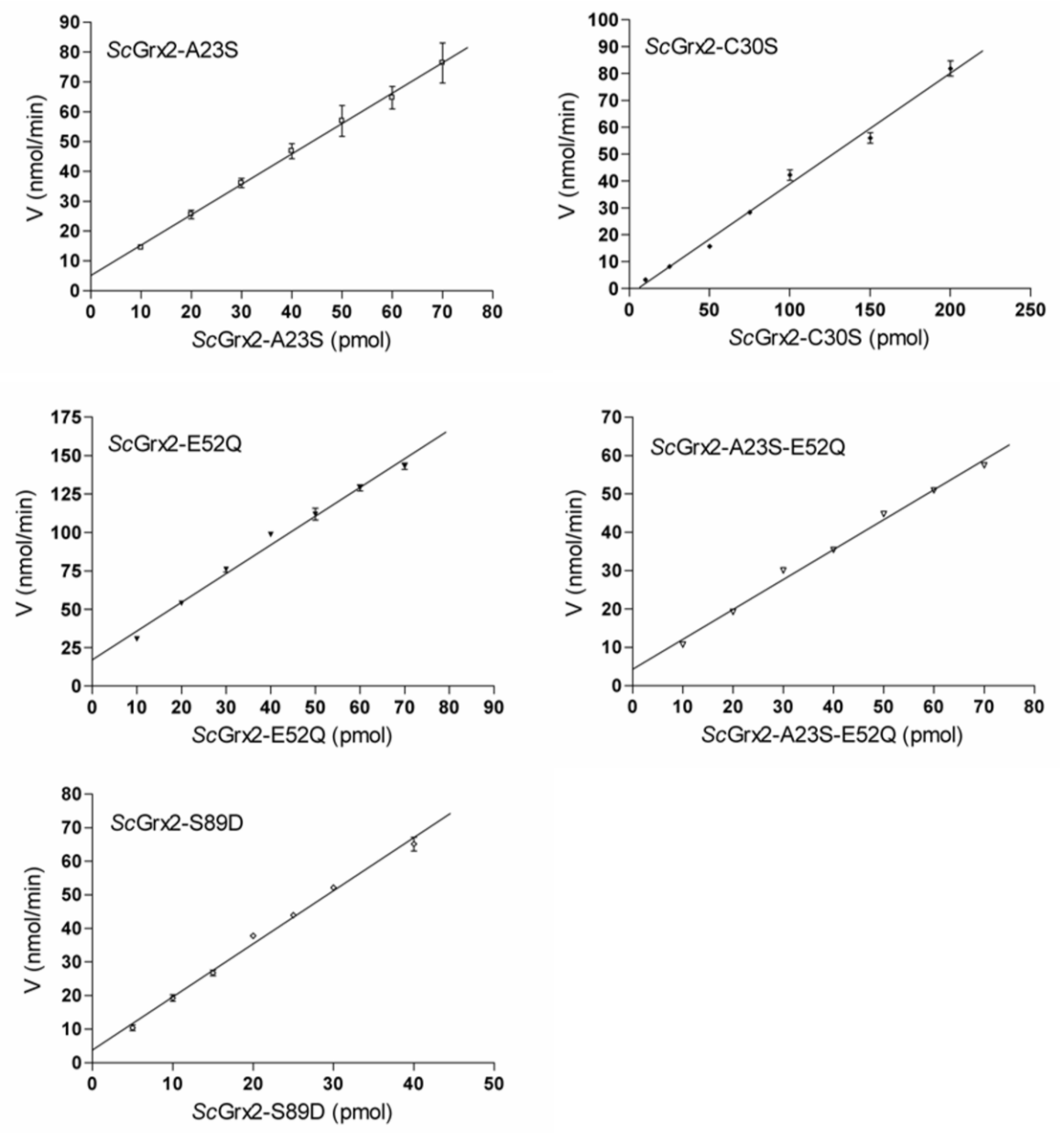

Figura 41: Gráficos de velocidade de consumo de NADPH (nmol/min) versus quantidade de Grx (pmol) adicionada, usados para a determinação das atividades específicas dos mutantes de *Sc*Grx2.

Como pode ser observado na Tabela 15, *Sc*Grx1-S23A e *Sc*Grx1-S23A-Q52E apresentam atividades específicas 3,4, e 3,0 vezes maiores que a *Sc*Grx1 selvagem,

respectivamente. Enquanto, os mutantes *Sc*Grx2-A23S e *Sc*Grx2-A23S-E52Q exibem atividades específicas 1,7, e 2,3 vezes menores que *Sc*Grx2, respectivamente.

A maior atividade apresentada por *Sc*Grx1-S23A e a menor atividade de *Sc*Grx2-A23S, em comparação à suas respectivas isoformas selvagens, indicam que estes resíduos estão, de alguma forma, relacionados à diferença de atividade observada entre *Sc*Grx1 e *Sc*Grx2. Mas para reforçar nossas hipóteses, as atividades específicas dos duplos mutantes *Sc*Grx1-S23A-Q52E e *Sc*Grx2-A23S-E52Q deveriam ser maior e menor, respectivamente, que a dos mutantes *Sc*Grx1-S23A e *Sc*Grx2-A23S, indicando que os resíduos Ser/Ala23 e Gln/Glu52 possuem um efeito conjunto sobre a atividade das Grxs ditiólicas de levedura. Entretanto, este efeito conjunto dos resíduos Ser/Ala23 e Gln/Glu52 pôde ser observado apenas com o duplo mutante *Sc*Grx2-A23S-Q52E, e não com *Sc*Grx1-S23A-Q52E (Tabela 15). Conseqüentemente, não ficou claro se os resíduos Gln/Glu52 têm realmente influência sobre a atividade de *Sc*Grx1 e *Sc*Grx2, ou se apenas Ser/Ala23 têm.

Também os resultados obtidos com os mutantes simples *Sc*Grx2-E52Q e *Sc*Grx1-Q52E geram dúvidas a respeito das influências de Gln/Glu52 sobre as atividades das Grxs ditiólicas. O mutante *Sc*Grx2-E52Q possui a mesma atividade que sua isoforma selvagem, enquanto o mutante *Sc*Grx1-Q52E é 1,5 vezes mais ativo que *Sc*Grx1 selvagem. Na verdade, era esperado que ambos estes mutantes simples não apresentassem diferença de atividade em relação às suas isoformas selvagens, mas que nos duplos, a interação mais ou menos efetiva da Cys30 com Gln52 ou Glu52 resultasse em atividades diferentes. Pois em *Sc*Grx2-E52Q, a Gln52 poderia interagir com a Cys30 do mesmo modo que o Glu52 o fazia, já que Ala23 permite esta interação. Deste modo, em *Sc*Grx2-E52Q, a conformação do resíduo de Cys30 não seria alterada em comparação à *Sc*Grx2. E em *Sc*Grx1-Q52E, a Ser23 poderia estabelecer ligações de hidrogênio com o Glu52, assim como mantinha com a Gln52, evitando a interação deste resíduo com a Cys30, de modo que a conformação da Cys30 não seria alterada.

A alteração da carga neutra da Gln52 para a carga negativa do Glu52 pode ter causado outras alterações na estrutura, as quais levaram ao pequeno aumento da atividade de *Sc*Grx1-Q52E em comparação à proteína selvagem. Ainda estamos procurando

características estruturais que expliquem o fato de termos dois mutantes simples (*Sc*Grx1-S23A e *Sc*Grx1-Q52E) mais ativos e um duplo (*Sc*Grx1-S23A-Q52E) não mais ativo que um dos simples (*Sc*Grx1-S23A), e um mutante duplo (*Sc*Grx2-A23S-Q52E) menos ativo que o simples (*Sc*Grx2-A23S), sendo que a segunda mutação (*Sc*Grx2-E52Q) parece não alterar a atividade da enzima.

Outra possibilidade seria que a Ser23 em *Sc*Grx1 poderia agir com uma base, retirando o próton da Cys30, favorecendo a formação do tiolato e, conseqüentemente, o ataque da Cys30 sobre o intermediário *Sc*Grx1-SG. Desta forma, a formação do dissulfeto intramolecular seria favorecida em *Sc*Grx1, ocorrendo, então, uma mudança do mecanismo monotiólico para o ditiólico, o que levaria a uma diminuição da velocidade da reação no ensaio com HED. No caso de *Sc*Grx2, a cadeia lateral do resíduo Ala23, com seu caráter hidrofóbico, não influenciaria a reatividade da Cys30 sobre o intermediário *Sc*Grx2-SG, permitindo a redução do mesmo por uma segunda molécula de GSH e dessa forma, favoreceria o mecanismo monotiólico.

Esta última proposição está de acordo com a observação de que *Sc*Grx1 apresenta um valor de K_M maior para GSH e um menor k_{cat} em comparação a *Sc*Grx2 e também com os resultados de atividade dos mutantes. A substituição da Ala23 por Ser em *Sc*Grx2-A23S provoca uma diminuição da atividade específica, quando comparada com a proteína selvagem, enquanto que a substituição da Ser23 por Ala em *Sc*Grx1-S23A causa um aumento na atividade específica em comparação a *Sc*Grx1 selvagem (Tabela 15).

Como descrito anteriormente na literatura, a substituição da cisteína C-terminal do sítio ativo de Grxs não leva a perda de atividade como oxidorredutase no ensaio de redução do dissulfeto misto β-ME-SG, uma vez que o mesmo se processa segundo o mecanismo monotiólico (Bushweller *et al.,* 1992; Yang & Wells, 1991; Yang *et al.,* 1998). Foi descrito para a *Hs*Grx1 que a velocidade global da reação no ensaio com HED é determinada pela velocidade da redução do intermediário glutatiolado Grx-SG (Srinivasan *et al.,* 1997). A ocorrência da reação secundária, que resulta na formação do dissulfeto intramolecular (Figura 3, reação c), provoca uma diminuição da velocidade global da reação, provavelmente por que parte da enzima e da GSH ficam envolvidas em reações intercâmbio

tiol-dissulfeto desnecessárias (Yang *et al.,* 1998). Então, a substituição da cisteína C-terminal do sítio ativo por uma serina em Grxs ditiólicas elimina a possibilidade de esta reação secundária ocorrer e, em princípio, deveria levar a um aumento da velocidade da reação. De fato, este comportamento é observado para os mutantes da Grx de porco, *Hs*Grx1 e *Hs*Grx2, além do mutante *Sc*Grx1-C30S (Yang & Wells, 1991; Yang *et al.,* 1998; Johansson *et al.,* 2004). Entretanto, para *Sc*Grx2-C30S e os mutantes C14S de *Ec*Grx1 e *Ec*Grx3, a mesma mutação leva a um decréscimo na velocidade da reação (Bushweller *et al.,* 1992; Nordstrand *et al.,* 1999). As razões para este efeito não estão claras até o momento, mas características estruturais específicas destas Grxs devem estar relacionadas a este comportamento. Dados cinéticos, apresentados a seguir, indicam que Cys30 poderia ter papel na interação de GSH com *Sc*Grx2 glutatiolada.

Como descrito para a Grx do bacteriofago T4 (Nikkola *et al.*, 1991), a substituição do Asp89 por uma serina no mutante *Sc*Grx1-D89S dobrou a atividade deste mutante em relação à *Sc*Grx1 selvagem. O carboxilato do Asp89 interage com o grupo α-amino do γ-Glu_{GS} na estrutura de *Sc*Grx1-SG. Esta interação é relativa ao intermediário Grx-SG que foi formado após a reação com o primeiro substrato (β-ME-SG). A mutação D89S elimina a possível repulsão entre as cargas dos carboxilatos do Asp89 e do γ-$Glu_{GS,}$ o que pode implicar em uma maior afinidade de ScGrx1-D89S por β-ME-SG, e levar ao observado aumento de atividade. Porém, o mutante *Sc*Grx2-S89D não apresentou alteração significativa de atividade em comparação à *Sc*Grx2 selvagem. Provavelmente, a diferente composição de aminoácidos ao redor do resíduo 89 em *Sc*Grx1 e *Sc*Grx2 provoque o comportamento diferente para estes dois mutantes. Também neste caso, ainda estamos estudando as estruturas de *Sc*Grx1 e *Sc*Grx2 para tentar entender melhor o papel de Asp/Ser89 nestas enzimas.

IV.12 - Análise cinética dos mutantes de *Sc*Grx1 e *Sc*Grx2

Com o objetivo de entender melhor o papel dos resíduos Cys30, Ser/Ala23 e Gln/Glu52 em *Sc*Grx1 e *Sc*Grx2, realizamos estudos de cinética bi-substrato com os mutantes *Sc*Grx1-S23A, *Sc*Grx1-S23A-Q52E, *Sc*Grx1-C30S, *Sc*Grx2-A23S, *Sc*Grx2-C30S

e *Sc*Grx2-A23S-E52Q. As Figuras 42-47 mostram os gráficos de Michaelis-Menten e os gráficos secundários usados para determinar as constantes cinéticas aparentes e reais (Tabelas 16 a 21) dos mutantes de *Sc*Grx1 e *Sc*Grx2. Estes dados cinéticos ainda são preliminares e precisam ser reproduzidos. Através deles, ainda não conseguimos determinar o K_M^{HED} real, mas conseguimos estimar o K_M^{GSH} real e o k_{cat} real. Os erros mostrados foram gerados pelo programa GraphPad Prism4, de acordo com a congruência dos dados em relação às respectivas equações das curvas. Como para *Sc*Grx1 e *Sc*Grx2 selvagens, obtivemos as constantes cinéticas aparentes dos mutantes a partir da regressão não-linear dos gráficos de Michaelis-Menten e usamos estas constantes para fazer os gráficos secundários e determinar os parâmetros cinéticos reais (Segel, 1975).

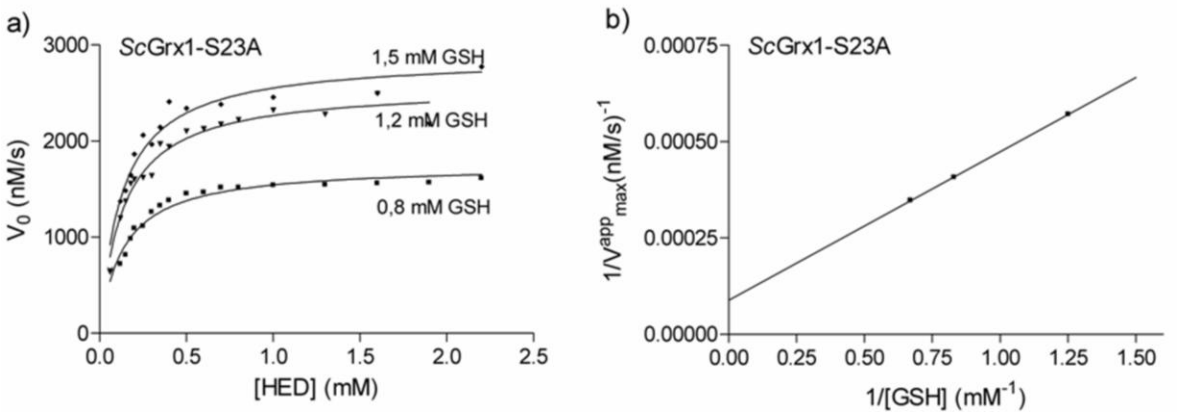

Figura 42: (a) Gráficos de velocidade inicial versus a concentração de HED para *Sc*Grx1-S23A em concentrações diferentes de GSH. A concentração de *Sc*Grx1-S23A utilizada foi 150 nM. (b) Gráfico secundário $1/V_{max}^{app}$ versus 1/[GSH] para *Sc*Grx1-S23A.

Tabela 16: Constantes cinéticas aparentes e reais para a redução do dissulfeto misto β-ME-SG por *Sc*Grx1-S23A.

[GSH] (mM)	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	K_M^{GSH} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M $(M.s)^{-1}$
0,8	0,13 ± 0,01	11,7 ± 0,2	4,3 ± 0,2	75 ± 2	(1,73 ± 0,07) x 10^4
1,2	0,13 ± 0,01	17,1 ± 0,4			
1,5	0,13 ± 0,02	19,2 ± 0,6			

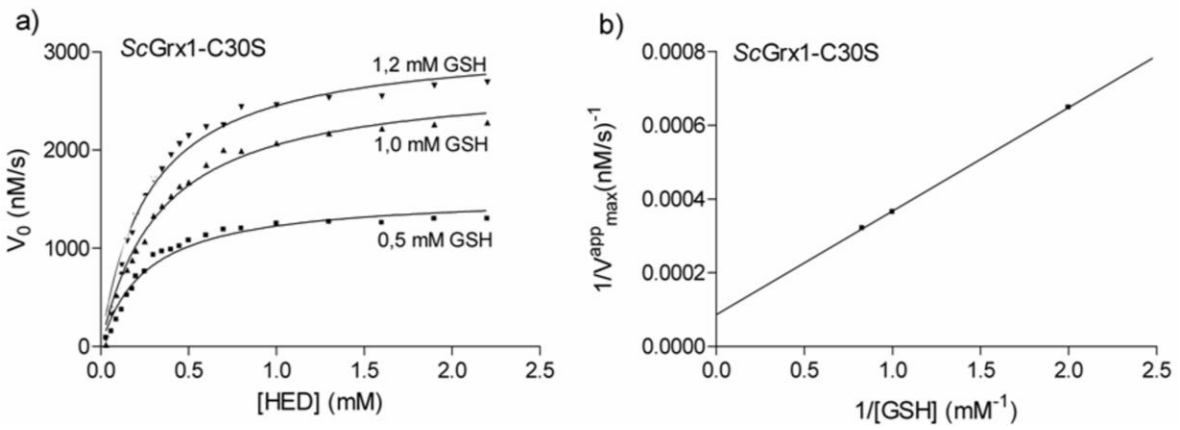

Figura 43: (a) Gráficos de velocidade inicial versus a concentração de HED para *Sc*Grx1-C30S em concentrações diferentes de GSH. A concentração de *Sc*Grx1-C30S utilizada foi 148 nM. (b) Gráfico secundário $1/V_{max}^{app}$ versus 1/[GSH] para *Sc*Grx1-C30S.

Tabela 17: Constantes cinéticas aparentes e reais para a redução do dissulfeto misto β-ME-SG por *Sc*Grx1-C30S.

[GSH] (mM)	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	K_M^{GSH} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M $(M.s)^{-1}$
0,5	0,26 ± 0,03	10,4 ± 0,4	3,2 ± 0,2	78 ± 4	(2,1 ± 0,2) x 10^4
1,0	0,33 ± 0,02	18,5 ± 0,5			
1,2	0,27 ± 0,02	21,0 ± 0,5			

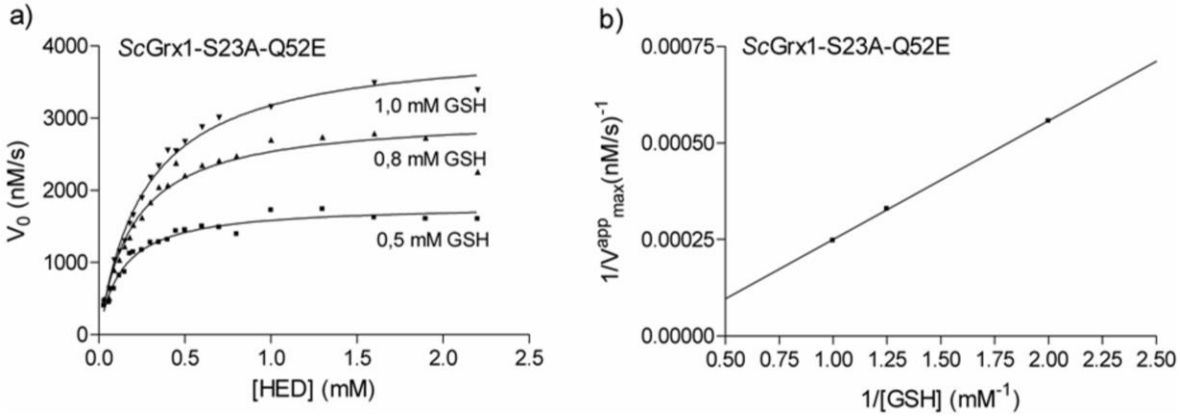

Figura 44: (a) Gráficos de velocidade inicial versus a concentração de HED para *Sc*Grx1-S23A-Q52E em concentrações diferentes de GSH. A concentração de *Sc*Grx1-S23A-Q52E utilizada foi 200 nM. (b) Gráfico secundário $1/V_{max}^{app}$ versus 1/[GSH] para *Sc*Grx1-S23A-Q52E.

Tabela 18: Constantes cinéticas aparentes e reais para a redução do dissulfeto misto β-ME-SG por *Sc*Grx1-S23A-Q52E.

[GSH] (mM)	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	K_M^{GSH} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M $(M.s)^{-1}$
0,5	0,13 ± 0,01	9,0 ± 0,2	5,3 ± 0,5	86 ± 9	(1,6 ± 0,3) x 10^4
0,8	0,20 ± 0,02	15,2 ± 0,5			
1,0	0,27 ± 0,02	20,2 ± 0,4			

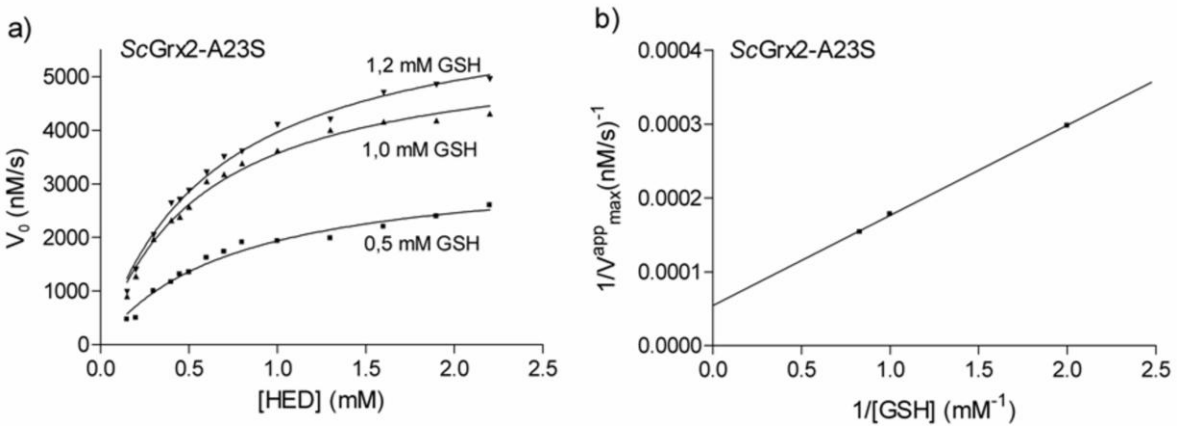

Figura 45: (a) Gráficos de velocidade inicial versus a concentração de HED para *Sc*Grx2-A23S em concentrações diferentes de GSH. A concentração de *Sc*Grx2-A23S utilizada foi 100 nM. (b) Gráfico secundário $1/V_{max}^{app}$ versus 1/[GSH] para *Sc*Grx2-A23S.

Tabela 19: Constantes cinéticas aparentes e reais para a redução do dissulfeto misto β-ME-SG por *Sc*Grx2-A23S.

[GSH] (mM)	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	K_M^{GSH} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M $(M.s)^{-1}$
0,5	0,73 ± 0,05	34 ± 2	2,2 ± 0,2	124 ± 13	(8,2 ± 0,8) x 10^4
1,0	0,57 ± 0,05	56 ± 2			
1,2	0,64 ± 0,04	65 ± 2			

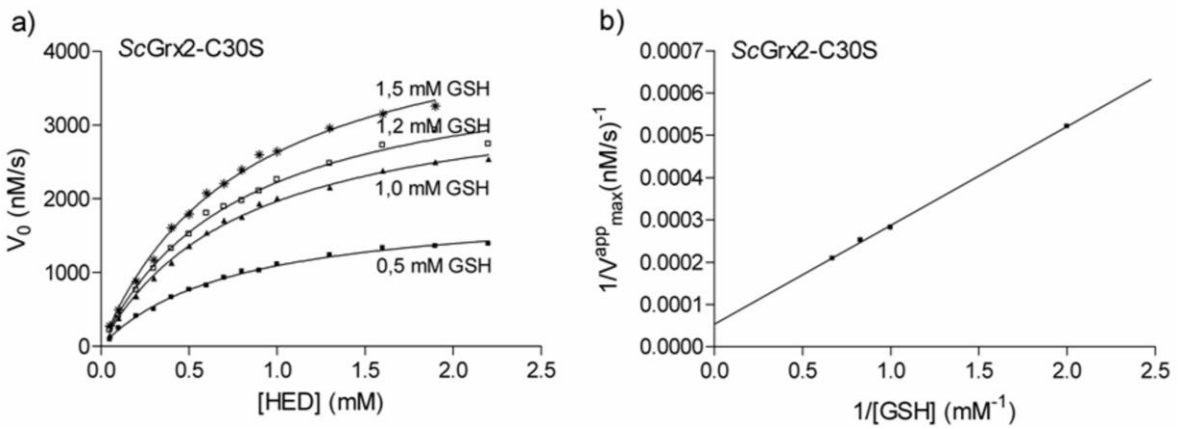

Figura 46: (a) Gráficos de velocidade inicial versus a concentração de HED para *Sc*Grx2-C30S em concentrações diferentes de GSH. A concentração de *Sc*Grx2-C30S utilizada foi 150 nM. (b) Gráfico secundário $1/V_{max}^{app}$ versus 1/[GSH] para *Sc*Grx2-C30S.

Tabela 20: Constantes cinéticas aparentes e reais para a redução do dissulfeto misto β-ME-SG por *Sc*Grx2-C30S.

[GSH] (mM)	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	K_M^{GSH} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M $(M.s)^{-1}$
0,5	0,75 ± 0,04	12,8 ± 0,3	4,5 ± 0,5	125 ± 14	(2,8 ± 0,5) x 10^4
1,0	0,79 ± 0,04	23,6 ± 0,5			
1,2	0,78 ± 0,05	26,5 ± 0,8			
1,5	0,82 ± 0,04	31,8 ± 0,8			

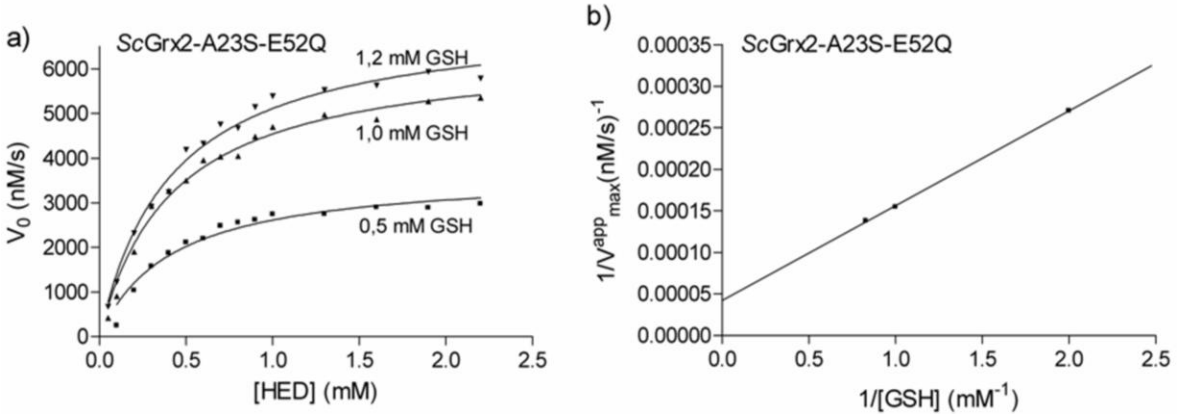

Figura 47: (a) Gráficos de velocidade inicial versus a concentração de HED para *Sc*Grx2-A23S-E52Q em concentrações diferentes de GSH. A concentração de *Sc*Grx2-A23S-E52Q utilizada foi 200 nM. (b) Gráfico secundário $1/V_{max}^{app}$ versus 1/[GSH] para *Sc*Grx2-A23S-E52Q.

Tabela 21: Constantes cinéticas aparentes e reais para a redução do dissulfeto misto β-ME-SG por *Sc*Grx2-A23S-E52Q.

[GSH] (mM)	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	K_M^{GSH} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M $(M.s)^{-1}$
0,5	0.41 ± 0.06	18 ± 1	2,7 ± 0,2	119 ± 9	(4,4 ± 0,5) x 10^4
1,0	0.43 ± 0.04	32 ± 1			
1,2	0.42 ± 0.03	39 ± 1			

A Tabela 22 mostra os resultados de atividade específica e cinética bi-substrato obtidos para *Sc*Grx1 e *Sc*Grx2 e seus mutantes para facilitar a comparação. As constantes cinéticas obtidas para essas proteínas seguem a tendência indicada pelos resultados de atividade específica, indicando que não há incongruência nos dados.

Tabela 22: Dados de atividade específica e constantes cinéticas reais para *Sc*Grx1, *Sc*Grx2 e seus mutantes.

	Atividade específca ($\mu mol.min^{-1}.mg^{-1}$)	K_M^{GSH} (mM)	k_{cat} (s^{-1})	k_{cat}/K_M $(M.s)^{-1}$
***Sc*Grx1**	8,2 ± 0,3	6,2 ± 2,5	17 ± 7	(2,7 ± 1,5) x 10^3
*Sc*Grx1-S23A	28,2 ± 0,3	4,3 ± 0,2	75 ± 2	(1,73 ± 0,07) x 10^4
*Sc*Grx1-C30S	39 ± 1	3,2 ± 0,2	78 ± 4	(2,1 ± 0,2) x 10^4
*Sc*Grx1-S23A-Q52E	24,5 ± 0,4	5,3 ± 0,5	86 ± 9	(1,6 ± 0,3) x 10^4
***Sc*Grx2**	125 ± 7	0,9 ± 0,2	129 ± 20	(1,5 ± 0,4) x 10^5
*Sc*Grx2-A23S	67 ± 1	2,2 ± 0,2	124 ± 13	(8,2 ± 0,8) x 10^4
*Sc*Grx2-C30S	38 ± 2	4,5 ± 0,5	125 ± 14	(2,8 ± 0,5) x 10^4
*Sc*Grx2-A23S-E52Q	54 ± 2	2,7 ± 0,2	119 ± 9	(4,4 ± 0,5) x 10^4

Podemos observar que todos os mutantes de *Sc*Grx1 analisados apresentam, considerando os erros, um mesmo valor de k_{cat}, o qual é cerca de 4,5 vezes maior do que o obtido para *Sc*Grx1 selvagem. Em relação aos valores de K_M^{GSH}, dados os erros, podemos afirmar que apenas o mutante *Sc*Grx1-C30S possui uma afinidade maior por GSH em comparação a *Sc*Grx1 selvagem. Deste modo, a maior eficiência catalítica de *Sc*Grx1-C30S (k_{cat}/K_M) deve ser resultado tanto de um maior *turnover*, quanto de uma maior

afinidade por GSH. Já para os mutantes *Sc*Grx1-S23A e *Sc*Grx1-S23A-Q52E, a maior eficiência catalítica apresentada, em relação à isoforma selvagem, deve ser determinada principalmente por um *turnover* maior. Lembrando mais uma vez, nos referimos à afinidade do intermediário Grx-SG por GSH, e não à afinidade de Grx reduzida por GSH.

Era esperado que os mutantes *Sc*Grx1-C30S, *Sc*Grx1-S23A e *Sc*Grx1-S23A-Q52E apresentassem maior k_{cat} que *Sc*Grx1, uma vez que a reação secundária de ataque da Cys30 sobre o dissulfeto misto Cys27-SG é abolida em *Sc*Grx1-C30S, e deve ocorrer com freqüência menor em *Sc*Grx1-S23A e *Sc*Grx1-S23A-Q52E, já que a Cys30, supostamente, adota uma conformação desfavorável ao ataque, ou não tem mais sua desprotonação favorecida nestes mutantes. Conseqüentemente, a redução do intermediário Grx-SG por GSH é favorecida, a enzima cicla mais rapidamente e é necessária uma concentração menor de GSH para atingir metade da velocidade máxima da reação, uma vez que moléculas de GSH não são desviadas para reduzir o dissulfeto intramolecular, que não está mais sendo formado.

Comparando as constantes cinéticas obtidas para os mutantes de *Sc*Grx2 e aquelas obtidas para *Sc*Grx2 selvagem, observamos que os mutantes *Sc*Grx2-A23S, *Sc*Grx2-C30S e *Sc*Grx2-A23S-E52Q apresentam todos K_M^{GSH} maior que a isoforma selvagem e, considerando os erros, valores de k_{cat} iguais. Portanto, a queda de atividade observada para estes mutantes está relacionada a uma menor afinidade destes por GSH, e não a um *turnover* menor.

Esperávamos que os mutantes *Sc*Grx2-A23S e *Sc*Grx2-A23S-E52Q apresentassem uma queda no *turnover*, pois segundo nossa hipótese, a presença da Ser23 favoreceria a desprotonação da Cys30 ou permitiria a sua alteração de conformação, favorecendo a reação secundária, que resulta na formação do dissulfeto intramolecular e retarda a ciclagem da enzima (Figura 3, reação c). Porém, esses mutantes não apresentaram uma modificação no *turnover* em relação à *Sc*Grx2 selvagem, mas sim uma menor afinidade por GSH. E claramente, *Sc*Grx2-C30S é o mutante que possui menor afinidade por GSH.

Como descrito anteriormente, esperávamos um aumento da atividade específica de *Sc*Grx2 ao substituirmos a Cys30 por uma serina, pois dessa forma, a ocorrência do mecanismo ditiólico não seria possível. Os resultados cinéticos apresentados aqui

possibilitaram elaborar uma possível explicação para o fato da eliminação da cisteína C-terminal diminuir a atividade monotiólica: a interação de *Sc*Grx2-SG com glutationa poderia ser afetada, como será discutido a seguir.

Havia sido mostrado para *Ec*Grx1, *Hs*Grx1 e Grx de porco que a especificidade por substratos glutatiolados, na primeira etapa da reação no mecanismo monotiólico (Figura 3, reação f), é dependente da ligação do tipo γ entre o Glu_{GS} e a Cys_{GS} (Peltoniemi *et al.*, 2006; Yang *et al.*, 1998; Rabenstein & Millis, 1995). Mais recentemente, foi descrito para *Ec*Grx1 que a especificidade por GSH, na segunda etapa da reação no mecanismo monotiólico (redução do intermediário Grx-SG; Figura 3, reação g), também depende da ligação do tipo γ presente em GSH. E que a Cys14, C-terminal, está relacionada à especificidade pela ligação do tipo γ da GSH na segunda etapa da reação, uma vez que a mutação C14S leva à perda desta especificidade (Saaranen *et al.*, 2009).

O mutante *Ec*Grx1-C14S apresenta um comportamento similar ao mutante *Sc*Grx2-C30S, mantendo ambos apenas cerca de 30% da atividade de suas isoformas selvagens. Considerando esta semelhança e o fato de que o mutante *Sc*Grx2-C30S apresenta um aumento de K_M^{GSH}, podemos levantar a hipótese de que em *Sc*Grx2, o resíduo de Cys30 poderia também estar envolvido na especificidade por GSH, dependente da ligação do tipo γ, na segunda etapa da reação no mecanismo monotiólico. Já os mutantes *Sc*Grx2-A23S e *Sc*Grx2-A23S-E52Q podem apresentar uma menor afinidade por GSH, possivelmente, por que estes resíduos influenciam a conformação adotada pela Cys30, como proposto anteriormente.

IV.13 - Determinação do pK_a da Cys27 dos mutantes de *Sc*Grx1 e *Sc*Grx2

Como mostrado anteriormente, não foi constatada uma diferença nos valores de pK_a da Cys27 de *Sc*Grx1 e *Sc*Grx2 que correspondesse à diferença de atividade observada entre essas proteínas. Porém foram identificados resíduos de aminoácidos que modificam as atividades destas enzimas como oxidorredutases, conforme discutido nas seções IV.10 e IV.11. Então resolvemos verificar se estas substituições alteram o pK_a da Cys27 dos mutantes *Sc*Grx1-S23A, *Sc*Grx1-Q52E, *Sc*Grx1-S23A-Q52E, *Sc*Grx1-C30S, *Sc*Grx2-

A23S, *Sc*Grx2-E52Q e *Sc*Grx2-A23S-E52Q, de modo que as mudanças nas atividades destes mutantes esteja relacionada a uma diferença de pK_a em relação às proteínas selvagens. Neste caso, utilizamos apenas o método de alquilação com MBB, como descrito em "Materiais e Métodos".

Na Tabela 23 são mostrados os valores de pK_a obtidos para os mutantes, e a razão esperada para as constantes de segunda ordem da reação, a partir da variação do pK_a da proteína mutante (A) em relação ao da selvagem (B), como discutido anteriormente na seção IV.4 ($k_A/k_B = 4^{(pKaB - pKaA)}$) (Gilbert, 1990; Szajewski & Whitesides, 1980). Para comparação, calculamos a razão entre a eficiência catalítica de cada mutante e a eficiência catalítica de suas respectivas isoformas selvagens, levando em consideração a aproximação de que a constante de segunda ordem da reação corresponde à k_{cat}/K_M.

Tabela 23: Valores de pK_a obtidos para a Cys27 de *Sc*Grx1, *Sc*Grx2 e seus mutantes, e a razão esperada entre as constantes de segunda ordem da reação catalisada por estes mutantes em comparação às suas enzimas selvagens, calculada com base na diferença de pK_a e com base na eficiência catalítica.*

	pK_a	k_A/k_B esperado	k_A/k_B obtido
***Sc*Grx1**	3,2 ± 0,2	-	-
*Sc*Grx1-S23A	3,5 ± 0,2	0,7	6,4
*Sc*Grx1-C30S	3,7 ± 0,2	0,5	7,7
*Sc*Grx1-Q52E	3,8 ± 0,2	0,4	-
*Sc*Grx1-S23A-Q52E	3,7 ± 0,2	0,5	5,9
***Sc*Grx2**	3,1 ± 0,2	-	-
*Sc*Grx2-A23S	3,7 ± 0,2	0,4	0,5
*Sc*Grx2-C30S	4,0 ± 0,2	0,3	0,2
*Sc*Grx2-E52Q	3,5 ± 0,2	0,6	-
*Sc*Grx2-A23S-E52Q	3,3 ± 0,2	0,8	0,3

* k_A/k_B esperado foi determinado através da equação $4^{(pKaB - pKaA)}$ como descrito anteriormente (Gilbert, 1990; Szajewski & Whitesides, 1980). k_A/k_B obtido foi determinado pelas eficiências catalíticas das proteínas mutante e selvagem $[(k_{cat}/K_M)_A/(k_{cat}/K_M)_B]$, respectivamente.

Comparando os valores de pK_a para a Cys27 obtidos para os mutantes de *Sc*Grx1 e *Sc*Grx2, vemos que todos os mutantes apresentaram um pequeno aumento no pK_a em

relação às isoformas selvagens. Em todos os casos, este aumento de pK_a, de acordo com a equação $k_A/k_B = 4^{(pKaB - pKaA)}$, leva à predição de uma diminuição da constante de segunda ordem da reação (Tabela 23). Porém, todos os mutantes de *Sc*Grx1 apresentam atividade maior do que *Sc*Grx1 selvagem, indicando que outros fatores além do pK_a do tiol devem estar afetando a reatividade da enzima.

Porém, para os mutantes de *Sc*Grx2, quando comparamos a variação predita, baseada na diferença de pK_a, para a constante de segunda ordem da reação e a variação estimada pelos valores de k_{cat}/K_M, observamos que estes valores são próximos entre si. Então, é possível que a queda de atividade dos mutantes de *Sc*Grx2 esteja ao menos parcialmente relacionada ao maior pK_a da Cys27 nestes mutantes em comparação ao pK_a da Cys27 da proteína selvagem.

Portanto, nossas análises indicam que, no caso das Grxs ditiólicas de levedura, resíduos de aminoácidos específicos de *Sc*Grx1 e *Sc*Grx2, como Ser/Ala23 e Cys30, estão intimamente relacionados à reatividade destas enzimas no mecanismo monotiólico. Outros resíduos como Gln/Gln52 e Asp/Ser89 também parecem ter influência sobre a atividade destas Grxs, mesmo que em menor proporção. O pK_a da cisteína reativa é claramente importante para a reatividade de ambas Grxs ditiólicas, uma vez que é bastante baixo nas duas, mas não parece ser um fator que esteja relacionado com as diferenças de atividade entre *Sc*Grx1 e *Sc*Grx2.

Já foram descritos diversos fatores que podem determinar a reatividade de oxidorredutases. Os mais estudados são: pK_a da cisteína reativa, potencial redox, e interações com substratos e proteína-proteína, determinadas por características estruturais e resíduos de aminoácidos específicos de cada enzima. Porém, os estudos realizados até o momento indicam que a reatividade de oxidorredutases resulta de um balanço entre este conjunto de fatores e possivelmente outros ainda não estudados, sendo difícil analisá-las observando cada fator isoladamente.

As diferenças estruturais e funcionais entre *Sc*Grx1 e *Sc*Grx2, mostradas nesse trabalho, podem refletir variações em termos de afinidade a diferentes substratos. Nesse contexto, é importante mencionar que recentemente mostramos que a desglutatiolação do proteassoma 20S de levedura é modulado por *Sc*Grx2 (Silva *et al*., 2008 - anexo 2).

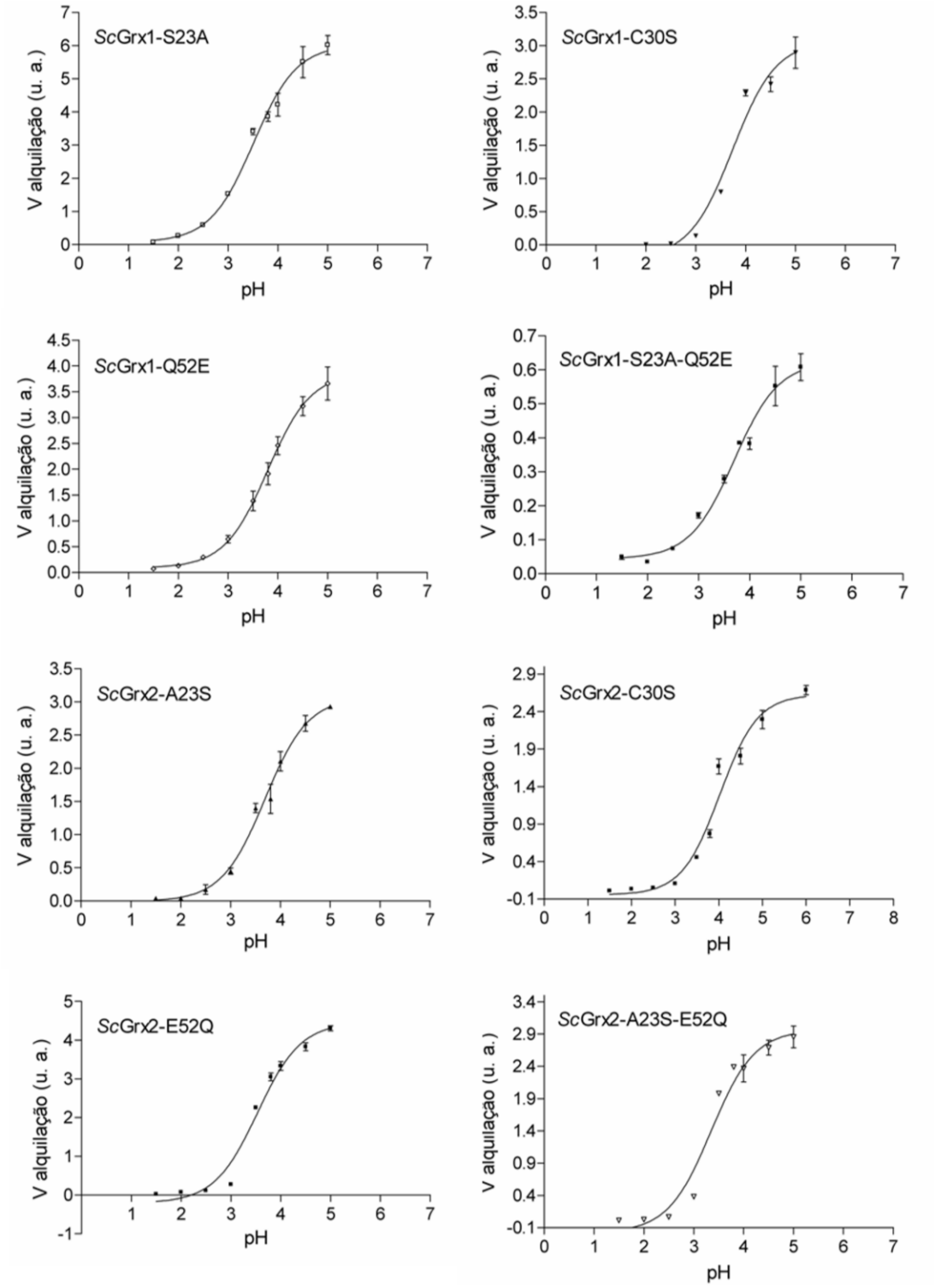

Figura 48: Gráficos utilizados para a determinação do pK_a dos mutantes de *Sc*Grx1 e *Sc*Grx2 através do método de alquilação com MBB (u.a.: unidades arbitrárias).

V - CONCLUSÕES E PERSPECTIVAS

O principal objetivo deste trabalho foi caracterizar funcional e estruturalmente as Grxs ditiólicas de *S. cerevisiae* e encontrar características relacionadas à diferença de atividade observada entre elas como oxidorredutases. Nossas principais conclusões estão listadas abaixo, seguidas de perspectivas para novos estudos.

- *Sc*Grx1 e *Sc*Grx2 não possuem atividade peroxidásica frente a peróxido de hidrogênio, *tert*-butil-hidroperóxido e hidroperóxido de cumeno. Em estudos futuros, seria interessante fazer mutações em *Sc*Grx1 e *Sc*Grx2, como, por exemplo, a substituição da Cys N-terminal por uma Thr, e verificar se estas Grxs adquirem atividade peroxidásica, uma vez que em Prxs a Cys N-terminal do motivo CXXC foi substituída por uma Thr.

- As Tiorredoxinas Redutases de levedura *Sc*Trr1 e *Sc*Trr2 não são capazes de reduzir *Sc*Grx1 e *Sc*Grx2.

- *Sc*Grx1 e *Sc*Grx2 não reduzem as pontes dissulfeto da insulina de pâncreas bovino. Futuramente, pretendemos encontrar um substrato protéico com dissulfeto para *Sc*Grx1 e *Sc*Grx2, e medir suas atividades no mecanismo ditiólico para verificar se a diferença de atividade observada para estas enzimas no mecanismo monotiólico também existe para o mecanismo ditiólico.

- *Sc*Grx1 e *Sc*Grx2 são ativas no ensaio clássico de redução do dissulfeto misto formado entre HED e glutationa, e apresentam uma diferença de eficiência catalítica neste ensaio de cerca de 55 vezes, sendo que *Sc*Grx2 apresenta K_M^{GSH} real menor e turnover maior que *Sc*Grx1.

- A cisteína reativa N-terminal (Cys27) do sítio ativo de *Sc*Grx1, *Sc*Grx2 e seus mutantes possui um valor de pK_a bastante baixo (entre 3,1 e 4,0), característico de Grxs. Porém, neste trabalho, não foi encontrada uma relação clara entre o valor de pK_a da Cys27 e a atividade destas enzimas.

- Os resíduos Ser/Ala23 estão relacionados à diferença de atividade observada entre *Sc*Grx1 e *Sc*Grx2, ou por permitirem a orientação da Cys30 por Gln/Glu52 em relação ao dissulfeto Cys27-SG, ou por promoverem, ou não, a desprotonação da Cys30. Ainda não temos certeza do papel dos resíduos Gln/Glu52. Para caracterizar melhor a questão da orientação da Cys30 por Gln/Glu52 seria interessante, futuramente, construir mutantes nos quais não seria possível haver interação com a Cys30 (substituindo Gln/Glu52 por Leu, por exemplo), independentemente dos resíduos Ser/Ala23. E para verificar se Ser23 pode estar envolvida na desprotonação da Cys30, seria interessante determinar o pK_a da Cys30 nos mutantes *Sc*Grx1-S23A e *Sc*Grx2-A23S. A determinação das estruturas dos mutantes *Sc*Grx1-S23A e *Sc*Grx2-A23S também poderia esclarecer a questão da influência de Ser/Ala23 sobre a orientação da Cys30.

- A Cys30 em *Sc*Grx2 parece estar relacionada ao reconhecimento da segunda molécula de GSH no mecanismo monotiólico, dada a menor afinidade do mutante *Sc*Grx2-C30S por GSH. O mesmo não pode ser dito sobre a Cys30 de *Sc*Grx1, uma vez que o mutante *Sc*Grx1-C30S apresenta maior afinidade por GSH. Independentemente do papel específico desempenhado pela Cys30 nestas Grxs, fica claro que estas cisteínas têm um papel modulador importante na atividade destas proteínas.

- A mutação D89S em *Sc*Grx1 promove um aumento de atividade, enquanto a mutação S89D em *Sc*Grx2 parece não ter influência sobre a atividade desta enzima. Em estudos futuros, a determinação dos parâmetros cinéticos para os mutantes *Sc*Grx1-D89S e *Sc*Grx2-S89D seria interessante para verificar se há diferença de afinidade pelo primeiro substrato da reação do mecanismo monotiólico.

- Todos os resultados deste trabalho indicam que *Sc*Grx1 e *Sc*Grx2 são enzimas com propriedades bastante distintas, sendo difícil fazer generalizações sobre elas. Acreditamos que as diferenças observadas entre *Sc*Grx1 e *Sc*Grx2 devem refletir em variações na especificidade por substratos nos diferentes compartimentos celulares nos quais estas enzimas se localizam: citosol e mitocôndria, respectivamente; e indicam que estas enzimas possuem funções biológicas não redundantes em *S. cerevisiae*.

VI - REFERÊNCIAS BIBLIOGRÁFICAS

Ausebel F. M., Brent R., Kingston R. E., Moore D. D., Seidman J. G., Smith J. A. & Struhl K. (1987) "Currents Protocols in Molecular Biology*"* John Wiley & Sons, N.Y.

Bacik, J.P & Hazes, B. (2007) "Crystal structures of a poxviral glutaredoxin in the oxidized and reduced states show redox-correlated structural changes" *J. Mol. Biol.* **365**, 1545-1558.

Bellí, G., Polaina, J., Tamarit, J., de la Torre, M. A., Rodríguez-Manzaneque, M. T., Ros, J. & Herrero, E. (2002) "Structure-function analysis of yeast Grx5 monothiol glutaredoxin defines essential amino acids for function of the protein" *J. Biol. Chem.* **277,** 37590-37596.

Benesch, R.E. & Benesch, R. (1955) "The acid strength of the –SH group in cysteine and related compounds" *J. Am. Chem. Soc.* **77,** 5877-5881.

Bergfors, T. (2003) "Seeds to crystals" *J. Struct. Biol*. **142,** 66-76.

Biteau, B., Labarre, J. & Toledano, M. B. (2003) "ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin" *Nature* **425,** 980-984.

Blessing, R. H. (1995) "An empirical correction for absorption anisotropy" *Acta Crystallog. sect. A*, **51**, 33-38.

Bollag, D.M., Rozycki, M.D. & Edelstein, S.J. (1996) *"*Protein methods" John Wiley & Sons Inc., New York, USA.

Bushweller, J. H., Åslund, F., Wüthrich, K. & Holmgren, A. (1992) "Structural and functional characterization of the mutant *Escherichia coli* glutaredoxin (C14→S) and its mixed disulfide with glutathione" *Biochemistry,* **31**, 9288-9293.

Bushweller, J. H., Billeter, M., Holmgren, A. & Wüthrich, K. (1994) "The nuclear magnetic resonance solution structure of the mixed disulfide between *Escherichia coli* glutaredoxin (C14→S) and glutathione" *J. Mol. Biol.* **235,** 1585-1597.

Brunger, A.T., Krukowski, A. and Erickson, J. (1990) "Slow-Cooling Protocols for Crystallographic Refinement by Simulated Annealing" *Acta Crystallog*. *sect A*, **46,** 585-593.

Brunger, A.T., Kuriyan, J. & Karplus, M. (1987) "Crystallographic R factor Refinement by Molecular Dynamics" *Science* **235,** 458-460.

Camier, S., Ma, E., Leroy, C., Pruvost, A., Toledano, M. & Marsolier-Kergoat, M. C. (2007) "Visualization of ribonucleotide reductase catalytic oxidation establishes thioredoxins as its major reductants in yeast" *Free Radic. Biol. Med.* **42,** 1008-1016.

Carvalho, A. P., Fernandes, P. A. & Ramos, M. J. (2006) "Similarities and differences in the thioredoxin superfamily" *Prog. Biophys. Mol. Biol*. **91,** 229-248.

Chae, H. Z., Chung, S. J. & Rhee, S. G. (1994) "Thioredoxin-dependent peroxide reductase from yeast" *J. Biol. Chem.* **269,** 27670-27678.

Chenna, R., Sugawara, H., Koile, T., Lopez, R., Gibson, T. J., Higgins, D. G. & Thompson, J. D. (2003) "Multiple sequence alignment with the Clustal series of programs" *Nucleic Acids Res.* **31,** 3497-3500.

Clamp, M., Cuff, J., Searle, S. M. & Barton, G. J. (2004) "The Jalview Java alignment editor" *Bioinformatics* **20,** 426-427.

Claude, J. B., Suhre, K., Notredame, C., Claverie, J. M. & Abergel, C. (2004) "CaspR: a web server for automated molecular replacement using homology modeling" *Nucleic Acid Res.* **32,** W606-609.

Collaborative Computational Project, Number 4. (1994) *Acta Crystallog. sect. D*, **50,** 760-763.

Collinson, E. J. & Grant, C. M. (2003) "Role of yeast glutaredoxins as glutathione s-transferases" *J. Biol. Chem*. **278,** 22492-22497.

Collinson, E. J., Wheeler, G. L., Garrido, E. O., Avery, A. M., Avery, S. V. & Grant, C. M. (2002) "The yeast glutaredoxins are active as glutathione peroxidases" *J. Biol. Chem*. **277,** 16712-16717.

Copley, S. D., Novak, W.R.P. & Babbitt, P.C. (2004) "Divergence of function in the thioredoxin fold suprafamily: evidence for evolution of peroxiredoxins from a thioredoxin-like ancestor" *Biochemistry* **43**, 13981-13995.

Corpet, F. (1988) "Multiple sequence alignment with hierarchical clustering" *Nucl. Acids Res.* **16,** 10881-10890.

Creighton, T. E. & Darby, N. J. (1993) "Dissecting the disulfide couple folding pathway of bovine pancreatic inhibitor: forming the first disulfide bonds in analogues of the reduced protein" *J. Mol. Biol.* **232,** 873-896.

Delano, W. L. (2002) "The Pymol Molecular Graphics System" Delano Scientific, San Carlos, CA., USA. (http://www.pymol.org).

Discola, K. F., Oliveira, M. A., Cussiol, J.R.R., Monteiro, G., Bárcena, J. A., Porras, P., Padilla, A., Guimarães, B. G. & Netto, L. E. S. (2009) "Structural aspects of the distinct biochemical properties of Glutaredoxin 1 and Glutaredoxin 2 from *Saccharomyces cerevisiae*" *J. Mol. Biol.* **385,** 889-901.

Discola, K. F., Oliveira, M. A., Monteiro, G. S., Bárcena, J. A., Porras, P., Padilla, A., Netto, L. E. S. & Guimarães, B. G. (2005) "Crystallization and preliminary X-ray crystallographic studies of glutaredoxin 2 from *Saccharomyces cerevisiae* in different oxidation states" *Acta Crystallog*. *sect. F*, **61,** 445-447.

Eckers, E., Bien, M., Stroobant, V., Herrmann, J. M. & Deponte, M. (2009) "Biochemical characterization of dithiol glutaredoxin 8 from *Saccharomyces cerevisiae*: the catalytic redox mechanism redux" *Biochemistry* **48,** 1410-1423.

Eklund, H., Ingelman, M., Söderberg, B.O., Uhlin, T., Nordlund, P., Nikkola, M., Sonnerstam, U., Joelson, T. & Petratos, K. (1992) "Structure of oxidized bacteriophage T4 glutaredoxin (thioredoxin)" *J. Mol. Biol*. **228,** 596-618.

Emsley, P. & Cowtan, K. (2004) "Coot: model-building tools for molecular graphics" *Acta Crystallog. sect. D*, **60**, 2126-2132.

Epp, O., Ladenstein, R. & Wendel, A. (1983) "The refined structure of the selenoenzyme glutathione peroxidase at 0.2-nm resolution" *Eur. J. Biochem.* **133,** 51-69.

Feng, Y., Zhong, N., Rouhier, N., Hase, T., Kusunoki, M., Jacquot, J.P., Jin, C. & Xia, B. (2006) "Structural Insight into Poplar Glutaredoxin C1 with a Bridging Iron-Sulfur Cluster at the Active Site" *Biochemistry* **45**, 7998-8008.

Fernandes, A. P., Fladvad, M., Berndt, C., Andrésen, C., Lillig, C. H., Neubauer, P., Sunnerhagen, M., Holmgren, A. & Vlamis-Gardikas, A. (2005) "A novel monothiol glutaredoxin (Grx4) from *Escherichia coli* can serve as substrate for thioredoxin reductase" *J. Biol. Chem.* **280,** 24544-24552.

Fernandes, A. P. & Holmgren, A. (2004) "Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system" *Antioxid. Redox Signal.* **6,** 63-74.

Gallogly, M.M., Starke, D.W., Leonberg, A.K., Ospina, S.M.E. & Mieyal, J.J. (2008) "Kinetic and mechanistic characterization and versatile catalytic properties of mammalian glutaredoxin 2: implications for intracellular roles" *Biochemistry* **47**, 11144-11157.

Gallogly, M. M., Starke, D. W. & Mieyal, J.J. (2009) "Mechanistic and kinetic details of catalysis of thiol-disulfide exchange by glutaredoxins and potential mechanisms of regulation" *Antioxid. Redox Signal.* **11,** 1059-1081.

Gan, Z., Sardana, M. K., Jacobs, J. W. & Polokoff, M. A. (1990) "Yeast thioltransferase – active site cysteines display differential reactivity" *Arch. Biochem. Biophys.* **282,** 110-115.

Gan, Z. & Wells, W.W. (1987) "Identification and reactivity of the catalytic site of pig liver thioltransferase" *J. Biol. Chem*. **262**, 6704-6707.
Garrido, E. O. & Grant, C. M. (2002) "Role of thioredoxins in the response of *Saccharomyces cerevisiae* to oxidative stress induced by hydroperoxides" *Mol. Microbiol*. **43**, 993-1003.

Gilbert, H. F. (1990) "Molecular and cellular aspects of thiol-disulfide exchange" *Adv. Enzymol. Relat. Areas Mol. Biol.* **63,** 69-172.

Grant, C. M. (2001) "Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions" *Mol. Microbiol*. **39**, 533-541.

Gravina, S.A. & Mieyal, J.J. (1993) "Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase" *Biochemistry* **32**, 3368-3376.

Håkansson, K. O. & Winther, J. R. (2007) "Structure of glutaredoxin Grx1p C30S mutant from yeast" *Acta Crystallog. sect. D*, **63,** 288-294.

Holmgren, A. (1985) "Thioredoxin" *Annu. Rev. Biochem*. **54**, 237-271.

Holmgren, A. (1989) "Thioredoxin and Glutaredoxin systems" *J. Biol. Chem*. **264**, 13963-13966.

Holmgren, A. & Åslund, F. (1995) "Glutaredoxin" *Methods Enzymol.* **252,** 283–292.

Huber-Wunderlich, M. & Glockshuber, R. (1998) "A single dipeptide sequence modulates the redox properties of a whole enzyme family" *Fold. Des.* **3,** 161-171.

Jacob, C., Giles, G.I., Giles, N.M. & Sies, H. (2003) "Sulfur and Selenium: The role of oxidation state in protein structure and function" *Angew. Chem. Int. Ed.* **42,** 4742-4758.

Jao, S., Ospina, S. M. E., Berdis, A. J., Starke, D. W., Post, C. B. & Mieyal, J. J. (2006) "Computational and mutational analysis of human glutaredoxin (thioltransferase): probing the molecular basis of the low pK_a of cysteine 22 and its role in catalysis" *Biochemistry* **45,** 4785-4796.

Jeng, M. F., Holmgren, A. &Dyson, J. (1995) "Proton sharing between cysteine thiols in *Escherichia coli* thioredoxin: implications for the mechanism of protein disulfide reduction" *Biochemistry* **34,** 10101-10105.

Jeong, J. S., Kwon, S. J., Kang, S. W., Rhee, S. G. & Kim, K. (1999) "Purification and characterization of a second type thioredoxin peroxidase (type II TPx) from *Saccharomyces cerevisiae*" *Biochemistry* **38,** 776-783.

Johansson, C., Kavanagh, K. L., Gileadi, O. & Oppermann, U. (2007) "Reversible sequestration of active site cysteines in a 2Fe2S-bridge dimmer provides a mechanism for glutaredoxin 2 regulation in human mitochondria" *J. Biol. Chem.* **282,** 3077-3082.

Johansson, C., Lillig, C. H., & Holmgren, A. (2004) "Human mitochondrial glutaredoxin reduces S-glutathionylated proteins with high affinity accepting electrons from either glutathione or Thioredoxin reductase" *J. Biol. Chem*. **279**, 7537-7543.

Jones, T. Zou, J., Cowan, S. & Kjeldgaard, M. (1991) "Improved methods for binding protein models in electron density maps and the location of errors in these models" *Acta Crystallog. sect. A*, **47**, 110-119.

Kabush, W. (1988) "Evaluation of single-crystal X-ray diffraction data from a position-sensitive detector" *J. Appl. Crystallog.* **21**, 916-924.

Katti, S. K., Robbins, A. H., Yang, Y., Wells, W. W. (1995) "Crystal structure of thioltransferase at 2.2 Å resolution" *Protein Sci*. **4,** 1998-2005.

Kemmink, J., Darby, N.J., Dijkstra, K., Nilges, M. & Creighton, T.E. (1997) "The folding catalyst protein disulfide isomerase is constructed of active and inactive thioredoxin modules" *Curr. Biol.* **7**, 239-245.

Kortemme, T. & Creigthon, T.E. (1995) "Ionisation of cysteine residues at the termini of model α-helical peptides. Relevance to unusual thiol pK_a values in proteins of the Thioredoxin family" *J. Mol. Biol.* **253,** 799-812.

Kosower, N. S., Kosower, E. M., Newton, G. L. & Ranney, H. M. (1979) "Bimane fluorescent labels: labeling of normal human red cells under physiological conditions" *Proc. Natl Acad. Sci. USA* **76,** 3382–3386.

Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993) "PROCHECK: a program to check the stereochemical quality of protein structures" *J. Appl. Crystallog.* **26,** 283-291.

Lazzeretti, P & Zanasi, R. (1997) "On the calculation of the parity-violating energies in hydrogen peroxide and hydrogen disulphide molecules within the random-phase approximation" *Chem. Phys. Lett.* **279,** 349-354.

Leslie, A.G.W. (1992) *Joint CCP4/ESF-BCB News. Protein Crystallogr.* ***26***. Daresbury Laboratory, Warrington, UK.

Lillig, C. H., Berndt, C. & Holmgren, A. (2008) “Glutaredoxin Systems” *Biochim. Biophys. Acta*. **1780,** 1304-1317.

López-Maribal, H.R. & Winther, J. R. (2008) “Redox characteristics of the eukaryotic cytosol” *Biochim. Biophys. Acta.* **1783,** 629-640.

Lopreiato, R., Facchin, S., Sartori, G., Arrigoni, G., Casonato, S., Ruzzene, M., Pinna, L. A. & Carignani, G. (2004) “Analysis of the interaction between piD261/Bud32, na evolutionarily conserved protein kinase of *Saccharomyces cerevisiae*, and the Grx4 glutaredoxin” *Biochem. J.* **377,** 395-405.

Luikenhuis, S., Perrone, G., Dawes, I. W. & Grant, C. M. (1998) “The yeast *Saccharomyces cerevisiae* contains two glutaredoxin genes that are required for protection against reactive oxygen species” *Mol. Biol. Cell.* **9,** 1081-1091.

Martin, J.L. (1995) “Thioredoxin - a fold for all reasons” *Structure* **3,** 245-250.

Martin, J. L., Bardwell, J. C. & Kuriyan, J. (1993) “Crystal structure of DsbA protein required for disulfide bond formation in vivo” *Nature* **365,** 464-468.

Matthews, B. W. (1968) “Solvent content of protein crystals” *J. Mol. Biol*. **33,** 491–497.

Mesecke, N., Spang, A., Deponte, M. & Herrmann, J, M. (2008a) “A novel group of glutaredoxins in the cis-Golgi critical for oxidative stress resistance” *Mol. Biol. Cell.* **19,** 2673-2680.

Mesecke, N., Mittler, S., Eckers, E., Herrmann, J. M. & Deponte, M. (2008b) ”Two novel monothiol glutaredoxins from *Saccharomyces cerevisiae* provide further insight into Iron-Sulfur cluster binding, oligomerization, and enzymatic activity of glutaredoxins” *Biochemistry* **47,** 1452-1463.

Mieyal, J. J., Starke, D. W., Gravina, S. A. & Hocevar, B. (1991) “Thioltransferase in human red blood cells: kinetics and equilibrium” *Biochemistry* **30,** 8883-8891.

Molina, M. M., Bellí, G., Torre, M. A., Rodríguez-Manzaneque, M. T. & Herrero, E. (2004) “Nuclear monothiol glutaredoxins of *Saccharomyces cerevisiae* can function as mitochondrial glutaredoxins” *J. Biol. Chem.* **279,** 51923-51930.

Mössner, E., Huber-Wunderlich, M. & Glockshuber, R. (1998) “Characterization of *Escherichia coli* thioredoxin variants mimicking the active-sites of other thiol-disulfide oxidoreductases” *Protein Sci.* **7,** 1233-1244.

Muller, E. G. D. (1996) “A glutathione reductase mutant of yeast accumulates high levels of oxidized glutathione and requires thioredoxin for growth” *Mol. Biol. Cell*. **7**, 1805-1813.

Munhoz, D. C. & Netto, L. E. S. (2004) “Cytosolic thioredoxin peroxidase I and II are important defenses of yeast against organic hydroperoxide insult” *J. Biol. Chem.* **279,** 35219-35227.

Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997) “Refinement of macromolecular structures by the maximum-likehood method” *Acta Crystallog. sect. D*, **53,** 240-255.

Navaza, J. (1994) “AmoRe: an automated package for molecular replacement” *Acta Crystallog. sect. D*, **57,** 1367-1372.

Nikkola, M., Gleason, F. K., Saarinen, M., Joelson, T., Björnberg, O. & Eklund, H. (1991) ”A putative glutathione-binding site in T4 glutaredoxin investigated by site-directed mutagenesis” *J. Biol. Chem*. **266,** 16105-16112.

Nordstrand, K., Åslund, F., Holmgren, A., Otting, G. & Berndt, K. D. (1999) ”NMR structure of *Escherichia coli* glutaredoxin 3 – glutathione mixed disulfide complex: implications for the enzymatic mechanism” *J. Mol. Biol.* **286**, 541-552.

Nordstrand, K., Sandström, A., Aslund, F., Holmgren, A., Otting, G. & Berndt, K.D. (2000) “NMR structure of oxidized glutaredoxin 3 from *Escherichia coli*” *J. Mol. Biol.* **303**, 423-432.

Ogusucu, R., Rettori, D., Munhoz, D.C., Netto, L.E.S. & Augusto, O. (2007) “Reactions of yeast thioredoxin peroxidases I and II with hydrogen peroxide and peroxinitrite: rate constants by competitive kinetics” *Free Radic. Biol. Med.* **42,** 326-334.

Ojeda, L., Keller, G., Muhlenhoff, U., Rutherford, J. C., Lill, L. & Winge, D. R. (2006) “Role of glutaredoxin 3 and glutaredoxin 4 in the iron regulation of Aft1 transcriptional activator in *Saccharomyces cerevisiae” J. Biol. Chem.* **281,** 17661-17669.

Outten, C. E. & Culotta, V. C. (2004) “Alternative start sites in the *Saccharomyces cerevisiae GLR1* gene are responsible for mitochondrial and cytosolic isoforms of glutathione reductase” *J. Biol. Chem.* **279,** 7785-7791.

Pan, J.L. & Bardwell, J.C.A. (2006) “The origami of thioredoxin-like folds” *Protein Sci.* **15,** 2217-2227.

Pedrajas, J. R., Kosmidou, E., Miranda-Vizuete, A., Gustafsson, J., Wright, A. P. H. & Spyrou, G. (1999) “Identification and functional characterization of a novel mitochondrial thioredoxin system in *Saccharomyces cerevisiae*” *J. Biol. Chem.* **274,** 6366-6373.

Pedrajas, J. R., Porras, P., Martínez-Galisteo, E., Padilla, C. A., Miranda-Vizuete, A. & Bárcena, J. A. (2002) “Two isoforms of *Saccharomyces cerevisiae* glutaredoxin 2 are expressed *in vivo* and localize to different subcellular compartments” *Biochem. J.* **364,** 617-623.

Peltoniemi, M. J., Karala, A. R., Jurvansuu, J. K., Kinnula, V. L. & Ruddock, L. W. (2006) "Insights into deglutathionylation reactions. Different intermediates in the glutaredoxin and protein disulfide isomerase catalysed reactions are defined by the γ-linkage present in glutathione" *J. Biol. Chem.* **281,** 33107-33114.

Penninckx, M. J. (2002) "An overview on glutathione in *Saccharomyces* versus non-conventional yeasts" *FEMS Yeast Res.* **2**, 295-305.

Po, H. N., Senozan, N. M. (2001) "Henderson-Hasselbalch Equation: Its History and Limitations" *J. Chem. Edu*. **78,** 1499-1503.

Poole, L. B., Karplus, P. A. & Claiborne, A. (2004) "Protein sulfenic acids in redox signaling" *Annu. Rev. Pharmacol. Toxicol.* **34,** 325-347.

Porras, P., Padilla, C. A., Krayl, M., Voos, W. & Barcena, J. A. (2006) "One single in-frame AUG codon is responsible for a diversity of subcellular localizations of glutaredoxin 2 in *Saccharomyces cerevisiae*" *J. Biol. Chem.* **281**, 16551-16562.

Pujol-Carrion, N., Bellí, G., Herrero, E., Nogues, A. & Torre-Ruiz, M. A. (2006) "Glutaredoxins Grx3 e Grx4 regulate nuclear localisation of Aft1 and the oxidative stress response in *Saccharomyces cerevisiae*" *J. Cell Sci.* **119,** 4554-4564.

Rabenstein, D. L. & Millis, K. K. (1995) "Nuclear magnetic resonance study of the thioltransferase-catalysed glutathione/glutathione disulfide interchange reaction" *Biochem. Biophys. Acta* **1249,** 29-36.

Read, R. J. (2001) "Pushing the boundaries of molecular replacement with maximum likelihood" *Acta Crystallog. sect. D*, **57,** 1373-1382.

Ren, G., Stephan, D., Xu, Z., Zheng, Y., Tang, D., Harrison, R. S., Kurz, M., Jarrot, R., Shouldice, S. R., Hiniker, A., Martin, J. L., Heras, B. & Bardwell, J. C. (2009) "Properties of the thioredoxin fold superfamily are modulated by a single amino acid residue" *J. Biol. Chem*. **284,** 10150-10159.

Riestch, A., Beckwith, J. (1998) "The genetics of disulfide bond metabolism" *Annu. Rev. Genet*. **32,** 163-184.

Ritz, D. & Beckwith, J. (2001) "Roles of thiol-redox pathways in bacteria" *Annu. Rev. Microbiol.* **55,** 21-48.

Rodríguez-Manzaneque, M. T., Ros, J., Cabiscol, E., Sorribas, A. & Herrero, E. (1999) "Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in *Saccharomyces cerevisiae" Mol. Cell Biol.* **19,** 8180-8190.

Rodríguez-Manzaneque, M. T., Tamarati, J., Belli, G., Ros, J. & Herrero, E. (2002) "Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes" *Mol. Biol. Cell.* **13,** 1109-1121.

Rouhier, N., Unno, H., Bandyopadhyay, S., Masip, L., Kim, S., Hirasawa, M., Gualberto, J.M., Lattard, V., Kusunoki, M., Knaff, D.B., Georgiou, G., Hase, T., Johnson, M.K. & Jacquot, J.P. (2007) "Functional, structural, and spectroscopic characterization of a glutathione-ligated [2Fe-2S] cluster in poplar glutaredoxin C1" *Proc. Natl. Acad. Sci. USA* **104,** 7379-7384.

Saaranen, M. J., Salo, K. E. H., Latva-Ranta, M. K., Kinnula, V. L. & Ruddock, L. W. (2009) "The C-terminal active site cysteine of *Escherichia coli* glutaredoxin 1 determines the glutathione specificity of the second step of peptide deglutathionylation" *Antioxid. Redox Signal.* **11,** DOI: 10.1089/ars.2008.2387.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) "Molecular Cloning: A Laboratory Manual" 2nd ed. Cold Spring Harbor Laboratory, New York.

Schafer, F. Q. & Buettner, G. R. (2001) "Redox environment of the cell as viewed through the redox state of glutathione disulfide/glutathione couple" *Free Rad. Biol. Med.* **30,** 1191-1212.

Segel, H. I. (1975) "Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-States Enzyme Systems" Wiley, New York.

Shelton, M. D., Chock, P. B. & Mieyal, J. J. (2005) "Glutaredoxin: role in reversible protein S-Glutathionylation and regulation of redox signal transduction and protein translocation" *Antioxid. Redox Signal.* **7,** 348-366.

Shenton, D., Perrone, G., Quinn, K. A., Dawes, I. W. & Grant, C. M. (2002) "Regulation of protein S-thiolation by glutaredoxin 5 in the yeast *Saccharomyces cerevisiae*" *J. Biol. Chem*. **277,** 16853-16859.

Silva, G. M., Netto, L. E. S., Discola, K. F., Piassa-Filho, G. M., Pimenta, D. C., Bárcena, J. A. & Demasi, M. (2008) "Role of glutaredoxin 2 and cytosolic thioredoxins in cysteinyl-based redox modification of the 20S proteasome" *FEBS J.* **275,** 2942-2955.

Sodano, P., Xia, T.H., Bushweller, J. H., Björnberg, O., Holmgren, A., Billeter, M. & Wüthrich, K. (1991) "Sequence-specific 1H n.m.r. assignments and determination of the three-dimensional structure of reduced *Escherichia coli* glutaredoxin" *J. Mol. Biol*. **221,** 1311-1324.

Srinivasan, U., Mieyal, P. A. & Mieyal, J. J. (1997) "pH profiles indicative of rate-limiting nucleophilic displacement in thioltransferase catalysis" *Biochemistry* **36,** 3199-3206.

Streitwieser, A., Heathcock, C.H. & Kosower, E.M. (1998) "Introduction to organic chemistry" (Revised printing) 4th ed. Prentice-Hall.

Sun, C., Berardi, M. J. & Bushweller, J. H. (1998) "The NMR solution structure of human glutaredoxin in the fully reduced form" *J. Mol. Biol*. **280**, 687-701.

Szajewski, R. P. & Whitesides, G. M. (1980) "Rate constants and equilibrium constants for thiol-disulfide interchange reactions involving oxidized glutathione" *J. Am. Chem. Soc.* **102,** 2011-2026.

Trotter, E. W., Grant, C. M. (2003) "Non-reciprocal regulation of the redox state of the glutathione-glutaredoxin and thioredoxin systems" *EMBO reports* **4**, 184-188.

Trujillo, M., Clippe, A., Manta, B., Ferrer-Sueta, G., Smeets, A., Declercq, J., Knoops, B. & Radi, R. (2007) "Pre-steady state kinetic characterization of human peroxiredoxin 5: Taking advantage of Trp84 fluorescence increase upon oxidation" *Arch. Biochem. Biophys.* **467,** 95-106.

Vilella, F., Alves, R., Rodríguez-Manzaneque, M. T., Bellí, G., Swaminathan, S., Sunnerhagen, P. & Herrero, E. (2004) "Evolution and cellular function of monothiol glutaredoxins: involvement in iron-sulphur cluster assembly" *Comp. Funct. Genom.* **5,** 328-341.

Vlamis-Gardikas, A., Åslund, F., Spyrou, G., Bergman, T. & Holmgren, A. (1997) "Cloning, overexpression and characterization of glutaredoxin 2, an atypical glutaredoxin from *Escherichia coli*" *J. Biol. Chem.* **272,** 11236-11243.

Voet, D. & Voet, J.G. (2004) "Biochemistry" 3rd Ed. John Wiley & Sons.

Xia, T.H., Bushweller, J.H., Sodano, P., Billeter, M., Björnberg, O., Holmgren, A. & Wüthrich, K. (1992) "NMR structure of oxidized *Escherichia coli* glutaredoxin: comparison with reduced *E. coli* glutaredoxin and functionally related proteins" *Protein Sci*. **1**, 310-321.

Wells, W. W., Xu, D. P., Yng, Y. & Rocque, P. A. (1990) "Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity" *J. Biol. Chem.* **265**, 15361-15364.

Winn, M. D., Isupov, M. N. & Murshudov, G. N. (2001) "Use of TLS parameters to model anisotropic displacements in macromolecular refinement" *Acta Crystallog. sect. D*, **57**, 122-133.

Wood, Z.A., Schröder, E., Harris, J.R. & Poole, L.B. (2003) "Structure, mechanism and regulation of peroxiredoxins" *Trends Biochem. Sci*. **28,** 32-40.

Yang, Y., Jao, S., Nanduri, S., Starke, D. W., Mieyal, J. & Qin, J. (1998) "Reactivity of the human thioltransferase (glutaredoxin) C7S, C25S, C78S, C82S mutant and NMR solution structure of its glutathionyl mixed disulfide intermediate reflect catalytic specificity" *Biochemistry* **37,** 17145-17156.

Yang, Y. & Wells, W. W. (1991) "Identification and characterization of the functional aminoacids at the active center of pig liver thioltransferase by site-directed mutagenesis" *J. Biol. Chem.* **266,** 12759-12765.

Yu, J., Zhang, N., Yin, P., Cui, P. & Zhou, C. (2008) "Glutathionylation-triggered conformational changes of glutaredoxin Grx1 from the yeast *Saccharomyces cerevisiae*" *Prot. Struct. Funct. Bioinf.* **72,** 1077-1083.

Anexo 1

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Structural Aspects of the Distinct Biochemical Properties of Glutaredoxin 1 and Glutaredoxin 2 from *Saccharomyces cerevisiae*

Karen Fulan Discola[1,2], Marcos Antonio de Oliveira[2,3], José Renato Rosa Cussiol[2], Gisele Monteiro[2], José Antonio Bárcena[4], Pablo Porras[4], C. Alicia Padilla[4], Beatriz Gomes Guimarães[5] and Luis Eduardo Soares Netto[2]*

[1]*Departamento de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas, 13083-970 Campinas, Brazil*

[2]*Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, 05508-900 São Paulo, Brazil*

[3]*Departamento de Biologia, Universidade Estadual Paulista, 11330-900 São Vicente, Brazil*

[4]*Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, 14071 Córdoba, Spain*

[5]*Laboratório Nacional de Luz Síncrotron, Campinas, Brazil*

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Glutaredoxins (Grxs) are small (9–12 kDa) heat-stable proteins that are ubiquitously distributed. In *Saccharomyces cerevisiae*, seven Grx enzymes have been identified. Two of them (yGrx1 and yGrx2) are dithiolic, possessing a conserved Cys-Pro-Tyr-Cys motif. Here, we show that yGrx2 has a specific activity 15 times higher than that of yGrx1, although these two oxidoreductases share 64% identity and 85% similarity with respect to their amino acid sequences. Further characterization of the enzymatic activities through two-substrate kinetics analysis revealed that yGrx2 possesses a lower K_M for glutathione and a higher turnover than yGrx1. To better comprehend these biochemical differences, the pK_a of the N-terminal active-site cysteines (Cys27) of these two proteins and of the yGrx2-C30S mutant were determined. Since the pK_a values of the yGrx1 and yGrx2 Cys27 residues are very similar, these parameters cannot account for the difference observed between their specific activities. Therefore, crystal structures of yGrx2 in the oxidized form and with a glutathionyl mixed disulfide were determined at resolutions of 2.05 and 1.91 Å, respectively. Comparisons of yGrx2 structures with the recently determined structures of yGrx1 provided insights into their remarkable functional divergence. We hypothesize that the substitutions of Ser23 and Gln52 in yGrx1 by Ala23 and Glu52 in yGrx2 modify the capability of the active-site C-terminal cysteine to attack the mixed disulfide between the N-terminal active-site cysteine and the glutathione molecule. Mutagenesis studies supported this hypothesis. The observed structural and functional differences between yGrx1 and yGrx2 may reflect variations in substrate specificity.

Keywords: glutaredoxin; *Saccharomyces cerevisiae*; glutathione; disulfide; X-ray structure

*Corresponding author. E-mail address: nettoles@ib.usp.br.
Present address: Synchrotron SOLEIL, Saint-Aubin, France.

Abbreviations used: Grx, glutaredoxin; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione, glutathione disulfide; HED, β-hydroxyethyl disulfide; β-ME-SG, glutathionylated β-mercaptoethanol; *t*-BOOH, *tert*-butyl-hydroperoxide; yGrx1, glutaredoxin 1 from yeast; $yGrx1_{GS}$, yeast Grx1-C30S mutant glutathionylated; $yGrx1_{red}$, yeast Grx1 reduced; yGrx2, glutaredoxin 2 from yeast; $yGrx2_{GS}$, yeast Grx2-C30S mutant glutathionylated; $yGrx2_{ox}$, yeast Grx2 oxidized; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

Introduction

Glutaredoxins (Grxs) are small, heat-stable oxidoreductases with conserved cysteine residues present in CXXC motifs.[1] These oxidoreductases are reduced by glutathione (GSH), producing glutathione disulfide (GSSG) that is then reduced by NADPH in a reaction catalyzed by glutathione reductase (GR).[1] Grxs were first discovered in *Escherichia coli* as dithiol, GSH-dependent hydrogen donors for ribonucleotide reductase in a mutant lacking thioredoxin.[2] Later, it was described that Grxs can also reduce mixed disulfides between proteins or low molecular weight thiols and GSH in reactions that require only their N-terminal active-site cysteine.[3–5] It is important to note that the reduction of glutathionylated substrates through the monothiol mechanism seems to be the major activity of Grxs; all dithiol Grxs described so far catalyze these reactions, but not all dithiol Grxs catalyze the reduction of protein disulfides by the dithiol mechanism.[6] Therefore, Grxs are receiving increasing attention in redox regulation processes due to their ability to catalyze the reduction of disulfides.[7]

In the monothiol mechanism, Grxs specifically interact with the GSH moiety of the protein-SG mixed disulfide target, using only the N-terminal cysteine of the CXXC motif (Fig. 1, reaction a).[5,9] A covalent Grx-SG mixed intermediate is formed and the target protein is released in its reduced form. Then, a second molecule of GSH reduces the Grx-SG mixed intermediate, generating reduced Grx and GSSG (Fig. 1, reaction b).[10] GSSG is then reduced by GR at the expense of NADPH.

The reduction of the Grx-SG mixed disulfide by the second GSH molecule (Fig. 1, reaction b) is the rate-determining step of the overall process.[8] If the reduction of the Grx-SG by an external nucleophile such as GSH is favored, the reaction tends to proceed by the monothiol mechanism. However, if the nucleophilic attack of the C-terminal cysteine on the Grx-SG is favored, a side reaction can occur and produce oxidized Grx with an intramolecular disulfide bond (Fig. 1, reaction c). In this case, the reaction proceeds through the dithiol mechanism, and the disulfide bond of Grx is reduced by two GSH molecules.[11,12]

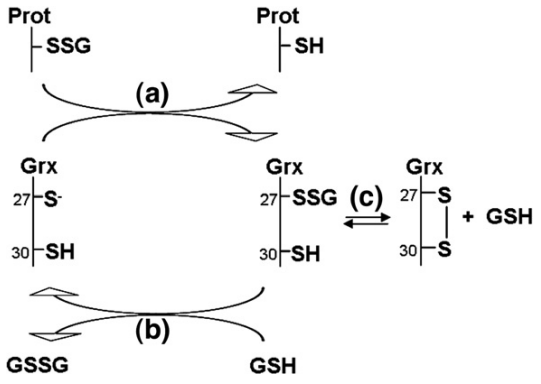

Fig. 1. Scheme of Grx monothiol mechanism. (a) The N-terminal cysteine of the Grx active site interacts with the GSH moiety of the protein-SG mixed disulfide target, then a covalent Grx-SG mixed intermediate is formed and the target protein is released in its reduced form. (b) A second molecule of GSH reduces the Grx-SG mixed intermediate, generating reduced Grx and glutathione disulfide (GSSG). (c) The oxidized form of Grx with an intramolecular disulfide bond can be formed by the nucleophilic attack of Cys30 and is reduced back by two GSH molecules. Adapted from Srinivasan *et al.*[8]

Another common feature of Grxs is that the pK_a of their N-terminal active-site cysteine thiol is generally very low (around 3.0 to 4.0). Therefore, most of these cysteine residues are deprotonated at physiological pH, whereas the C-terminal active-site cysteine is usually protonated.[12–14] The thiolate form of the N-terminal cysteine of Grx appears to contribute to its catalytic activity because it is a good leaving group (Fig. 1, reaction b) rather than a good nucleophile (Fig. 1, reaction a), which is in line with the observation that reaction b is the rate-limiting step.[8,12]

In *Saccharomyces cerevisiae*, seven Grx genes have been identified (*GRX1–7*). Glutaredoxin 1 and 2 from yeast (yGrx1 and yGrx2) are dithiolic Grxs, which contain the conserved CPYC motif in their active sites.[15] yGrx2 is present in two molecular weight isoforms (11,900 and 15,900), each synthesized by one of two in-frame translation initiation start sites.[16] The cytosolic isoform is synthesized from the second AUG and lacks an N-terminal extension, while the long isoform that carries a mitochondrial targeting presequence is translated from the first AUG.[17] The latter is translocated to the mitochondria where it is either processed by the mitochondrial processing peptidase to a short soluble isoform that localizes to the matrix or left unprocessed and retained in the outer mitochondrial membrane.[17] yGrx3, yGrx4 and yGrx5 are monothiol isoforms that contain the motif CGFS in their active sites.[18] yGrx3 and yGrx4 are required for Aft1 iron regulation in the nucleus, whereas yGrx5 is thought to be involved in iron–sulfur cluster metabolism and assembly.[19,20] Recently, two other monothiolic enzymes, yGrx6 and yGrx7, were described in *S. cerevisiae*.[21]

The current work is focused on the two dithiolic enzymes, yGrx1 and the short isoform of yGrx2. These proteins are highly similar (64% identity and 85% similarity), although single deletions of their genes have rendered yeast mutants with distinct phenotypes.[15] Δ*GRX1* is sensitive to oxidative stress caused by superoxide anion, whereas Δ*GRX2* is sensitive to stress induced by hydrogen peroxide.[15] Furthermore, studies with yeast knockouts have indicated that yGrx2 accounts for most of the GSH-dependent oxidoreductase activity in the cell.[15] Since yGrx1 and yGrx2 expression levels are similar,[15] the exhibited phenotypes in knockout strains could reflect that yGrx2 is a more efficient enzyme than yGrx1.

We compared recombinant dithiolic Grxs from yeast and observed that the specific activity of yGrx2

was 15 times higher than that of yGrx1 in the standard β-hydroxyethyl disulfide (HED) assay, despite their high amino acid sequence similarity. Bisubstrate kinetics analysis indicated that both K_M toward GSH and turnover rate account for this enzymatic difference. In an attempt to better understand the functional differences between yGrx1 and yGrx2, we elucidated the crystallographic structure of yGrx2 in the oxidized state and of the yGrx2-C30S mutant in complex with GSH through a mixed disulfide. Comparisons between these structures and those of yGrx1[22] revealed that residues near the active-site region could be directly implicated in the functional differences of yGrx1 and yGrx2. Mutagenesis studies supported this hypothesis.

Results and Discussion

yGrx2 is more active as an oxidoreductase than yGrx1

Yeast cell-free extracts from Δ*GRX2* but not from Δ*GRX1* lose most of their GSH-dependent oxidoreductase activity,[15] indicating that yGrx2 accounts for the majority of this enzymatic activity. Based on this observation, we measured the specific activities of pure recombinant yeast dithiolic Grxs, using the standard assay of reduction of the mixed disulfide formed between HED and GSH (glutathionylated β-mercaptoethanol, β-ME-SG).[23] yGrx2 was approximately 15 times more active than yGrx1 (Table 1, Supplementary Data), although they share a high level of sequence similarity (Fig. 2a).

To eliminate any artifacts due to metal contamination, which could result from leaching of nickel during affinity chromatography, we compared the activity results of yGrx1 and yGrx2 purified with cobalt-affinity chromatography. Although cobalt binds more strongly to a Talon affinity column (Clontech) than nickel to a Hi-Trap affinity column (GE Healthcare), the specific activities were essentially the same. Furthermore, we also removed the N-terminal his-tag from the yGrx1 and yGrx2 recombinant proteins, but their oxidoreductase activities remained the same. Therefore, yGrx2 is intrinsically more active than yGrx1, which explains results of previous studies on yeast knockout strains.[15]

Two-substrate kinetics for yGrx1 and yGrx2

In order to better characterize the activity of yeast dithiol Grxs, we varied the concentration of HED at different concentrations of GSH and determined the apparent K_M and k_{cat} of yGrx1 and yGrx2 for β-ME-SG (Table 2).[21,26] β-ME-SG was formed during a 3-min preincubation and the reactions were started with the addition of Grx. We confirmed in separate experiments that the GR concentration used was not rate limiting (data not shown). The apparent K_M and V_{max} values were determined by non-linear regression of Michaelis–Menten plots (see Supplementary Data). yGrx1 presented higher affinity (lower apparent K_M values) for β-ME-SG than yGrx2, but a lower turnover number (lower k_{cat} values) than yGrx2 (Table 2). These parameters are related to the formation of the Grx-SG mixed disulfide (Fig. 1, reaction a). As a consequence, the catalytic efficiency (k_{cat}/K_M in M^{-1} s^{-1}) of yGrx1 is approximately five times lower compared with yGrx2.

Kinetic data linearization by Lineweaver–Burk resulted in intersecting lines on double-reciprocal plots (data not shown), which indicates a sequential mechanism for both yGrx1 and yGrx2. Similar patterns were obtained for yGrx7 and human Grx from red blood cells in the HED assay, which confirm that the β-ME-SG formation in the preincubation reaction is nonenzymatic.[21,26]

We built secondary plots with the reciprocal values of V_{max}^{app} *versus* the reciprocal of GSH concentrations to obtain the "true" kinetics parameters (Fig. 3a and b).[27] yGrx2 has a lower K_M for GSH than yGrx1 and a higher turnover number, resulting in a

Table 1. Values of specific activity* and pK_a of Cys27 for yGrx1, yGrx2, and the mutants yGrx1-C30S, yGrx1-S23A, yGrx1-S23A-Q52E, yGrx2-C30S, yGrx2-A23S and yGrx2-A23S-E52Q

Protein	Specific activity (μmol min^{-1} mg^{-1})	pK_a Cys27[a]	
		Monobromobimane alkylation	Iodoacetamide inactivation
Grx1	8.2±0.3	3.2±0.2	4.0±0.2
Grx1-C30S	38.7±0.5	N.D.	N.D.
Grx1-S23A	27.8±0.5	N.D.	N.D.
Grx1-S23A-Q52E	24.5±0.4	N.D.	N.D.
Grx2	125±7	3.1±0.2	3.5±0.2
Grx2-C30S	38±1	N.D.	3.2±0.2
Grx2-A23S	74±5	N.D.	N.D.
Grx2-A23S-E52Q	54±2	N.D.	N.D.

* The reaction mixture for the determination of specific activities contained 100 mM Tris–HCl pH 7.4, 6 μg/ml GR, 1 mM GSH, 0.7 mM HED, 0.1 mg/ml BSA, 0.2 mM NADPH and 2 mM EDTA. For more details, see Materials and Methods. N.D., not determined.

[a] pK_a values were obtained from inflection points in sigmoidal dose–response curves with Hill slope equal to 1 as described in Materials and Methods and Supplementary Material.

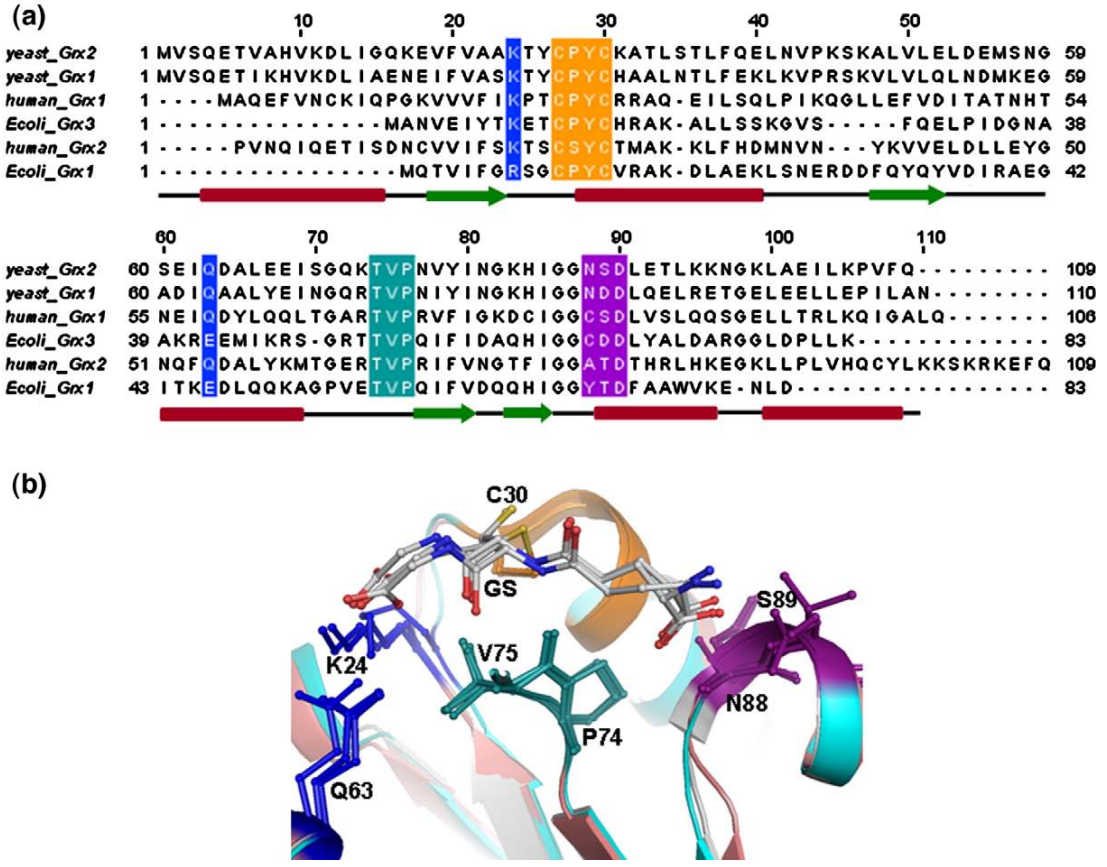

Fig. 2. Alignment of Grxs with glutathionylated structures. (a) Sequences were aligned using ClustalW[24] and the alignment representation was produced with Jalview.[25] The active-site residues of Grxs are highlighted with an orange rectangle and residues within the blue rectangles are involved in interactions with the Gly residue from GSH. The green rectangle indicates residues that form the TVP motif involved in interactions with the Cys residue from GSH, and within the violet rectangle are the residues participating in interactions with the GSH γ-Glu residue. The secondary-structure elements of yGrx2 are indicated at the bottom of the figure (arrows representing β-sheets and cylinders for α-helices). (b) Structural superposition of yGrx2 (cyan), yGrx1 (gray) and human Grx2 (pink) glutathionylated structures. The residues involved in interactions with the GSH moiety are identified with the sequence number of yGrx2, and residues highlighted in (a) are shown in the same colours.

catalytic efficiency 2 orders of magnitude higher than that of yGrx1 (Table 3). Since these parameters are related to the rate-limiting step (Fig. 1, reaction b) of the overall monothiol mechanism, both the lower K_M for GSH and higher turnover number are responsible for the high specific activity of yGrx2 (Table 3).

Properties of the yGrx1-C30S and yGrx2-C30S mutants

As previously described, the substitution of the C-terminal active-site cysteine by a serine residue in dithiolic Grxs does not abolish the GSH-dependent oxidoreductase activity in the HED assay because it

Table 2. Apparent kinetic constants for the reduction of the mixed disulfide formed between GSH and HED by yGrx1 and yGrx2, determined from Michaelis–Menten plots

yGrx1			yGrx2		
[GSH] (mM)	K_M^{app} (mM)	k_{cat}^{app} (s^{-1})	[GSH] (mM)	K_M^{app} (mM)	k_{cat} (s^{-1})
0.5	0.12	1.3	0.5	0.9	47
1.0	0.12	2.2	1.0	0.7	66
1.5	0.14	3.5	1.5	0.6	85
2.0	0.13	4.2			

The reaction mixture contained 100 mM Tris–HCl, pH 7.4, 6 μg/ml GR, 0.1 mg/ml BSA, 0.2 mM NADPH and 2 mM EDTA. The concentration of HED was varied from 0.03 to 2.2 mM at fixed concentrations of GSH, and the reactions were started by the addition of 500 or 50 nM of yGrx1 or yGrx2, respectively.

proceeds via the monothiol mechanism.[5,13,28] The yGrx1-C30S mutant presented higher specific activity than wild-type yGrx1, similarly to the mutants for the C-terminal active-site cysteine of human Grx1 and Grx2 and pig Grx (Table 1).[13,28,29] Nevertheless, the specific activity of yGrx2-C30S decreased to 38±2 μmol min^{-1} mg^{-1}, which corresponds to 30% of the wild-type yGrx2 activity (Table 1). This observation is very consistent with data previously reported for C14S mutants of Grx1 and Grx3 from *E. coli*.[5,11]

As mentioned before, it was described for human Grx1 that the overall reaction rate in the HED assay is determined by the rate of Grx-SG intermediate reduction.[8] The occurrence of the side reaction (Fig. 1, reaction c), which results in the formation of the intramolecular disulfide bond, is implicated in a decrease in the reaction rate, probably because part of the enzyme and GSH are involved in a nonproductive exchange reaction.[28] The substitution of the C-terminal active-site cysteine by a serine residue in dithiolic Grxs eliminates the possibility of this side reaction and in principle should increase the reaction rate. This was the case for yGrx1-C30S, pig Grx and the human Grx1 and Grx2 mutants.[13,28,29]

However, for yGrx2-C30S and *E. coli* Grx1 and Grx3, the same mutation causes a decrease in specific activity.[5,11] The reasons for this effect are not clear but might be related to differential structural features among different Grx isoforms. Future studies are necessary in order to better understand the particularities of these enzymes.

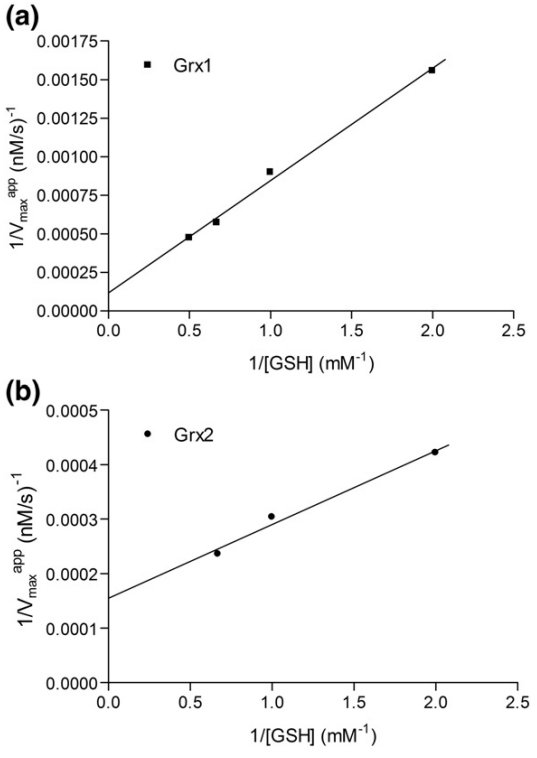

Fig. 3. Secondary plots (reciprocal values of V_{max}^{app} *versus* the reciprocal of GSH concentration) used for the determination of the kinetic constants of yGrx1 (a) and yGrx2 (b) for GSH.

Table 3. Kinetic constants for the reduction of the mixed disulfide formed between GSH and HED by yGrx1 and yGrx2, obtained with the secondary plots of $1/V_{max}^{app}$ *versus* 1/[GSH]

	GSH		
Protein	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M $(M.s)^{-1}$
Grx1	6.2	17.1	2.75×10^3
Grx2	0.9	129	1.43×10^5

The pK_a of reactive cysteines cannot explain the difference between the oxidoreductase activities of the dithiolic yGrxs

In the previous sections, it was demonstrated that yGrx2 possesses higher activity than yGrx1, which is related to both a lower K_M for GSH and higher turnover (k_{cat}). It is well known that the N-terminal active-site cysteine residue of Grxs possesses a very low pK_a value and that this feature is important for catalysis.[8,12–14] Therefore, a significant difference in the pK_a values of yGrx1 and yGrx2 might explain the variation in the reactivities between these two enzymes.

The pK_a relative to the thiol group of the N-terminal active-site cysteine (Cys27) of both yGrx2 and yGrx1 were determined by two independent methods (Table 1, Fig. 4). The values obtained by iodoacetamide inactivation are very similar to those previously determined for yGrx2 and human Grx1 using the same approach.[12,14] The substitution of the C-terminal cysteine to a serine in the yGrx2-C30S mutant resulted in a slight decrease (not significant) in the pK_a value of Cys27 (Table 1, Fig. 4a). Furthermore, analysis by specific thiolate monobromobimane alkylation (which generates a fluorescent adduct)[30] indicated that the pK_a values of Cys27 from yGrx1 and yGrx2 are even more similar if not identical (Table 1, Fig. 4b). In the case of yGrx1, although the pK_a values for yGrx1 Cys27 determined by the independent approaches presented some divergence (Table 1), both of them indicated that the corresponding sulfhydryl moiety should be present mostly as thiolate under physiological conditions.

Thiol–disulfide exchange reactions are expected to proceed as S_N2-type, in which the rate is dependent on the basicity (pK_as) of the nucleophile and the leaving groups[31,32] and the reactive specie is the ionized thiolate.[33] For a thiol–disulfide exchange reaction where the thiolate anion (SR_{nuc}) attacks one of the two sulfur atoms of the disulfide bond, the central sulfur (SR_c) will have the higher pK_a value.[8,12] As described by the following reaction,

$$SR_{nuc} + SR_{lg}SR_c \rightarrow SR_{nuc}SR_c + SR_{lg}$$

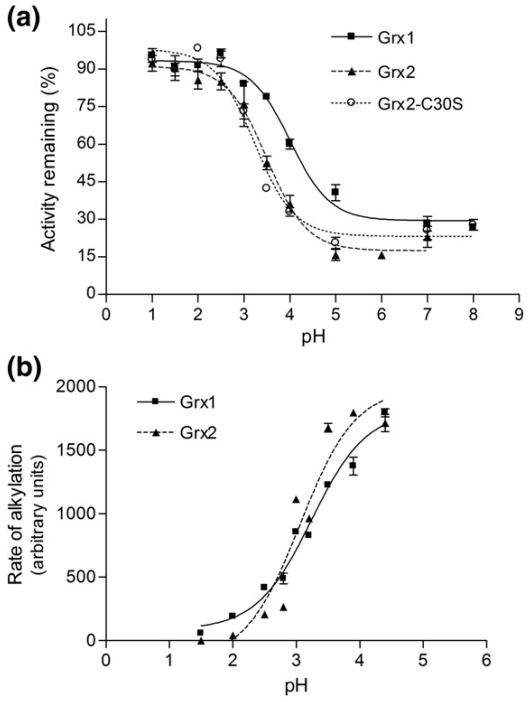

Fig. 4. Determination of pK_a values for reactive cysteines. (a) Iodoacetamide inactivation. yGrx1 (filled squares), yGrx2 (filled triangles) and yGrx2-C30S (open circles). The enzymes (250 nM yGrx1, 20 nM yGrx2, or 100 nM yGrx2-C30S) were incubated with 300 μM iodoacetamide at different pH values for 3 min at room temperature and then assayed using the standard assay for Grxs after a 100-fold dilution. The percentage of remaining activity at each pH was determined by comparing the activity of the enzyme incubated with and without iodoacetamide. All buffers were used at a concentration of 10 mM and ionic strength was adjusted with the addition of 0.5 M NaCl. (b) Monobromobimane alkylation. yGrx1 (filled squares) and yGrx2 (filled triangles). The prereduced yGrx1 and yGrx2 enzymes (10 μM) were alkylated with 2 μM of monobromobimane at different pH values, as described in Materials and Methods.

the lower the pK_a of the leaving sulfur (SR_{lg}), the faster the rate will be, according to the Brønsted equation: $\log k = c + \beta_{nuc} pK_{a(nuc)} + \beta_c pK_{a(c)} + \beta_{lg} pK_{a(lg)}$, where β_{nuc}, β_c and β_{lg} are the Brønsted base coefficients for the nucleophile, central group and leaving group, respectively.[8,12] Accordingly, for a given homologous set of thiol–disulfide exchange reactions, the second-order rate constant increases by a factor of four for each unit decrease in the pK_a of the leaving group ($\Delta k = 4\Delta^{pKa}$).[31,32] Assuming that GSH-mediated reduction of the Grx-SG intermediate is the rate-limiting step in the HED assay,[8] the difference in the pK_a values of yGrx1 and yGrx2 Cys27 residues would result in an increase of the rate constant of yGrx2 by 2.0-fold relative to that of yGrx1 when the results from iodoacetamide inactivation are considered, and an increase of 1.15-fold when the results from monobromobimane alkylation are considered (Table 1). However, the difference observed in the enzymatic activities is much higher than expected due to the difference in the pK_a values, suggesting the involvement of other factors, possibly structural features. Although the Brønsted equation applies quite well to low molecular weight thiols,[32] some deviation occurs in proteins,[12] further indicating that structural features besides pK_a values are important in controlling thiol–disulfide exchange rates.

It is important to mention that in this work we have assumed that the rate-limiting reaction in the HED assay for yGrxs is the reduction of the Grx-SG mixed disulfide intermediate, as described for human Grx1.[8] This is because yGrx2 activity presented a pH dependence similar to that of human Grx1, with a pH optimum value near 9.0.[14] Also, Cys27 from yGrx1 and yGrx2 presented pK_a values similar to that of human Grx1, suggesting that the thiolate pK_a of the second substrate (GSH) is involved in the rate-determining step.[8,26] Therefore, when all the information are taken together, pK_a values for yGrx1 and yGrx2 Cys27 should not be an important factor in the control of the overall reaction rate.

Crystal structures of yGrx2

Since the pK_a values of the reactive cysteines did not correlate with their intrinsic activities, we decided to compare the structural features of yGrx1 and yGrx2. The crystal structures of yGrx1 in the reduced and glutathionylated forms (named here as $yGrx1_{red}$ and $yGrx1_{GS}$, respectively) were recently reported,[22] and to perform comparisons, we crystallized yGrx2 and solved its structure in two different forms.

Before crystallization, yGrx2 was subjected to treatments with oxidizing [*tert*-butyl-hydroperoxide (*t*-BOOH), diamide and hydrogen peroxide] and reducing (DTT and GSH) agents in an attempt to obtain its structure in different oxidation states. The first structure was solved and refined at 2.05 Å resolution, corresponding to yGrx2 pretreated with *t*-BOOH (named herein as $yGrx2_{ox}$). The $yGrx2_{ox}$ structure was used as a search model to solve the other structures of yGrx2 under different treatments. After structure solving and refinement of all data sets, we observed that in all cases (even in proteins treated with reductants), the two cysteines in the active site were oxidized, forming an intramolecular disulfide bond. The alignment of these structures showed that they were identical, and electron density map analyses indicate that no oxidation of any other residue, except the active-site cysteines, was observed, not even in the structure of yGrx2 pretreated with *t*-BOOH. We then performed analysis on the oxidized structure obtained from the data set that presented the best resolution and statistics (Table 4). Other attempts were made to obtain yGrx2 structure in the reduced form, but all of them failed.

In another attempt to obtain the reduced structure of yGrx2, we constructed the yGrx2-C30S mutant, which was also used to obtain the yGrx2 structure

Table 4. Diffraction and refinement statistics of oxidized and glutathionylated yeast Grx2

	Oxidized ($yGrx2_{ox}$)	Glutathionylated ($yGrx2_{GS}$)
Diffraction data statistics		
Space group	$P4_12_12$	$P2_12_12_1$
Unit cell dimensions (Å)	$a=b=47.63$, $c=94.59$	$a=37.0076$, $b=45.2051$, $c=105.0348$
Resolution range (Å)	42.56–2.05 (2.16–2.05)	37.01–1.91 (2.01–1.91)
Measured reflections	90,136	247,826
Unique reflections	7295	14,326
Completeness (%)	99.8 (99.3)	99.7 (98.3)
Multiplicity (%)	12.3 (11.2)	8.6 (8.2)
R_{sym}	0.08 (0.294)	0.070 (0.389)
$\langle I/\sigma(I)\rangle$	28.9 (7.8)	23.6 (4.5)
Refinement statistics		
R_{factor}	0.198	0.196
R_{free}	0.230	0.262
No. of nonhydrogen atoms	899	1829
No. of water molecules	65	137
rmsd		
Bond lengths (Å)	0.012	0.012
Bond angles (Å)	1.450	1.392
Average *B*-factor (Å^2)		
Main chain	18.7	13.0
Side chain	22.2	16.9
Ramachandran analysis (%)		
Favored regions	97.9	95.8
Additionally allowed regions	2.1	4.2

The data for oxidized yGrx2 crystal were previously published and are shown here again for completeness.[34]

in a complex with GSH. Crystals of yGrx2-C30S with a glutathionyl mixed disulfide ($yGrx2_{GS}$) were obtained, and the structure was refined at 1.91 Å resolution (Table 4). However, again, we could not obtain crystals of reduced yGrx2-C30S after several trials.

$yGrx2_{ox}$ crystallizes in space group $P4_12_12$, with one molecule in the asymmetric unit, whereas glutathionylated yGrx2-C30S crystallizes in space group $P2_12_12_1$, with two molecules in the asymmetric unit (referred to as chains A and B). Structures were refined to final *R* values of *R*-factor = 0.198 and $R_{free}=0.230$ ($yGrx2_{ox}$) and *R*-factor = 0.196 and $R_{free}=0.262$ ($yGrx2_{GS}$). The high quality of the electron density maps allowed an accurate assignment of the entire polypeptide chain of $yGrx2_{ox}$ (109 residues). Chain A of $yGrx2_{GS}$ and residues 2 through 108 of $yGrx2_{GS}$ chain B were also assigned. Molecules of GSH were modeled as bound to each of the chains A and B in the $yGrx2_{GS}$ structure. Ramachandran analyses showed that 97.9% of the residues in the $yGrx2_{ox}$ and 95.8% of the residues in the $yGrx2_{GS}$ structures were in the most favored regions and that no residues were in disallowed regions.

The overall yGrx2 structure is similar to previously determined Grx structures with a central mixed four-stranded β-sheet flanked by five α-helices (Fig. 5). The active site is localized to the second α-helix and the loop that precedes it. The N-terminal active site cysteine, Cys27, is solvent exposed and the C-terminal Cys30 is buried one turn later along the helix α2 in the interior of the molecule. The distance between the γ-sulfur atoms of Cys27 and Cys30 in the intramolecular disulfide bond is 2.07 Å (Fig. 6a) and the distance between the γ-sulfur atoms of Cys27 and the glutathione cysteine (Cys_{GS}) in the mixed disulfide bond is 2.08 Å (Fig. 6b).

The $yGrx2_{ox}$ and $yGrx2_{GS}$ models have very similar tertiary structures (Fig. 5). The alignment of the $yGrx2_{ox}$ and $yGrx2_{GS}$ structures results in an overall rmsd of 0.59 Å for all 109 C^{α} atoms. Despite

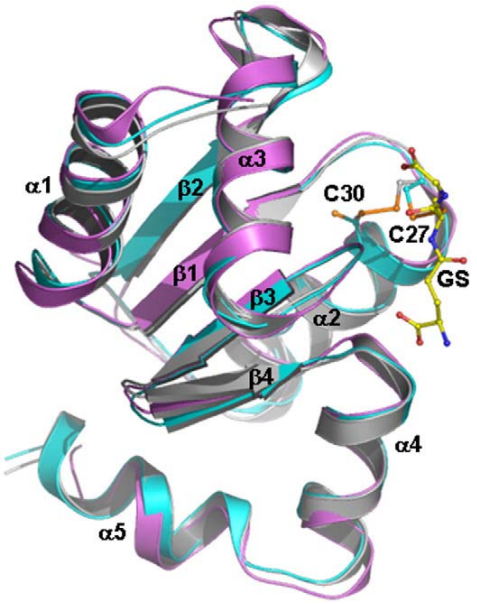

Fig. 5. Cartoon representation of the overall fold of yGrx1 and yGrx2 showing the structural alignment of $yGrx1_{GS}$ (α-carbon atoms in violet; PDB code 2JAC),[22] $yGrx2_{ox}$ (α-carbon atoms in gray) and $yGrx2_{GS}$ (α-carbon atoms in cyan). The GSH molecule bonded to the yGrx2-C30S mutant is shown in yellow.

the global similarity between $yGrx2_{ox}$ and $yGrx2_{GS}$, local conformational changes between structures of yGrx2 in the oxidized and glutathionylated forms occur in the active-site loop that is situated between the strand β1 and the helix α2. Several residues changed their conformation in the $yGrx2_{GS}$ structure, in comparison to the $yGrx2_{ox}$ structure, and interact with the GSH moiety (Fig. 7).

Both yGrx2 structures are very similar to those determined for yGrx1 in the reduced [Protein Data Bank (PDB) code 2JAD] and glutathionylated (PDB code 2JAC) forms (Fig. 5).[22] The alignment of $yGrx2_{GS}$ with $yGrx1_{GS}$ results in an rmsd of 0.7 Å for 108 C^{α} atoms of yGrx2, and the interactions between these proteins and the GSH moiety are very similar (see Supplementary Data). Other Grx structures have been determined with a glutathionyl mixed disulfide: human Grx1 (PDB code 1B4Q),[28] human Grx2 (PDB code 2FLS),[35] *E. coli* Grx1 (PDB code 1GRX)[9] and *E. coli* Grx3 (PDB code 3GRX).[11] Comparison of these structures shows that the Grx residues that interact with the GSH moiety are highly conserved (Fig. 2a and b). Nevertheless, there are differences among Grx enzymes and also between yGrx1 and yGrx2 (see below).

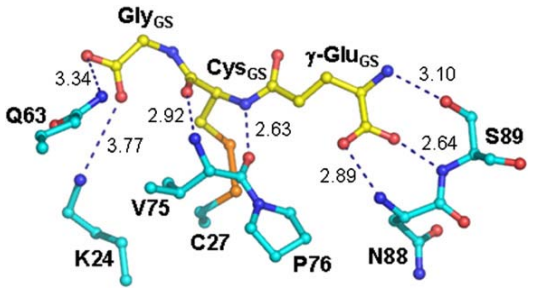

Fig. 7. yGrx2 interactions with GSH in the $yGrx2_{GS}$ structure. yGrx2 residues involved in interactions with the GSH moiety are represented in cyan and the GSH is shown in yellow. The hydrogen bonds and salt bridges are indicated by blue dashed lines, with the atomic distances in angstroms.

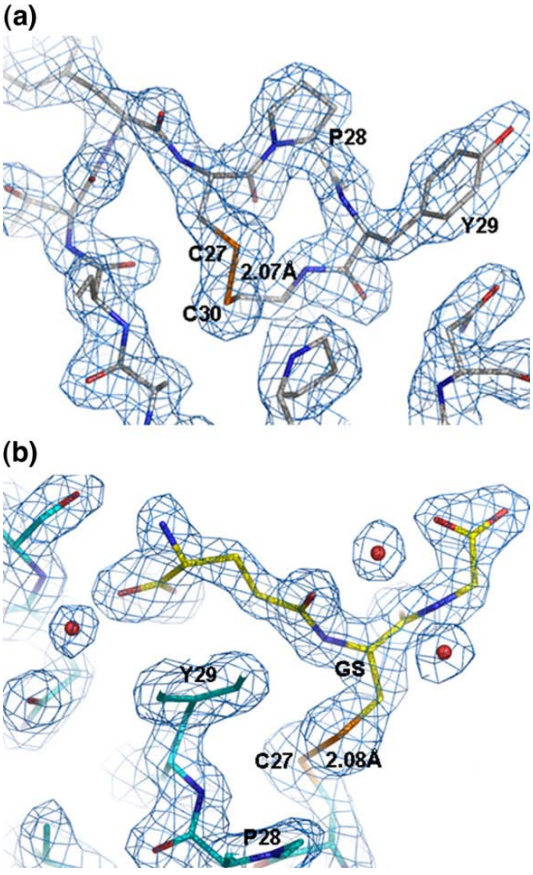

Fig. 6. Electron density maps ($2F_o - F_c$, contoured at 1.0σ) for the active site of (a) $yGrx2_{ox}$ and (b) $yGrx2_{GS}$. The continuous electron density between the sulfur atoms of Cys27 and Cys30 and Cys27 and the GSH cysteine residue show the disulfide bonds. The bond lengths of the intramolecular disulfide and the mixed disulfide with GSH are 2.07 and 2.08 Å, respectively.

The alignment of the $yGrx2_{GS}$ structure with the glutathionylated structures of human Grx1, human Grx2, *E. coli* Grx1 and *E. coli* Grx3 resulted in an overall rmsd of 1.67 Å (for 99 C^{α} atoms of yGrx2), 1.20 Å (for 97 C^{α} atoms of yGrx2), 2.08 Å (for 64 C^{α} atoms of yGrx2) and 1.68 Å (for 79 C^{α} atoms of yGrx2), respectively. Therefore, the overall structure of yGrx2 is more similar to the yGrx1 and human Grx1 and Grx2 structures than to the bacterial counterparts. These eukaryotic Grx structures have the same secondary structural elements, whereas *E. coli* Grx structures do not possess the first helix found in the eukaryotic Grxs, and *E. coli* Grx1 does not possess the last helix found in the other Grxs.

Comparison between yGrx2 and yGrx1 structures

Although the overall structures of yGrx1 and yGrx2 are very similar, an evident difference between the $yGrx2_{GS}$ and $yGrx1_{GS}$ structures is the conformation adopted by Ser30. In $yGrx1_{GS}$, the Ser30 side chain is directed toward Cys27, whereas in $yGrx2_{GS}$ the Ser30 side chain is turned to the opposite side (Fig. 8). In fact, the distance between the Ser30 hydroxyl oxygen and the sulfur atom of Cys27 is 3.47 Å in $yGrx1_{GS}$ and 5.14 Å in $yGrx2_{GS}$. Assuming that a cysteine at position 30 could have a side-chain conformation similar to that of serine in the C30S mutants of both $yGrx1_{GS}$ and $yGrx2_{GS}$, Cys30 of $yGrx1_{GS}$ would be able to attack the Cys27 and form an intramolecular disulfide bond, whereas the Cys30 of $yGrx2_{GS}$ would be in an unfavorable configuration for an attack on the corresponding mixed disulfide between Cys27 and GSH. If the formation of the intramolecular disulfide bond is disfavored in yGrx2, reduction of the glutathionyl mixed disulfide by an external nucleophile, such as

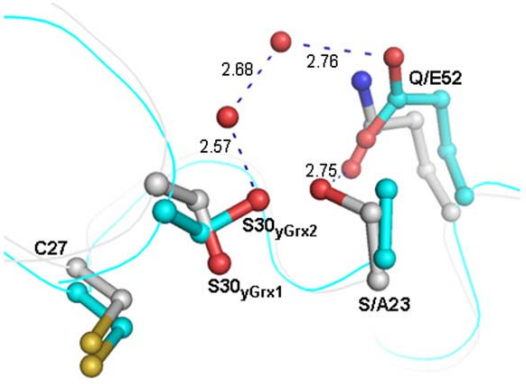

Fig. 8. Side-chain conformation of Ser30 in the structures of the C30S mutants of yGrx1[22] (gray) and yGrx2 (cyan) in their glutathionylated forms. It is clear that the distances from the Cys27 sulfur atoms (yellow) to the Ser30 oxygen atom (red) are very different between yGrx1 and yGrx2, indicating that the conformation of a Ser30 residue in yGrx2 is less favorable to attack the mixed disulfide.

GSH, is then favored (Fig. 1). Consequently, the monothiol mechanism (involved in the HED assay) is also favored.

Corroborating the above interpretation, in *E. coli* Grx3 glutathionylated structure,[11] Ser14 (which replaces the C-terminal active-site cysteine in this structure) presents a more buried conformation similar to that of $yGrx2_{GS}$ Ser30. In all other Grx structures reported in the glutathionylated form (all obtained with mutations of the C-terminal active-site cysteine to serine), including the *E. coli* Grx1,[9] the conformation of this Ser is similar to that found in $yGrx1_{GS}$. The more buried conformation of Ser30 in the $yGrx2_{GS}$ structure appears to be related to the interaction with the Glu52 carboxylate via two water molecules (Fig. 8). The distance between the oxygen of the carboxylate of Glu52 and the hydroxyl of Ser30 is 5.02 Å in $yGrx2_{GS}$. Similarly, *E. coli* Grx3 possesses the Glu30 residue, and an oxygen atom of its carboxylate group is located at 5.74 Å from the hydroxyl of Ser14 in its glutathionylated structure. Glu30 of *E. coli* Grx3 does not occupy the same position as yGrx2 Glu52, although the distances between the carboxylate groups of these Glu residues and the hydroxyl of the Ser are similar in both structures. Also, in both structures, the Ser adopts a more buried conformation than that adopted in other Grx glutathionylated structures. Remarkably, like yGrx2, *E. coli* Grx3 possesses higher monothiolic activity than *E. coli* Grx1, although to a much lower extent (twofold).

Other Grxs, such as pig Grx and human Grx1, possess an Asp residue at the equivalent position of yGrx2 Glu52. In these Grxs, however, there is an Ile residue that prevents the contact (direct or indirect) between the Asp and the C-terminal active-site residue. In the equivalent position of the mammalian Grx isoleucine residue, yGrx2 possesses an alanine residue (Ala23), which, due to the short side chain, does not prevent interaction between Ser30 and Glu52 (Fig. 8). *E coli* Grx3 also does not possess bulky residues that could prevent interactions between Ser14 and Glu30. Yeast Grx1 possesses a Gln52 replacing Glu52; however, this residue makes a hydrogen bond with the hydroxyl of Ser23 (yGrx2 Ala23) and does not interact with Ser30 (Fig. 8).

Our analyses suggest that the unfavorable conformation of yGrx2 Cys30 to form intramolecular disulfide could be related to interactions established with Glu52, which are possible due to the non-interfering side chain of Ala23. In contrast, the favorable conformation of yGrx1 Cys30 to attack the Grx-SG is probably related to the fact that those interactions present in yGrx2 are not possible in yGrx1 due to the substitution of Ala23 by Ser23, which forms a hydrogen bond with Gln52, avoiding the interaction between Cys30 and Gln52.

In order to test this hypothesis, we constructed mutants of both yGrx1 and yGrx2 (yGrx1-S23A, yGrx1-S23A-Q52E, yGrx2-A23S and yGrx2-A23S-E52Q). yGrx1-S23A and yGrx1-S23A-Q52E presented specific activities 3.4- and 3.0-fold higher than that of the wild-type yGrx1, respectively, whereas yGrx2-A23S and yGrx2-A23S-E52Q mutants exhibited specific activities 1.7- and 2.3-fold lower than that of the wild-type yGrx2, respectively (Table 1).

The higher activity of yGrx1-S23A and the lower activity of yGrx2-A23S compared with the activities of their respective wild-type isoforms indicated that these residues are in fact involved in the variation in the activities between dithiolic yGrxs. To further corroborate our hypothesis, the yGrx1 and yGrx2 double mutants should have higher and lower specific activities, respectively, than the single mutants yGrx1-S23A and yGrx2-A23S, indicating that the residues Ser/Ala23 and Gln/Glu52 have an additive effect in the activity of dithiolic yGrxs. However, this additive effect of Ser/Ala23 and Gln/Glu52 can be observed only in the yGrx2-A23S-Q52E double mutant, but not in yGrx1-S23A-E52Q (Table 1). Consequently, it is not clear if Gln/Glu52 is really related with the variation in the specific activity of dithiolic yGrxs.

Another possibility is that only Ser/Ala23 but not Gln/Glu52 exerts influence on yGrx1 and yGrx2 activities. In this case, Ser23 in yGrx1 could act as a base, taking away the proton from Cys30, favoring thiolate formation and, consequently, the attack of Cys30 on the Grx-SG intermediate (Fig. 1, reaction c). As a result, the formation of the intramolecular disulfide bond would switch the mechanism from the monothiol to the dithiol, which would result in a lower reaction rate in the HED assay, as discussed before. In the case of yGrx2, the more inert character of Ala23 should not influence the reactivity of Cys30 in the Grx-SG intermediate, allowing the reduction of the mixed disulfide by an external GSH molecule, therefore favoring the monothiol mechanism. This feature is consistent with the observation that yGrx1 presented higher K_M for GSH and lower k_{cat} values compared to yGrx2. This hypothesis is also in agreement with the mutagenesis experiments, since the

substitution of Ala23 by Ser in yGrx2-A23S mutant provokes a decrease in its specific activity compared with the yGrx2 wild-type enzyme, whereas the substitution of Ser23 by Ala in yGrx1-S23A causes an increase in its specific activity compared with the yGrx1 (Table 1).

It is important to mention here that in the structure of $yGrx1_{GS}$ the N-terminal active-site Cys27 residue is not bonded to the cysteine of GSH,[22] whereas in the $yGrx2_{GS}$ structure the glutathionyl mixed disulfide is observed (Fig. 6b). This difference might be a complicating factor for the comparison of these two structures, because it could have relevant consequences with respect to local structural conformations. However, as mentioned by the authors, the disulfide bond of yGrx1-SG was probably broken upon exposure to synchrotron radiation, and the orientation of GSH and yGrx1 residues probably was not affected, since the interactions with the GSH moiety were similar to those found in other glutathionylated Grx structures.[22,28,35] Furthermore, there are structures available in the PDB where the glutathionyl mixed disulfide is present and the serine residues equivalent to yGrx1 Ser30 presented the same conformation as in the $yGrx1_{GS}$ structure.[9,28] Additionally, the hypotheses raised here from structural comparisons were strengthened by biochemical assays with mutant proteins (Table 1).

Another aspect that is probably related to the different biochemical activities of yGrx1 and yGrx2 is their redox potential. In fact, Grx1 and Grx3 from *E. coli* also share high amino acid sequence and overall structure similarity, although they possess very distinct redox potentials ($\Delta E^{\circ\prime} = 35$ mV).[36] In contrast, Grx1 and Grx2 from humans possess more similar redox potentials ($\Delta E^{\circ\prime} = 11$ mV),[37] although they do not share the same degree of similarity. In this case, it is important to mention that human Grx2 presents several unusual features such as an additional disulfide bond with a very negative redox potential (−317 mV).[36] Several aspects of the relationships between redox potential and protein structure remain to be established.

In conclusion, the great enzymatic difference observed between yGrx1 and yGrx2 appears not to be related to the pK_a of their reactive cysteines, but to specific structural features of these enzymes. Considering the conformation of Ser30/Cys30 in dithiolic yGrxs glutathionylated structures and the influence of Ser23 and Ala23 in the yGrx1 and yGrx2 activities, respectively, we hypothesize that yGrx2 could be more specially adapted than yGrx1 to the monothiol mechanism. These structural and functional differences between yGrx1 and yGrx2 might reflect variations in substrate specificity.

Materials and Methods

Cloning, expression and purification

Wild-type yeast *GRX2* gene was cloned into the pET15b expression vector, expressed in *E. coli* and purified by cobalt-affinity chromatography as previously described.[34]

Site-directed mutagenesis was employed to mutate the cysteine 30 residue of Grx2 to a serine residue. The *GRX1* and *GRX2* genes were PCR amplified from *S. cerevisiae* genomic DNA of the strain W303 with the primers 5′-CGCGATCCATATGATGGTATCTCAAGAAAC-3′ (forward *GRX1*), 5′-CGCAAGCTTGGATCCTTAATTTGCAAGAATAGG-3′ (reverse *GRX1*), 5′-CGCGATCCATATGATGGTATCCCAGGAAACAGTTGCTCACGTAAAGGATCTGATTGGCCAAAAGGAAGTGTTTGTTGCAGCAAAGACATACTGCCCTTACAGCAAAGCTACTTTG-3′ (forward *GRX2-C30S*, mutated sequence underlined), 5′-CGCAAGCTTGGATCCCTATTGAAATACCGGCTTC-3′ (reverse *GRX2-C30S*). The forward primers contain NdeI restriction sites and the reverse primers contain BamHI restriction sites. The PCR products and the expression vector pET15b were first digested with NdeI and BamHI, and then the products were ligated into the pET15b. The *GRX1-S23A*, *GRX1-C30S* and *GRX2-A23S* mutants were prepared with the QuickChange Site-Directed Mutagenesis Kit from Stratagene according to the manufacturer's recommendations. The pET15b/*GRX1* and pET15b/*GRX2* plasmids were used as templates with two complementary primers containing the desired mutation (underlined): 5′-GAGATCTTCGTCGCAGCAAAAACGTACTGTCC-3′ (forward *GRX1-S23A*); 5′-CGTACTGTCCATACTCTCATGCAGCCCTAAAC-3′ (forward *GRX1-C30S*); 5′-GAAGTGTTTGTTGCATCCAAGACATACTGCCC-3′ (forward *GRX2-A23S*). The mutants *GRX1-S23A-Q52E* and *GRX2-A23S-E52Q* were also prepared using the same strategy, with the pET15b/*GRX1-S23A* and the pET15b/*GRX2-A23S* plasmids as templates and the following primers: 5′-GTT CTG GTT TTG GAG TTG AAT GAC ATG AAG-3′ (forward *GRX1-Q52E*); 5′-G GCC CTT GTG TTG CAG TTA GAT GAA ATG AGC-3′ (forward *GRX2-E52Q*). The gene sequences were confirmed by automated DNA sequencing and the resulting plasmids were used to transform *E. coli* BL21(DE3). Protein expression was induced by the addition of 1 mM IPTG to cultures grown in LB medium at 310 K to $OD_{600} = 0.8$. The cells were harvested after 3 h of incubation at 310 K.

Cell pellets were resuspended in 20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 20 mM imidazole and 1 mM PMSF and lysed by sonication. Subsequently, the cell extract was kept on ice for 20 min during a 1% streptomycin sulfate treatment. The extract was centrifuged at 16,000*g* for 45 min to remove cell debris, and the supernatant was further clarified by filtration. The proteins were purified by metal-affinity chromatography (Nickel-Hi-trap from GE Healthcare or Cobalt-Talon metal-affinity resin from Clontech) with imidazole gradients. Protein purity was confirmed by SDS-PAGE.

Yeast Grx1 and Grx2 his-tag fusion proteins were digested with the Thrombin Clean Cleave Kit by Sigma, following the manufacturer's protocol.

Assay for Grx activity

Specific activities of yGrx1, yGrx2 and of the mutants yGrx1-S23A, yGrx1-C30S, yGrx1-S23A-Q52E, yGrx2-A23S, yGrx2-C30S and yGrx2-A23S-E52Q were measured with the standard assay for Grxs, at 30 °C, using a mixed disulfide between HED and GSH as the substrate.[23] Concentrations of yGrx1, yGrx2 and yGrx2-C30S were varied from 50 to 400, 5 to 35 and 10 to 200 nM, respectively, and the concentrations of the mutants yGrx1-S23A, yGrx1-S23A-Q52E, yGrx1-C30S, yGrx2-A23S and yGrx2-A23S-

E52Q were varied from 10 to 100 nM. The reaction mixture, containing 100 mM Tris–HCl, pH 7.4, 6 μg/ml GR, 1 mM GSH, 0.7 mM HED, 0.1 mg/ml bovine serum albumin (BSA), 0.2 mM NADPH and 2 mM ethylenediaminetetraacetic acid (EDTA) in a volume of 1 ml, was incubated at 30 °C for 3 min for the formation of the mixed disulfide between GSH and HED. The reaction was started by the addition of Grx and followed by the decrease in the absorbance at 340 nm due to the oxidation of NADPH. Measured activities in all assays were corrected by subtracting the velocities of the control reactions without the enzyme, and three independent experiments were performed at each substrate concentration.

Two-substrate kinetics

Two-substrate kinetics were performed at 30 °C, varying the concentration of HED from 0.03 to 2.2 mM at fixed concentrations of GSH (0.5, 1.0, 1.5 and 2.0 for yGrx1; 0.5, 1.0 and 1.5 for yGrx2).[21] The reaction mixture contained the same components at the same concentrations as described for the measurement of Grx-specific activities. In this case, we also waited for 3 min for the formation of the mixed disulfide between GSH and HED before starting the reaction with the addition of Grx. The concentrations of yGrx1 and yGrx2 used in these assays were 500 and 50 nM, respectively. Measured activities in all assays were corrected by subtracting the velocities of the control reactions without the enzyme. Three independent experiments were performed at each substrate concentration, and the apparent K_M and V_{max} values were determined by non-linear regression of Michaelis–Menten plots. The reciprocal values of V_{max}^{app} were plotted *versus* the reciprocal of the GSH concentration to provide the kinetic parameters for GSH.[27]

pK_a determination of N-terminal cysteine

To determine the pK_a of the N-terminal active-site cysteine of yGrx1, yGrx2 and the yGrx2-C30S mutant, the prereduced enzymes were incubated at different pH values with or without 300 μM iodoacetamide for 3 min at room temperature.[12,14,26] After incubation, a 100-fold dilution in an ice-cold bath quenched the reaction mixture, and the protein was assayed for activity with the standard assay for Grx, described in Materials and Methods. The percentage of remaining activity at each pH was determined by comparing the activity of the enzyme incubated with and without iodoacetamide at the same pH. The concentration of enzyme used in the assays was 250, 20 and 100 nM for yGrx1, yGrx2 and yGrx2-C30S, respectively. Buffers for incubation of enzymes were used at 10 mM concentration (KCl–HCl, pH 1.0 and 1.5; citrate–phosphate, pH 2.0, 2.5, 3.0, 3.5, 4.0, 5.0 and 6.0; Tris–HCl, pH 7.0 and 8.0), and for the assay, 100 mM Tris–HCl buffer, pH 7.4, was used. The ionic strength was adjusted to 0.5 M by the addition of NaCl.

In a second, independent approach, the pK_a of the N-terminal active-site cysteine residues was determined by monobromobimane alkylation that generates a fluorescent product (λ_{exc} 396 nm, λ_{em} 482 nm).[30] yGrx1 and yGrx2 were previously reduced by excess DTT (100 mM) for 2 h in the presence of 0.1 mM diethylenetriamine pentaacetic acid at room temperature. Excess DTT was removed by size-exclusion chromatography (PD-10 columns, GE Healthcare), with 5 mM Tris–HCl, pH 7.5 (Grx2) or pH 8.5 (Grx1), as elution buffer. Protein concentration was determined by absorbance at 280 nm and specific extinction coefficient ($\varepsilon_{yGrx1} = 6085$, $\varepsilon_{yGrx2} = 4470$). yGrx1 and yGrx2 (10 μM) were incubated with monobromobimane (2 μM) at various pH values in 96-well plates in triplicate. All buffers used in the alkylation reactions were at 50 mM concentration (KCl–HCl, pH 1.0 and 1.5; citrate–phosphate, pH 2.0 to 5.0) and reactions proceeded at 37 °C with shaking. The ionic strength was adjusted to 0.5 M by the addition of NaCl. The rates of monobromobimane alkylation were determined by extrapolation of the maximum inclination in the kinetics. As a control, it was verified that the fluorescence of the same protein–monobromobimane adduct does not change as a function of pH.

The plots displayed in Fig. 4a and b were fitted by non-linear regression to sigmoidal dose–response curves with GraphPad Prism4 software. The Hill slope utilized was equal to 1.00. The pK_a values were obtained from the inflection point, in this case, log EC_{50}.

Crystallization and X-ray data collection

In an attempt to obtain the crystal structure of yGrx2 in different oxidative states, prior to crystallization, yGrx2 samples (10 mg/ml in 5 mM Tris–HCl, pH 7.4) were submitted to treatment for 1 h at 310 K with 1 mM hydrogen peroxide, 10 mM *t*-BOOH, 3 mM diamide, 10 mM DTT or 10 mM GSH. We also subjected freshly grown yGrx2 crystals to soaking experiments with 10 mM DTT and 10 mM GSH in an effort to obtain the structure of reduced yGrx2. Furthermore, to obtain yGrx2-C30S with a glutathionyl mixed disulfide for crystallization, the purified protein was first incubated with 50 mM DTT for 1 h at room temperature. Excess DTT was removed by gel-filtration chromatography using a PD10 column (GE Healthcare). Then, the reduced protein was treated with 25 mM GSSG for 1 h at room temperature followed by gel-filtration chromatography. The glutathionylated sample of yGrx2-C30S was concentrated to 13 mg/ml in 5 mM Hepes, pH 7.4.

Crystallization trials for yGrx2 were performed with the hanging-drop vapor-diffusion method, as previously described.[34] Crystals of yGrx2 suitable for X-ray diffraction measurements were obtained under all the treatments in condition 10 of the Crystal Screen kit [30% polyethylene glycol (PEG) 4000, 0.1 M sodium acetate, pH 4.6, and 0.2 M ammonium acetate]. Crystals of yGrx2-C30S with a glutathionyl mixed disulfide were obtained in condition 22 of the Crystal Screen kit; after optimization, we obtained crystals suitable for X-ray diffraction experiments under the following conditions: 30% PEG 4000/0.1 M sodium acetate, pH 5.4/0.2 M sodium acetate.

Crystals were produced from samples subjected to all treatments described and different data sets were collected. The crystals, cryoprotected by 20% glycerol, were cooled to 110 K and X-ray diffraction data were collected using synchrotron radiation at the protein crystallography beamline D03B MX1 at the Brazilian Synchroton Light Laboratory.

Data processing, structure solution and refinement

All data sets were indexed and integrated with MOSFLM[38] and scaled and merged using SCALA[39,40] from the CCP4 suite.[41] The first structure of yGrx2 (oxidized with *t*-BOOH) was solved by molecular replacement with the program AMORE,[42] using as a search model a polyalanine theoretical model constructed with the program MODELLER[43] and the coordinates of a thioltransferase from *Sus scrofa* (PDB code 1KTE),[44] as previously described.[34] The other structures of yGrx2 and

glutathionylated yGrx2-C30S were solved by molecular replacement with the refined structure of oxidized yGrx2 ($yGrx2_{ox}$) using the programs AMORE and PHASER.[42,45]

Refinements of all yGrx2 structures were performed with REFMAC 5.0,[46] and a TLS atomic displacement model[47] was used in the later stages of the structure refinement of yGrx2-C30S with a glutathionyl mixed disulfide. The programs O[48] and COOT[49] were used for visual inspection and manual model building between the refinement cycles. The stereochemical quality of the final models was assessed by PROCHECK.[50] Structural alignments were performed with the programs O[48] or COOT.[49] Molecular graphics figures were generated with the program PyMOL.[51]

Protein Data Bank accession numbers

Atomic coordinates of the *S. cerevisiae* Grx2 oxidized and glutathionylated structures have been deposited in the RCSB PDB with the accession codes 3D4M and 3D5J, respectively.

Acknowledgements

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.10.055

References

1. Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. *J. Biol. Chem.* **264**, 13963–13966.
2. Holmgren, A. (1976). Hydrogen donor system for *Escherichia coli* ribonucleoside–diphosphate reductase dependent upon glutathione. *Proc. Natl Acad. Sci. USA*, **73**, 2275–2279.
3. Holmgren, A. (1978). Glutathione-dependent enzyme reactions of the phage T4 ribonucleotide reductase system. *J. Biol. Chem.* **253**, 7424–7430.
4. Holmgren, A. (1979). Glutathione-dependent synthesis of deoxyribonucleotides. Characterization of the enzymatic mechanism of *Escherichia coli* glutaredoxin. *J. Biol. Chem.* **254**, 3672–3678.
5. Bushweller, J. H., Åslund, F., Wüthrich, K. & Holmgren, A. (1992). Structural and functional characterization of the mutant *Escherichia coli* glutaredoxin (C14→S) and its mixed disulfide with glutathione. *Biochemistry*, **31**, 9288–9293.
6. Lillig, C. H., Berndt, C. & Holmgren, A. (2008). Glutaredoxin systems. *Biochim. Biophys. Acta*, **1780**, 1304–1317.
7. Gallogly, M. M. & Mieyal, J. J. (2007). Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Curr. Opin. Pharmacol.* **7**, 381–391.
8. Srinivasan, U., Mieyal, P. A. & Mieyal, J. J. (1997). pH profiles indicative of rate-limiting nucleophilic displacement in thioltransferase catalysis. *Biochemistry*, **36**, 3199–3206.
9. Bushweller, J. H., Billeter, M., Holmgren, A. & Wüthrich, K. (1994). The nuclear magnetic resonance solution structure of the mixed disulfide between *Escherichia coli* glutaredoxin (C14→S) and glutathione. *J. Mol. Biol.* **235**, 1585–1597.
10. Fernandes, A. P. & Holmgren, A. (2004). Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antioxid. Redox Signal.* **6**, 63–74.
11. Nordstrand, K., Åslund, F., Holmgren, A., Otting, G. & Berndt, K. D. (1999). NMR structure of *Escherichia coli* glutaredoxin 3–glutathione mixed disulfide complex: implications for the enzymatic mechanism. *J. Mol. Biol.* **286**, 541–552.
12. Jao, S., Ospina, S. M. E., Berdis, A. J., Starke, D. W., Post, C. B. & Mieyal, J. J. (2006). Computational and mutational analysis of human glutaredoxin (thioltransferase): probing the molecular basis of the low pK_a of cysteine 22 and its role in catalysis. *Biochemistry*, **45**, 4785–4796.
13. Yang, Y. & Wells, W. W. (1991). Identification and characterization of the functional amino acids at the active center of pig liver thioltransferase by site-directed mutagenesis. *J. Biol. Chem.* **266**, 12759–12765.
14. Gan, Z., Sardana, M. K., Jacobs, J. W. & Polokoff, M. A. (1990). Yeast thioltransferase—active site cysteines display differential reactivity. *Arch. Biochem. Biophys.* **282**, 110–115.
15. Luikenhuis, S., Perrone, G., Dawes, I. W. & Grant, C. M. (1998). The yeast *Saccharomyces cerevisiae* contains two glutaredoxin genes that are required for protection against reactive oxygen species. *Mol. Biol. Cell*, **9**, 1081–1091.
16. Pedrajas, J. R., Porras, P., Martínez-Galisteo, E., Padilla, C. A., Miranda-Vizuete, A. & Bárcena, J. A. (2002). Two isoforms of *Saccharomyces cerevisiae* glutaredoxin 2 are expressed *in vivo* and localize to different subcellular compartments. *Biochem. J.* **364**, 617–623.
17. Porras, P., Padilla, C. A., Krayl, M., Voos, W. & Barcena, J. A. (2006). One single in-frame AUG codon is responsible for a diversity of subcellular localizations of glutaredoxin 2 in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **281**, 16551–16562.
18. Rodríguez-Manzaneque, M. T., Ros, J., Cabiscol, E., Sorribas, A. & Herrero, E. (1999). Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **19**, 8180–8190.
19. Ojeda, L., Keller, G., Muhlenhoff, U., Rutherford, J. C., Lill, L. & Winge, D. R. (2006). Role of glutaredoxin 3 and glutaredoxin 4 in the iron regulation of Aft1 transcriptional activator in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **281**, 17661–17669.
20. Rodríguez-Manzaneque, M. T., Tamarati, J., Belli, G., Ros, J. & Herrero, E. (2002). Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. *Mol. Biol. Cell*, **13**, 1109–1121.

21. Mesecke, N., Mittler, S., Eckers, E., Herrmann, J. M. & Deponte, M. (2008). Two novel monothiol glutaredoxins from *Saccharomyces cerevisiae* provide further insight into iron–sulfur cluster binding, oligomerization, and enzymatic activity of glutaredoxins. *Biochemistry*, **47**, 1452–1463.
22. Håkansson, K. O. & Winther, J. R. (2007). Structure of glutaredoxin Grx1p C30S mutant from yeast. *Acta Crystallog., Sect. D: Biol. Crystallogr.* **63**, 288–294.
23. Holmgren, A. & Åslund, F. (1995). Glutaredoxin. *Methods Enzymol.* **252**, 283–292.
24. Chenna, R., Sugawara, H., Koile, T., Lopez, R., Gibson, T. J., Higgins, D. G. & Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* **31**, 3497–3500.
25. Clamp, M., Cuff, J., Searle, S. M. & Barton, G. J. (2004). The Jalview Java alignment editor. *Bioinformatics*, **20**, 426–427.
26. Mieyal, J. J., Starke, D. W., Gravina, S. A. & Hocevar, B. (1991). Thioltransferase in human red blood cells: kinetics and equilibrium. *Biochemistry*, **30**, 8883–8891.
27. Segel, H. I. (1975). Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. Wiley, New York.
28. Yang, Y., Jao, S., Nanduri, S., Starke, D. W., Mieyal, J. J. & Qin, J. (1998). Reactivity of the human thioltransferase (glutaredoxin) C7S, C25S, C78S, C82S mutant and NMR solution structure of its glutathionyl mixed disulfide intermediate reflect catalytic specificity. *Biochemistry*, **37**, 17145–17156.
29. Johansson, C., Lillig, C. H. & Holmgren, A. (2004). Human mitochondrial glutaredoxin reduces *S*-glutathionylated proteins with high affinity accepting electrons from either glutathione or thioredoxin reductase. *J. Biol. Chem.* **279**, 7537–7543.
30. Kosower, N. S., Kosower, E. M., Newton, G. L. & Ranney, H. M. (1979). Bimane fluorescent labels: labeling of normal human red cells under physiological conditions. *Proc. Natl Acad. Sci. USA*, **76**, 3382–3386.
31. Gilbert, H. F. (1990). Molecular and cellular aspects of thiol–disulfide exchange. *Adv. Enzymol. Relat. Areas Mol. Biol.* **63**, 69–172.
32. Szajewski, R. P. & Whitesides, G. M. (1980). Rate constants and equilibrium constants for thiol–disulfide interchange reactions involving oxidized glutathione. *J. Am. Chem. Soc.* **102**, 2011–2026.
33. Creighton, T. E. & Darby, N. J. (1993). Dissecting the disulfide couple folding pathway of bovine pancreatic inhibitor: forming the first disulfide bonds in analogues of the reduced protein. *J. Mol. Biol.* **232**, 873–896.
34. Johansson, C., Kavanagh, K. L., Gileadi, O. & Oppermann, U. (2007). Reversible sequestration of active site cysteines in a 2Fe2S-bridge dimmer provides a mechanism for glutaredoxin 2 regulation in human mitochondria. *J. Biol. Chem.* **282**, 3077–3082.
35. Åslund, F., Berndt, K. D. & Homlgren, A. (1997). Redox potentials of glutaredoxins and other thiol–disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein–protein equilibria. *J. Biol. Chem.* **272**, 30780–30786.
36. Sagemark, J., Elgán, T. H., Bürglin, T. R., Johansson, C., Holmgren, A. & Berndt, K. D. (2007). Redox properties and evolution of human glutaredoxins. *Prot. Struct. Funct. Bioinf.* **68**, 879–892.
37. Discola, K. F., Oliveira, M. A., Monteiro, G. S., Bárcena, J. A., Porras, P., Padilla, A. *et al.* (2005). Crystallization and preliminary X-ray crystallographic studies of glutaredoxin 2 from *Saccharomyces cerevisiae* in different oxidation states. *Acta Crystallogr., Sect. F*, **61**, 445–447.
38. Leslie, A. G. W. (1992). Joint CCP4/ESF-EAMBCB. Newsletter Protein Crystallography 26. Daresbury Laboratory, Warrington, UK.
39. Kabush, W. (1988). Evaluation of single-crystal X-ray diffraction data from a position-sensitive detector. *J. Appl. Crystallogr.* **21**, 916–924.
40. Blessing, R. H. (1995). An empirical correction for absorption anisotropy. *Acta Crystallogr., Sect. A: Found. Crystallogr.* **51**, 33–38.
41. Collaborative Computational Project, Number 4. (1994). *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **50**, 760–763.
42. Navaza, J. (1994). AmoRe: an automated package for molecular replacement. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **57**, 1367–1372.
43. Claude, J. B., Suhre, K., Notredame, C., Claverie, J. M. & Abergel, C. (2004). CaspR: a Web server for automated molecular replacement using homology modelling. *Nucleic Acids Res.* **32**, W606–W609.
44. Katti, S. K., Robbins, A. H., Yang, Y. & Wells, W. W. (1995). Crystal structure of thioltransferase at 2.2 Å resolution. *Protein Sci.* **4**, 1998–2005.
45. Read, R. J. (2001). Pushing the boundaries of molecular replacement with maximum likelihood. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **57**, 1373–1382.
46. Murshudov, G. N., Vagin, A. A. & Dodson, E. J. (1997). Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **53**, 240–255.
47. Winn, M. D., Isupov, M. N. & Murshudov, G. N. (2001). Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **57**, 122–133.
48. Jones, T., Zou, J., Cowan, S. & Kjeldgaard, M. (1991). Improved methods for binding protein models in electron density maps and the location of errors in these models. *Acta Crystallogr., Sect. A: Found Crystallogr.* **47**, 110–119.
49. Emsley, P. & Cowtan, K. (2004). Coot: model-building tools for molecular graphics. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **60**, 2126–2132.
50. Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). PHOCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* **26**, 283–291.
51. DeLano, W. L. (2002). *The PyMOL Molecular Graphics System*. DeLano Scientific, San Carlos, CA.

Supplementary Data

Figure Sup1.

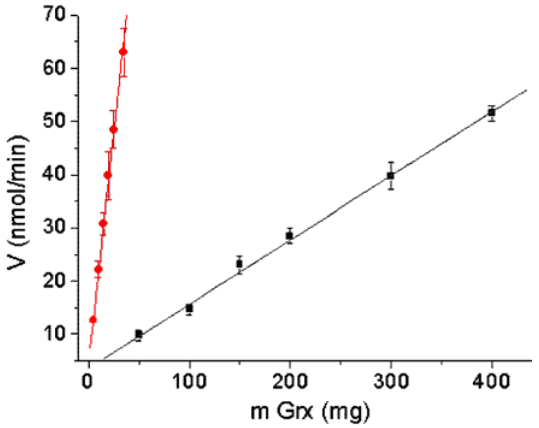

Figure 1. Specific activities of yGrx1 (black squares) and yGrx2 (red balls) in the HED assay.

Figure Sup2.

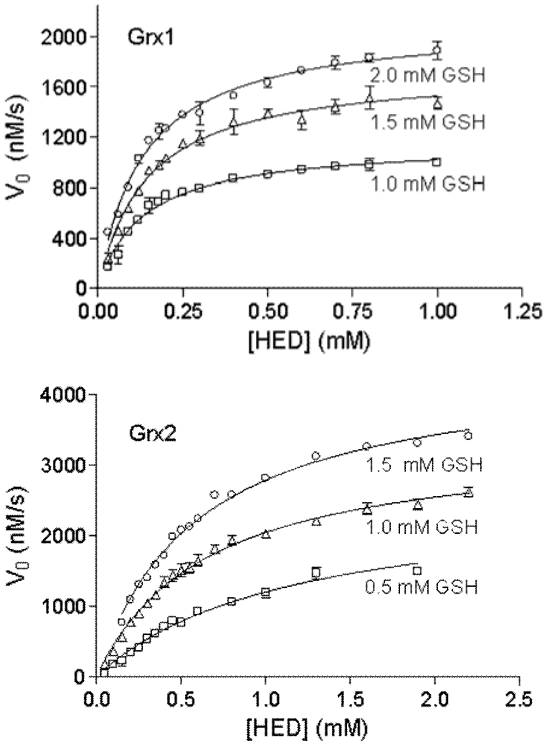

Figure 2. Michaelis-Menten plots from the reduction of the mixed disulfide between glutathione and HED by yGrx1 (a) and yGrx2 (b), at fixed concentrations of glutathione.

Figure Sup3.

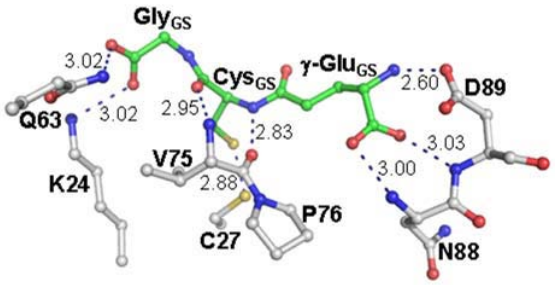

Figure 3. yGrx1 interactions with glutathione in the yGrx1$_{GS}$ structure. yGrx1 residues (carbon atoms in cyan) involved in interactions with the glutathione moiety are represented in pink, and glutathione is shown in green. The hydrogen bonds are indicated by blue dashed lines, with the atomic distances in angstroms. Cys^{27} of yGrx1 does not form a disulfide bond with the Cys residue of glutathione; the distance between their sulphur atoms is 2.88Å.

Anexo 2

the FEBS Journal

Role of glutaredoxin 2 and cytosolic thioredoxins in cysteinyl-based redox modification of the 20S proteasome

Gustavo M. Silva[1,2], Luis E.S. Netto[2], Karen F. Discola[2], Gilberto M. Piassa-Filho[1], Daniel C. Pimenta[1], José A. Bárcena[3] and Marilene Demasi[1]

1 Instituto Butantan, Laboratório de Bioquímica e Biofísica, São Paulo, Brazil
2 Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, Brazil
3 Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, Spain

Keywords
20S proteasome; deglutathionylation; glutaredoxin; S-glutathionylation; thioredoxins

Correspondence
M. Demasi, Instituto Butantan, Laboratório de Bioquímica e Biofísica, Avenida Vital Brasil, 1500, 05503 900 São Paulo, Brazil
Fax: +55 11 3726 7222 ext. 2018
Tel: +55 11 3726 7222 ext. 2101
E-mail: marimasi@butantan.gov.br

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The yeast 20S proteasome is subject to sulfhydryl redox alterations, such as the oxidation of cysteine residues (Cys-SH) into cysteine sulfenic acid (Cys-SOH), followed by S-glutathionylation (Cys-S-SG). Proteasome S-glutathionylation promotes partial loss of chymotrypsin-like activity and post-acidic cleavage without alteration of the trypsin-like proteasomal activity. Here we show that the 20S proteasome purified from stationary-phase cells was natively S-glutathionylated. Moreover, recombinant glutaredoxin 2 removes glutathione from natively or *in vitro* S-glutathionylated 20S proteasome, allowing the recovery of chymotrypsin-like activity and post-acidic cleavage. Glutaredoxin 2 deglutathionylase activity was dependent on its entry into the core particle, as demonstrated by stimulating S-glutathionylated proteasome opening. Under these conditions, deglutathionylation of the 20S proteasome and glutaredoxin 2 degradation were increased when compared to non-stimulated samples. Glutaredoxin 2 fragmentation by the 20S proteasome was evaluated by SDS–PAGE and mass spectrometry, and S-glutathionylation was evaluated by either western blot analyses with anti-glutathione IgG or by spectrophotometry with the thiol reactant 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. It was also observed *in vivo* that glutaredoxin 2 was ubiquitinated in cellular extracts of yeast cells grown in glucose-containing medium. Other cytoplasmic oxido-reductases, namely thioredoxins 1 and 2, were also active in 20S proteasome deglutathionylation by a similar mechanism. These results indicate for the first time that 20S proteasome cysteinyl redox modification is a regulated mechanism coupled to enzymatic deglutathionylase activity.

Oxidation of protein cysteine residues into sulfenic acid (Cys-SOH) and the subsequent S-glutathionylation of these residues during enzyme catalysis and redox signaling have been increasingly accepted as commonly occurring events in redox regulation [1–9]. This reversible mechanism is believed to play a regulatory role in enzyme catalysis and binding of transcription factors to DNA targets, among other processes. The first step in protein-Cys-SH oxidation generates Cys-SOH, which is prone to S-glutathionylation by

Abbreviations
20S PT, 20S proteasome core; AMC, 7-amido-4-methylcoumarin; CDL, cardiolipin; Cys-SOH, cysteine sulfenic acid; GR, glutathione reductase; Grx2, recombinant glutaredoxin 2; Grx2C30S, mutant glutaredoxin 2; GSH, glutathione; HED, hydroxyethyldisulfide; NBD, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; n-PT, natively S-glutathionylated 20S proteasome; PT-SG, *in vitro* S-glutathionylated 20S proteasome; PT-SH, dithiotreitol-treated 20S proteasome; RS, reductive system for Grx2 containing 2 mM NADPH, 0.3 U·mL^{-1} GR and 0.5 mM GSH; s-LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-AMC; Trr1, recombinant thioredoxin reductase 1; z-ARR-AMC, carbobenzoxy-Ala-Arg-Arg-AMC; z-LLE-AMC, carbobenzoxy-Leu-Leu-Glu-AMC.

sulfhydryls, e.g. glutathione (GSH); otherwise, the oxidation continues to further generate the cysteine sulfinic (Cys-SO_2H) and cysteine sulfonic (Cys-SO_3) acid forms [5,10]. Glutaredoxins [9,11,12], as well as thioredoxins [13], are postulated to be directly responsible for deglutathionylation in yeast cells. The first function assigned to glutaredoxins was the reduction of intramolecular disulfide bonds in the ribonucleotide reductase of thioredoxin-deleted *Escherichia coli* strains [14]. Since then, biochemical and genetic approaches have provided evidence for a protective role of glutaredoxins under oxidative conditions and during redox signaling, e.g. GSH-dependent reduction of protein-mixed disulfides by means of its so-called deglutathionylase activity in various eukaryotic cells [9,11,12,15–17].

Yeast possesses two dithiolic (Grx1 and Grx2) and five monothiolic glutaredoxins. These isoforms differ in their location and response to oxidative stress, among other factors [9,11,18–22]. Evidence indicates that Grx2 is the main glutathione-dependent oxidoreductase in yeast, whereas Grx1 and Grx5 may be required during certain stress conditions or after the formation of particular mixed disulfide substrates [11,12].

We have shown previously that yeast Cys-20S proteasomal residues are S-glutathionylated *in vitro* by reduced glutathione if previously oxidized to Cys-SOH [8]. Moreover, this mechanism was shown to be responsible for a decrease in proteasomal chymotrypsin-like activity. Here, we show that the 20S proteasome core purified from stationary-phase cells is also S-glutathionylated under basal conditions, and that Grx2 was able to dethiolate the 20S core. Another interesting finding is that the resulting deglutathionylation process restores proteasomal chymotrypsin-like activity and post-acidic cleavage concomitant with Grx2 degradation by the 20S particle. We also show that cytoplasmic thioredoxins 1 and 2 play similar roles. Both isoforms were able to deglutathionylate the 20S core, allowing rescue of proteasomal activities.

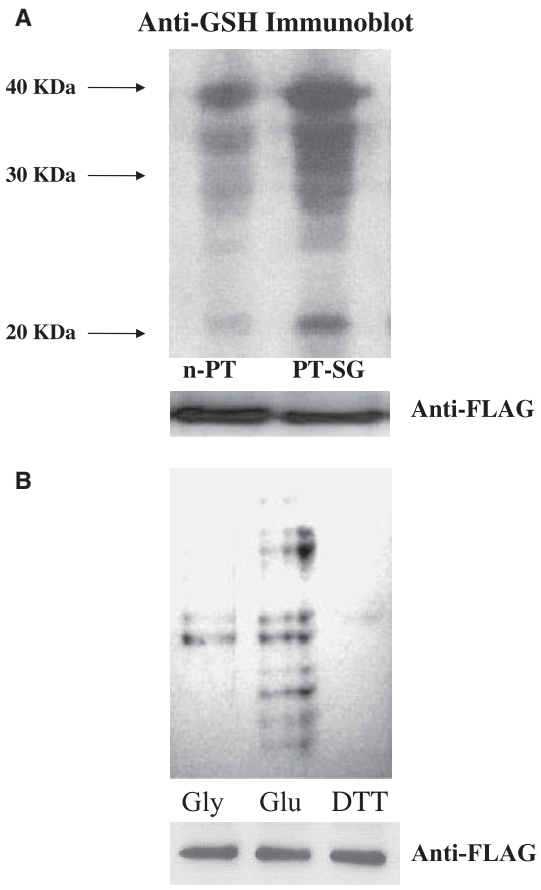

Fig. 1. Anti-GSH blotting of 20S proteasome preparations. After proteasome purification, samples (30 μg) were dissolved in gel loading buffer containing 10 mM *N*-ethylmaleimide and applied to SDS–PAGE. (A) Representative blots of natively (n-PT) and *in vitro* S-glutathionylated (PT-SG) proteasomal preparations. (B) 20S proteasome preparations obtained from cells grown to stationary phase in glycerol/ethanol- (Gly) or glucose-containing (Glu) media. DTT, sample of the n-PT preparation treated with 300 mM dithiothreitol. Anti-FLAG, loading control performed as described in Experimental procedures on the same membranes utilized for anti-GSH blotting.

Results

20S proteasome is natively S-glutathionylated

We demonstrated previously that the 20S proteasome core (PT) is S-glutathionylated when cells are challenged with H_2O_2 [8]. We began the present investigation by verifying whether the 20S PT is also natively S-glutathionylated. Remarkably, the 20S core purified from cells grown to stationary phase in glucose-enriched medium was natively S-glutathionylated, as assessed by western blotting using anti-GSH (Fig. 1A, n-PT). By comparing the *in vitro* proteasome S-glutathionylation (PT-SG) to that observed in preparations obtained from cells grown to stationary phase (n-PT), we observed that the 20S particle was not fully S-glutathionylated *in vivo* when compared to the *in vitro* process (Fig. 1A). The *in vitro* assay results indicated that the potential for S-glutathionylation of 20S proteasome subunits is much higher than that observed inside cells (Fig. 1A). Moreover, the 20S core purified from cells grown to stationary phase in glucose-containing medium was more greatly S-glutathionylated when compared to preparations obtained

from cells grown in glycerol/ethanol-containing medium (Fig. 1B, lanes Glu and Gly, respectively). As a control, samples purified from cells grown in glucose were treated with 10 mM dithiothreitol dithiothreitol before loading onto the gel utilized for the immunoblot assay (Fig. 1B, lane dithiothreitol). After dithiothreitol treatment, 20S proteasome S-glutathionylated bands were completely absent. The purified 20S PT SDS/PAGE profile is shown in supplementary Fig. S1 (lane 2).

As shown previously [23] and confirmed in our laboratory, intracellular reductive ability is higher when yeast cells are grown in glycerol/ethanol-enriched medium (data not shown). Glucose is known to repress expression of genes related to antioxidant defenses and mitochondrial biogenesis [24,25], but glycerol/ethanol growth conditions only support respiratory growth and maintain antioxidant defenses at increased levels [23]. Together with increased antioxidant parameters, we found that the chymotrypsin-like activity of purified 20S proteasome obtained from cells grown in glycerol/ethanol was five times that of preparations obtained from cells grown in glucose-containing medium, with no alteration of 20S proteasome levels (data not shown). These results suggest that proteasomal activity might be modulated according to intracellular redox modifications.

20S proteasome deglutathionylation by Grx2

The observation that the 20S core purified from stationary-phase cells was already S-glutathionylated, together with our data showing that S-glutathionylation of the 20S core particle varies according to the metabolic conditions of yeast cells (Fig. 2 and Demasi M & Silva GM unpublished results), provide strong evidences that this redox alteration plays an important physiological role. Our next goal was to identify an enzymatic mechanism that is able to modulate the proteasomal activity by redox modifications, e.g. deglutathionylation. Based on reports in the literature, Grx2 is one of the enzymes responsible for GSH-dependent deglutathionylase activity in yeast cells [11], and, in addition, Grx2 co-localizes with the proteasome in the cytosol. Thus, recombinant Grx2 was evaluated for its ability to deglutathionylate PT-SG obtained through a multi-step procedure as described in Experimental procedures. Preparations from each step (oxidized, *in vitro* S-glutathionylated and Grx2-treated samples) were reacted with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD), a sulfhydryl and sulfenic acid reagent [1], and the formation of Cys-S-NBD and Cys-S(O)-NBD adducts or their disappearance was followed by spectral

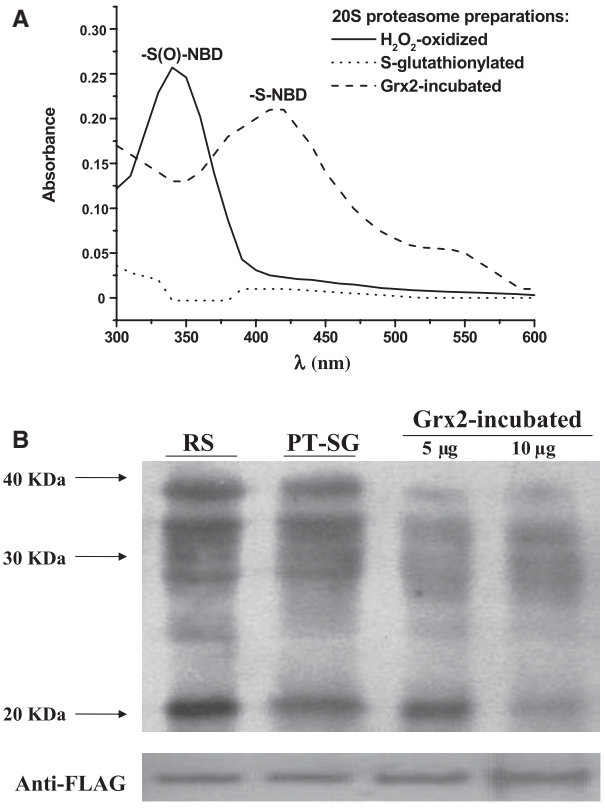

Fig. 2. Recombinant Grx2 deglutathionylase activity on S-glutathionylated 20S PT. (A) Assay with the sulfhydryl and sulfenic acid reactant 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD). The Cys-S(O)–NBD conjugate (solid line) or NBD-reacted S-glutathionylated 20S core (dotted line) were generated by reaction of 100 μM NBD with H_2O_2- or GSH-treated proteasome preparations (described in Experimental procedures) denatured using 5 M guanidine. The Cys-S–NBD conjugate (dashed line) was generated by incubation of S-glutathionylated 20S PT with Grx2 in the presence of the RS (2 mM NADPH, 0.3 U·mL^{-1} GR and 0.5 mM GSH), followed by reaction with NBD. Excess NBD was removed by filtration as described previously [8]. Spectra were recorded as indicated. (B) Anti-GSH blotting. The *in vitro* S-glutathionylated 20S PT was prepared as described in Experimental procedures. Samples (20 μg PT-SG) were incubated for 30 min at 37 °C under the indicated conditions in a final volume of 40 μL and applied to 12.5% SDS–PAGE for immunoblot analysis. RS, sample incubated in the presence of 0.5 mM GSH, 2 mM NADPH and 0.3 U·mL^{-1} GR without Grx2; PT-SG, sample incubated without the RS or Grx2; Grx2-incubated, samples incubated in the presence of the RS plus Grx2 at the indicated concentrations. Anti-FLAG, loading control performed as described in Experimental procedures on the same membranes utilized for anti-GSH blotting.

measurement. When the 20S core was oxidized with H_2O_2, sulfenic acid was formed (Fig. 2A, solid line). However, the sulfenic form of the 20S core cysteine residues completely disappeared when H_2O_2-oxidized 20S preparations were treated with GSH (Fig. 2A,

dotted line). This result is consistent with the idea that, under these conditions, cysteine residues of the 20S core are protected from NBD modification by S-glutathionylation. The S-glutathionylated 20S core was reduced to Cys-SH after incubation with recombinant Grx2 (Fig. 2A, dashed line), indicating that this thiol disulfide oxido-reductase is capable of removing GSH residues from the core. Similar S-glutathionylated 20S PT samples were also analyzed by immunoblot with anti-GSH IgG (Fig. 2B). PT-SG was incubated with two concentrations of recombinant Grx2 in the presence of the GSH-dependent reductive system, as described in Experimental procedures. As seen in Fig. 2B, S-glutathionylated bands of the 20S core (PT-SG) significantly decreased after incubation in the presence of Grx2 (Grx2-incubated), and incubation with 10 μg Grx2 increased proteasome deglutathionylation when compared to the incubation with 5 μg Grx2. The molar ratios of PT : Grx2 were 1 : 10 and 1 : 20, respectively. To evaluate the effect of the GSH-dependent reductive system on deglutathionylation, proteasomal preparations were incubated in standard buffer containing the reductive system but not Grx2 (Fig. 2B, RS). The reductive system had no effect on 20S PT deglutathionylation.

Taken together, the results shown in Fig. 2 provide direct evidence that Grx2 is capable of partly deglutathionylating the 20S proteasome.

Grx2 increases chymotrypsin-like activity and post-acidic cleavage of the S-glutathionylated 20S proteasome

To demonstrate to what extent S-glutathionylation interferes with proteasomal activity, site-specific activities were determined using n-PT and *in vitro* S-glutathionylated PT-SG and PT-SH preparations (Fig. 3). Chymotrypsin-like proteasomal activities from n-PT and PT-SG preparations were 62% and 45% of that observed in the PT-SH preparation, respectively, whereas the post-acidic cleavage in the n-PT and PT-SG preparations was 50% and 35%, respectively, of that in PT-SH preparations (Fig. 3; samples indicated by −). As observed previously [8], the trypsin-like activity was not modified by any redox modification of the core. The results shown in Fig. 3 (samples indicated by −) demonstrate that proteasomal activities are inversely correlated to the extent of S-glutathionylation.

As discussed above, chymotrypsin-like activity and post-acidic cleavage were decreased by S-glutathionylation. Next, our goal was to verify whether reduction of S-glutathionylated proteasome by Grx2 would increase

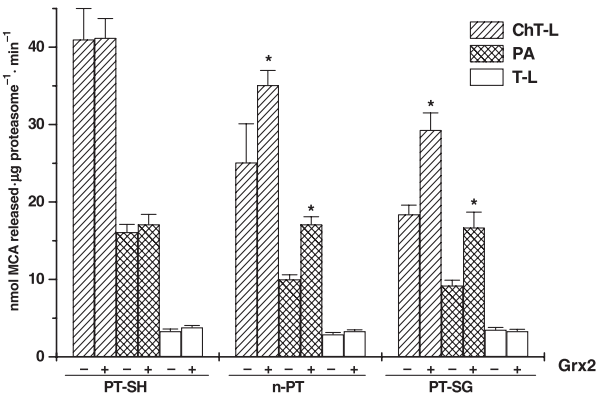

Fig. 3. Effect of Grx2 on proteasomal hydrolytic activities. To test for the recovery of proteasomal chymotrypsin-like activity and post-acidic cleavage after pre-incubation with Grx2, the indicated proteasomal preparations (50 μg·200 μL^{-1}) were immobilized on anti-FLAG affinity gel as described previously [8]. Grx2 (1 μg) plus the GSH-dependent reductive system (RS) were mixed with immobilized proteasome preparations, and the samples were incubated for 30 min at 37 °C with shaking. After incubation, control (–) and Grx2-incubated samples (+) were washed three times by centrifugation (8000 ***g*** × 15 mins at room temperature) and redilution with standard buffer through Microcon YM-100 filters. Final immobilized proteasome preparations were transferred to 96-well plates in 100 μL standard buffer. Indicated substrates (ChT-L, chymotrypsin-like; T-L, trypsin-like; PA, post-acidic) were added to a final concentration of 50 μM. Hydrolysis was followed for 45 min at 37 °C, and fluorescence (440 nm; excitation 365 nm) was recorded every 5 min. All results are means ± SD and are expressed as nmol AMC released per μg proteasome per min. Asterisk indicate a *P* value of < 0.0003 (ANOVA) compared to PT-SH samples.

modified proteasomal activities to the levels of the PT-SH preparation. As expected, Grx2 pre-incubation with S-glutathionylated forms of the 20S proteasome (n-PT and PT-SG) resulted in increased chymotrypsin-like activity and post-acidic cleavage (Fig. 3; samples indicated by +). The activities in the PT-SH preparation did not change after incubation with Grx2. If the dithiothreitol-reduced proteasomal activity (PT-SH) is taken as the maximum attainable (100%), chymotrypsin-like activity for n-PT was 63% recovered after incubation with Grx2, whereas the recovery was 48% for PT-SG. Post-acidic cleavage for the PT-SG and n-PT preparations was totally recovered after incubation with Grx2. Again, trypsin-like proteasomal activity was not modified by any of the treatments performed here. Taken together, the results presented so far indicate that S-glutathionylation and Grx2 modulate post-acidic cleavage and chymotrypsin-like activity by modifying the redox state of proteasomal cysteine residues.

Similar experiments to those described above were performed using cytosolic thioredoxins, and they also

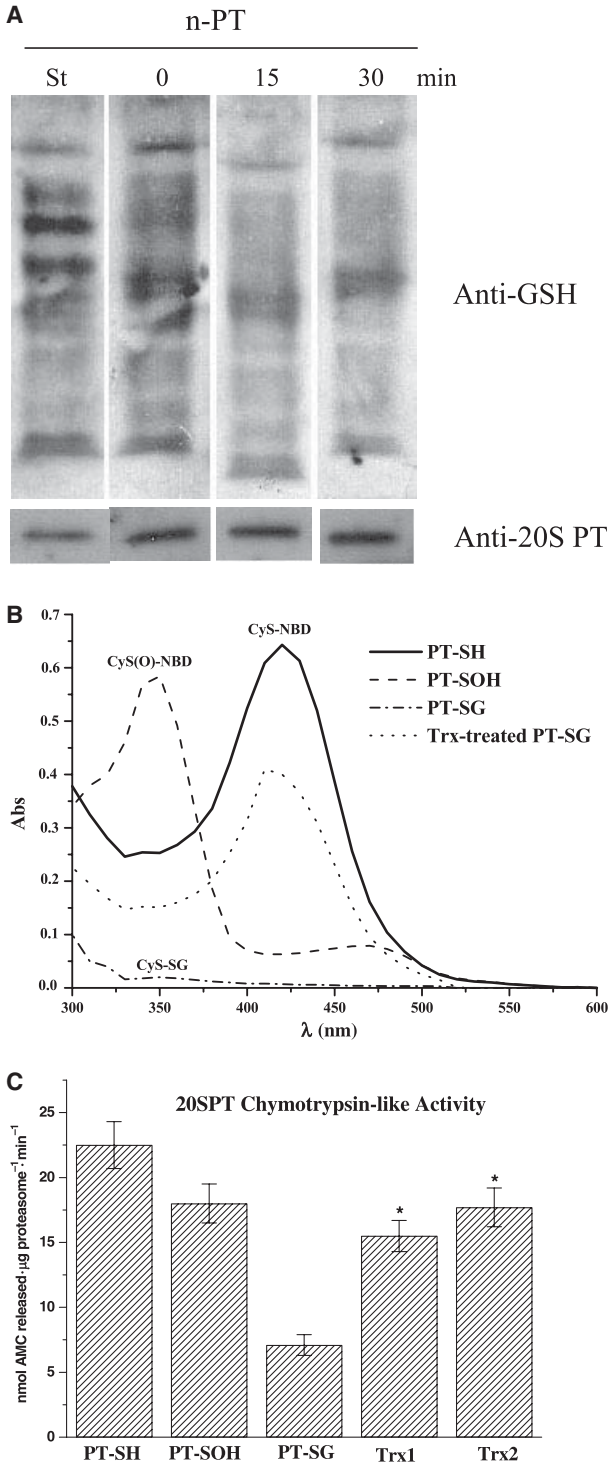

Fig. 4. Deglutathionylation of 20S proteasome preparations by recombinant Trx1 and Trx2. (A) n-PT preparations (20 μg) were mixed with Trx1 (3 μg) plus 2 mM NADPH and 0.5 μg Trr1 and incubated at 37 °C for 15 or 30 min (lanes indicated by 15 and 30, respectively) or kept on ice (lane indicated by 0). Samples were analyzed by western blotting with anti-GSH as described in Fig. 1. St, control n-PT preparation incubated for 30 min at 37 °C in the absence of Trx1. Anti-20SPT, loading control performed with the same membranes utilized for anti-GSH blotting. (B) PT-SH, PT-SOH (PT-SH after treatment with hydrogen peroxide) and PT-SG preparations were generated as described in Experimental procedures. The Cys-S–NBD (solid line), Cys-S(O)–NBD (dashed line) conjugates and the NBD-reacted S-glutathionylated 20S core (dashed/dotted line) were generated from 100 μg PT-SOH or PT-SG preparations. The Cys-S–NBD conjugate (dotted line) was obtained after incubation of PT-SG (100 μg) with Trx1 or Trx2 (1 μg) in the presence of 2 mM NADPH and 0.5 μg Trr1 per 100 μL (final concentration), followed by dilution in 5 M guanidine and reaction with NBD. Results shown are representative of three independent experiments. (C) Effect of Trx1 and Trx2 on the recovery of chymotrypsin-like proteasomal activity. One microgram of PT-SH, PT-SOH or PT-SG, as indicated, was assayed for hydrolysis of the fluorogenic peptide s-LLVY-AMC (10 μM), as described in Experimental procedures. PT-SG samples (50 μg) were incubated for 30 min in the presence of Trx1 (1 μg) or Trx2 (1 μg) plus 2 mM NADPH and 0.5 μg Trr1 per 100 μL. Aliquots (1 μg) of Trx1- and Trx2-treated PT-SG were removed for the hydrolytic assay. The results shown are means ± SD and represent six independent experiments. Asterisks indicate *P* values of < 0.000012 (ANOVA) compared to PT-SG samples.

exhibited deglutathionylase activity towards 20S PT as evaluated by both anti-GSH probing and NBD assay of similar proteasome preparations (Fig. 4A,B, respectively). An immunoblot analysis performed after incubation of n-PT preparations with Trx1 revealed that the time course of proteasomal deglutathionylation was as short as 15 min, and 30 min incubation did not change the extension of deglutathionylation when these blots (Fig. 4A, 15 and 30) were compared to the control sample of n-PT (Fig. 4A, St).

Figure 4B shows results obtained for an NBD assay performed with both Trx1 and Trx2. The molar ratio between thioredoxins and the *in vitro* S-glutathionylated core (PT-SG) was 10 : 1. As shown in Fig. 4B, incubation of PT-SG (Fig. 4B, Cys-S-SG) with either Trx1 or Trx2 promoted the appearance of the reduced Cys-S–NBD adduct. However, formation of proteasomal intraprotein sulfur bonds is expected during treatment with H_2O_2, as *in vitro* S-glutathionylation of proteasomal preparations occurs through formation of cysteine sulfenic acid, as described in supplementary material Doc. S1. To rule out the possibility that the Cys-S–NBD adduct formed after incubation of S-glutathionylated proteasome preparations with thioredoxins was formed by reduction of sulfur bonds instead of deglutathionylation, proteasome preparations were incubated with Trx1 just after treatment with H_2O_2 (molar ratio 20S PT : Trx1 of 1 : 20), followed by reaction with NBD. The results did not indicate formation of the Cys–NBD adduct

(data not shown). The proteasome concentration in the assays was five times the concentration utilized in the experiments shown in Fig. 4B. Thus, we concluded from this set of experiments that formation of the Cys–NBD adduct after incubation of PT-SG preparations with thioredoxins (as shown in Fig. 4B) most likely occurred through deglutathionylation.

Next we performed assays to test whether thioredoxins could recover the hydrolytic activity of S-glutathionylated proteasome preparations. Recovery of the chymotrypsin-like activity of the *in vitro* S-glutathionylated core (PT-SG) by Trx1 and Trx2 was very similar (Fig. 4C). The chymotrypsin-like activity of PT-SG preparations compared to that obtained from dithiothreitol-reduced preparations (PT-SH) was 71% and 77% after incubation with Trx1 and Trx2, respectively. These results were very close to those obtained with Grx2 (63%), as described above.

Mechanism of deglutathionylation

One question raised during the experiments described above was whether the oxido-reductases exerted their effects by reducing only mixed disulfides located on the surface of the 20S core particle, or whether they were also able to enter the latent 20S PT to reduce cysteine residues inside the catalytic chamber. By analyzing structural features of yeast 20S PT from the Protein Data Bank (PDB identification 1RYP), we determined that only a few cysteine residues among the total of 72 are exposed to the environment: 10 solvent-accessible cysteines were determined to be present on the surface, with some of them being totally exposed and others slightly buried but still solvent-accessible. All of the other cysteine residues are either buried in the skeletal structure or exposed to the internal catalytic chamber environment. Therefore, we investigated whether Grx2 enters the core particle. Assuming that Grx2 must be at least partially degraded to reach inside the proteasome, we first evaluated Grx2 degradation using SDS–PAGE (Fig. 5A). Degradation of Grx2 was achieved by incubating n-PT with Grx2 in standard buffer for 2 h (Fig. 5A, lane 2) or by proteasomal stimulation with 0.0125% SDS (Fig. 5A, lane 4). As a control, proteasomal preparations were heated to 100 °C (Fig. 5A, lane 3) prior to incubation with Grx2 and compared to standard Grx2 incubated in standard buffer lacking proteasome (Fig. 5A, lane 1); no proteolysis was seen. Degradation by the proteasome was determined by the decreased intensity of Grx2 bands as evaluated by measurement of optical density. When incubated in standard buffer, n-PT was able to degrade about 70% of Grx2

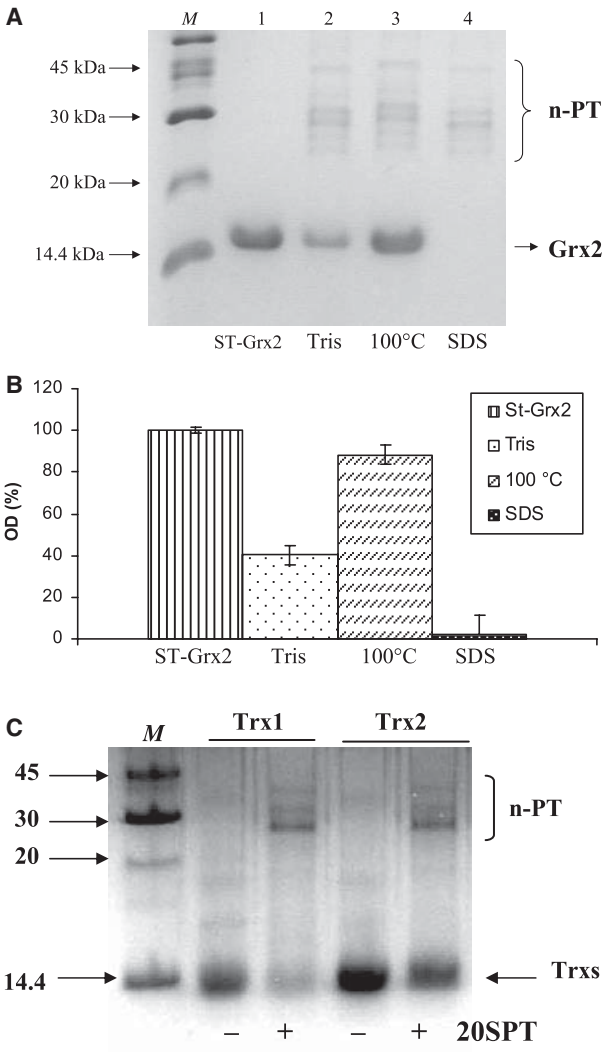

Fig. 5. Degradation of Grx2, Trx1 and Trx2 by n-PT preparations. (A) Grx2 (5 μg) was incubated in the presence of 2.5 μg n-PT for 2 h at 37 °C and afterwards applied to 20% SDS–PAGE. Lane 1 represents standard Grx2 (ST-Grx2) incubated in standard buffer without n-PT, and lanes 2–4 represent of Grx2 incubation in the presence of n-PT in standard buffer (Tris), heated at 100 °C or activated by 0.0125% SDS before addition of Grx2. *M*, molecular mass markers. (B) Optical density measurement of Grx2 bands. Grx2 bands shown in (A) were quantified using IMAGEQUANT software. Values are means ± SD from three independent experiments. The results are expressed as a percentage of the ST-Grx2 band, which was set as 100. (C) Trx1 and Trx2 aliquots (5 and 10 μg, respectively) were incubated with 2.5 μg 20SPT (+) in standard buffer for 30 min at 37 °C. After incubation, samples were applied to 20% SDS–PAGE. (–), Trx1 and Trx2 samples incubated under the same conditions in the absence of natively S-glutathionylated 20S PT. *M*, molecular mass markers.

(Fig. 5B). It is well established that 20S PT is activated by SDS at low concentrations [26]. When 0.0125% SDS was added to the buffer (Fig. 5A, lane 4), Grx2

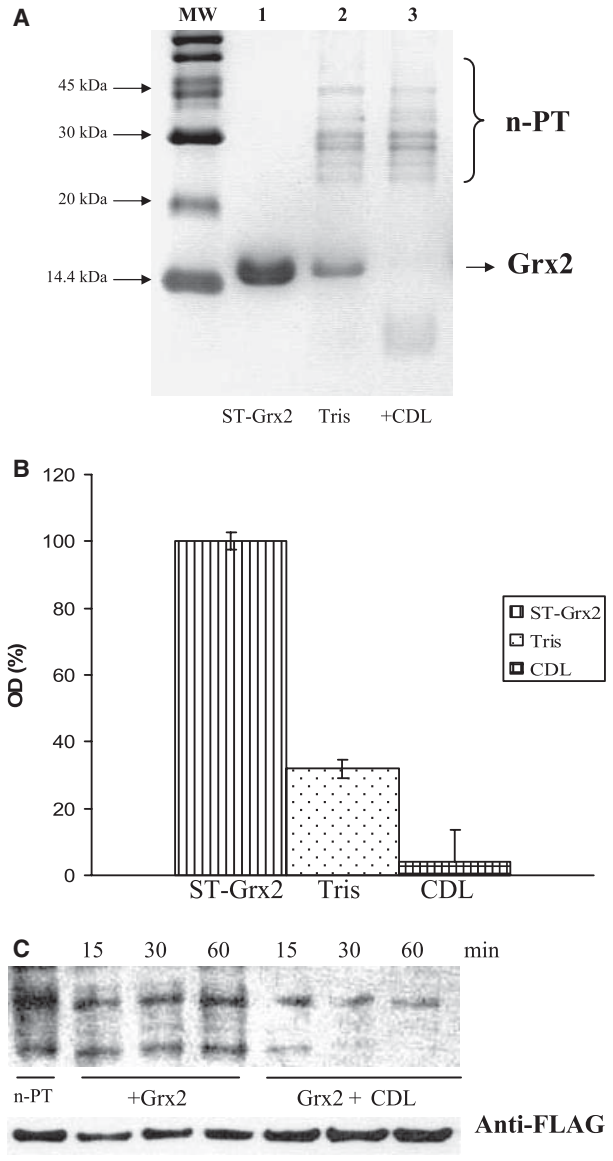

Fig. 6. Stimulation of Grx2-dependent proteasome deglutathionylation by cardiolipin. (A) Increased degradation of Grx2 in the presence of cardiolipin (CDL). 20% SDS–PAGE representative of n-PT preparations (2.5 μg) incubated for 2 h at 37 °C in standard buffer with Grx2 (5 μg). Lane 1, purified Grx2 incubated without n-PT; lane 2, Grx2 plus n-PT; lane 3, Grx2 plus CDL-activated n-PT (preincubation in the presence of 1.75 μg CDL per μg n-PT for 5 min at 37 °C). (B) Optical density quantification of Grx2 bands. Values are means ± SD for three independent experiments represented in (A). The results are expressed as a percentage of the ST-Grx2 band, which was set as 100%. (C) Anti-GSH immunoblot. N-PT (20 μg) samples were incubated with Grx2 in a final volume of 40 μL (10 μg; +Grx2) in the presence or absence of CDL (Grx2+ CDL) for the indicated durations. N-PT, 20S PT preparation incubated under the same conditions without Grx2 or CDL. Anti-FLAG, loading control performed as described in Experimental procedures on the same membranes utilized for anti-GSH blotting.

degradation was increased to 98% when compared to the standard band for Grx2. The same results were obtained with the other deglutathionylases assayed, Trx1 and Trx2. As shown in Fig. 6C, both Trx1 and Trx2 were degraded by the proteasome (molar ratios for n-PT : Trx1 and n-PT : Trx2 were 1 : 10 and 1 : 20, respectively).

To evaluate whether Grx2 degradation was a non-specific process, Grx2, commercially available cytochrome *c*, recombinant peroxidase Ohr (organic hydroperoxide resistance protein), ovalbumin and bovine casein at similar concentrations were incubated with n-PT (supplementary Fig. S2). We selected cytochrome *c* because of its well-known resistance to degradation by the latent form of the 20S particle [27,28], and because its molecular mass (12 kDa) is close to that of recombinant Grx2 (14.1 kDa), eliminating the possibility of size- or protein diameter-specific degradation. The organic hydroperoxide resistance protein Ohr (17 kDa) was tested because of its cysteinyl-based active site [29,30]. Ovalbumin is a larger protein (44 kDa) that known to be degraded *in vitro* by 20S PT only when denatured [31,32]. Moreover, we compared the degradation of all proteins with that of casein, which has a low secondary structure content and is easily hydrolyzed by the 20S core. After incubation and prior to application to SDS–PAGE, n-PT was removed by filtration. The only two proteins degraded by 20S PT were Grx2 and casein (supplementary Fig S2), indicating a specific proteolytic process, probably correlated to the structural characteristics of Grx2 and its interaction with 20S PT. All of the other proteins tested here were resistant to degradation, in agreement with the view that the latent form of the 20S PT recognizes specific features in target proteins. These results gave further support to the notion that Grx2 deglutathionylase activity plays a regulatory role in 20S PT activities.

We next analyzed Grx2 fragmentation using mass spectrometry, by incubating Grx2 in standard buffer for 30 min or 2 h in the presence of n-PT. After incubation, standard Grx2 and fragments recovered by filtering the incubation mixture through 100 kDa cut-off micro filters were processed for MS analysis, as described in Experimental procedures. Grx2 degradation by the core, as shown by SDS–PAGE (Fig. 5A), was confirmed by the MS analysis (Table 1 and supplementary Fig. S3). As expected, Grx2 fragmentation by 20S PT was increased after 2 h incubation compared to the 30 min incubation (supplementary Fig. S3B,C, respectively). MS analysis of purified recombinant Grx2 not incubated with the proteasome confirmed the high degree of purity and absence of

Table 1. Peptides derived from *in vitro* degradation of Grx2 by the 20S proteasome and identified by mass spectrometry. Samples were prepared as described in Experimental procedures. Results shown were obtained as described for supplementary Fig. S3.

Peak[a]	Residues	Parent ion mass	Peptide sequence
a	22–65	4898.75 ± 0.19	VSQETVAHVKDLIGQKEVFVAAKTY**47CPYC51**KATLSTLFQELNVPK
b	47–103	6255.05 ± 0.32	**47CPYC51**KATLSTLFQELNVPKSKALVLELDEMSNGSEIQDALEEISGQKTVPNVYINGK
c	33–72	4445.30 ± 0.31	LIGQKEVFVAAKTY**47CPYC51**KATLSTLFQELNVPKSKALVLE
d	41–75	3887.68 ± 0.20	VAAKTY**47CPYC51**KATLSTLFQELNVPKSKALVLELDE
e	33–65	3703.95 ± 0.65	LIGQKEVFVAAKTY**47CPYC51**KATLSTLFQELNVPK
f	45–75	3517.84 ± 0.42	TY**47CPYC51**KATLSTLFQELNVPKSKALVLELDE
g	66–93	3032.25 ± 0.29	SKALVLELDEMSNGSEIQDALEEISGQK

[a] Peaks shown in supplementary Fig. S3.

any fragmentation after 2 h incubation in standard buffer at 37 °C (supplementary Fig. S3A). As shown in supplementary Fig. S3B, after 30 min incubation with the proteasome, a 4898 kDa Grx2 fragment was generated (Table 1). Although Grx2 fragmentation was greatly increased after the 2 h incubation when compared to the 30 min incubation (supplementary Fig. S3C and Table 1), the 4898 kDa peptide remained intact. It is noteworthy that almost all the fragments detected after the 2 h incubation, possess the active site (47CPYC51; Table 1). Most probably, these N-terminal fragments are correctly structured and retain oxido-reductase activity as the CPYC domain appears in the inner core of most of them.

To corroborate the results shown above, we tested whether deglutathionylation by Grx2 is increased when its entry into the catalytic chamber is stimulated. Cardiolipin is a well-established proteasome activator that is capable of stimulating 20S core particle entry [33]. Our hypothesis was that cardiolipin would have a synergistic effect on Grx2-dependent deglutathionylation by increasing Grx2 core entry. Therefore, after incubation of 20S PT with cardiolipin and Grx2, samples were analyzed by SDS–PAGE (Fig. 6A,B) and western blot using antibody against GSH (Fig. 6C), in parallel with proteasomal activity measurement in order to confirm catalytic recovery (Table 2).

It was found that activation of the 20S core by cardiolipin increased Grx2 degradation by 30% according to optical density measurements when compared to its degradation by the 20S PT but not stimulated by cardiolipin (Fig. 6A, lanes 3 and 2, respectively, and Fig. 6B). In parallel, deglutathionylation by Grx2 (evaluated by anti-GSH blotting analysis) in the presence of cardiolipin was greatly enhanced (Fig. 6C). It is noteworthy that, with increasing incubation time, the effect of cardiolipin was much more pronounced when compared to proteasome samples solely incubated with Grx2 for the same duration of incubation (Fig. 6C). These results strongly suggest that proteasome deglutathionylation is dependent on Grx2 entry into the catalytic chamber. The results shown in Table 2 confirm the cardiolipin stimulatory effect on 20S PT deglutathionylation, showing increased chymotrypsin-like activity and post-acidic proteasomal cleavage after simultaneous incubation of proteasome preparations with cardiolipin and Grx2. The results obtained showed 25% and 65% increased chymotrypsin-like activity and 61% and 100% increased post-acidic cleavage of n-PT and PT-SG preparations, respectively, when compared to samples incubated solely in the presence of Grx2. In all of the experiments described, after a 30 min pre-incubation with 20S core particle, Grx2 and cardiolipin were removed

Table 2. Effect of Grx2 on chymotrypsin-like activity and post-acidic cleavage of the natively and *in vitro* S-glutathionylated 20S PT pre-incubated with cardiolipin. Natively (n-PT) and *in vitro* (PT-SG) S-glutathionylated proteasome preparations in 20 mM Tris/HCl, pH 7.5 (20 μg·100 μL^{-1}) were pre-incubated for 5 min with cardiolipin (1.75 μg·1 μg^{-1} proteasome) followed by addition of Grx2 plus the RS. After 30 min at 37 °C, samples were filtered through YM-100 microfilters and washed three times with standard buffer. Proteasome recovered on the microfilter membrane was incubated (1 μg·100 μL^{-1}) with the indicated substrates (each at 50 μM). Fluorescence emission (440 nm; excitation 365 nm) was determined after 45 min incubation at 37 °C. All results are means ± SD and are expressed as nmol AMC released per μg proteasome per min. As controls, n-PT preparations were incubated in standard buffer in the absence of Grx2 or pre-treatment with cardiolipin (CDL), or pre-incubated with CDL in the absence of Grx2. Asterisks indicate a *P* value < 0.00034 compared to same proteasomal samples incubated in the presence of Grx2 without CDL (ANOVA).

	Chymotrypsin-like (s-LLVY-AMC)	Post-acidic (z-LLE-AMC)
n-PT	28 ± 2	14 ± 1.1
Pre-incubated with CDL	30 ± 1.8	15.5 ± 0.9
n-PT/Grx2	40 ± 1.5	19 ± 0.7
+ CDL	50 ± 4*	30.5 ± 1.5*
PT-SG/Grx2	37 ± 2.5	18 ± 1.0
+ CDL	61 ± 4.5*	36 ± 3.5*

by cycles of filtration and re-dilution, as described in the legend to Table 2, immediately prior to hydrolytic activity measurement. This procedure ensured that the increased post-acidic cleavage and chymotrypsin-like activity observed after 20S PT incubation with Grx2 in the presence of cardiolipin were due to increased deglutathionylation rather than cardiolipin-dependent proteasomal-stimulated activity, as previously reported when 20S PT activity was determined during incubation with cardiolipin [33]. To control the cardiolipin washing procedure, proteasomal catalytic activity was determined with samples not incubated with Grx2. Under these conditions, proteasomal activity was not increased after washing cardiolipin from the reaction mixture when compared to proteasomal activity determined in samples of untreated 20S PT (Table 2). Our conclusion from this set of experiments was that cardiolipin-stimulated Grx2 entry into the core increased 20S PT deglutathionylation. These results suggest that cysteine residues located inside the core are critical for redox regulation through S-glutathionylation.

Glutaredoxins with two cysteines in the active site possess two activities: mono- and dithiolic [9]. Therefore, we performed experiments with the Grx2C30S mutant, which lacks the C-terminal cysteine residue and retains only monothiolic activity. Grx2C30S activity determined using hydroxyethyldisulfide (HED) as a substrate, as described in the Experimental procedures, was 70% of that with the wild-type protein (data not shown). Monothiolic Grx2C30S was also able to deglutathionylate n-PT, although to a lesser extent than wild-type Grx2 (supplementary Fig. S4, C30S and WT, respectively). The active C30S mutant was also degraded by the 20S PT (data not shown). Therefore, monothiolic glutaredoxins should be considered as potential proteasomal deglutathionylases.

Grx2 is ubiquitinated *in vivo*

To determine whether Grx2 ubiquitination takes place at the physiological level, we next analyzed the presence of Grx2–ubiquitin complexes in crude cellular extract from yeast grown to stationary phase in glucose-enriched medium. During ubiquitination, up to six molecules of ubiquitin (8.5 kDa) can be added to form a polyubiquitin chain. We performed the experiments by immunoprecipitating Grx2 from the crude cellular extracts, followed by anti-ubiquitin and anti-Grx2 western blotting analyses (Fig. 7). Blotting with anti-Grx2 serum under reducing conditions showed the short (11.9 kDa) and long (15.9 kDa) forms of Grx2 (Fig 7, anti-Grx2). The band at 20 kDa is compatible with the size of mono-ubiquitinated short Grx2 isoforms (cytosolic and mitochondrial matrix) [34], as the same band was seen in the anti-ubiquitin blot (Fig. 7, anti-Ub). Blotting of the same samples with anti-ubiquitin revealed the presence of higher molecular mass complexes (above 50 kDa), compatible with poly-ubiquitinated Grx2 isoforms (Fig. 7, anti-Ub). These bands were not visualized in the anti-Grx2 blotting, most probably because they represent poly-ubiquitinated isoforms with a low concentration of Grx2. These results are the first demonstration that Grx2 is ubiquitinated *in vivo*.

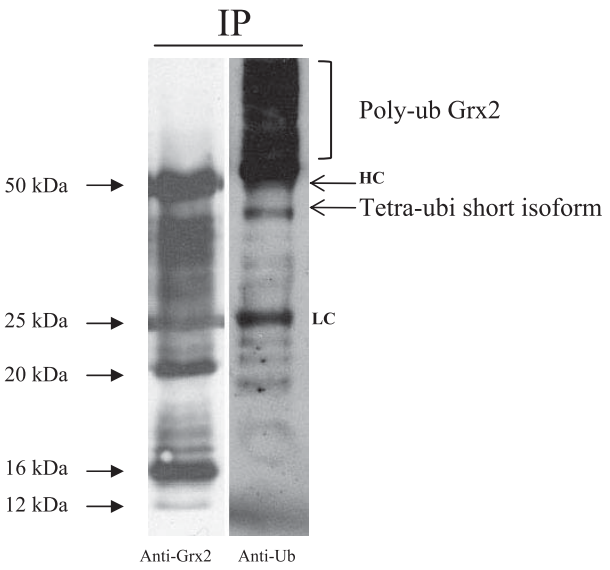

Fig. 7. *In vivo* Grx2 ubiquitination. Grx2 was immunoprecipitated with anti-Grx2 from crude a cellular extract of yeast cells grown to stationary phase in glucose-enriched medium, followed by blotting with anti-Grx2 (Anti-Grx2) or anti-ubiquitin (Anti-ub) as indicated. Immunoprecipitated samples were treated with 100 mM dithiothreitol prior to western blotting analyses. The molecular masses shown were deduced from a molecular mass standard ladder (Kaleidoscope; GE Biosciences, Piscataway, NJ, USA) by overlapping the membrane and overexposed blotted films (data not shown). LC and HC, light and heavy chains of IgG immunoglobulin.

Discussion

Sulfhydryl groups play a critical role in the function of many proteins, including enzymes, transcription factors and membrane proteins [35]. In a previous report, we concluded that oxidative stress induced proteasome glutathionylation and loss of chymotrypsin-like activity [8]. Now, we show that the S-glutathionylation and deglutathionylation processes represent biological redox regulation of 20S PT under basal conditions. We also showed the existence of regulatory mechanisms (best characterized in the case of Grx2) that are able to deglutathionylate the core particle, leading to

concomitant recovery of proteolytic activities. Our data show that two cytosolic thioredoxins also have the same effects on the 20S particle (Fig. 4). Furthermore, in principle, monothiolic glutaredoxins might also dethiolate the core, based on the ability of mutant Grx2C30S to perform this activity (supplementary Fig. S4). The existence of multiple pathways to dethiolate 20S PT may represent a highly tuned process to regulate this protease complex.

The data present in Figs 5 and 6 indicate that either Grx2, Trx1 and Trx2 must enter the latent 20S core to deglutathionylate proteasomal cysteine residues and recover proteasomal activities (Figs 3 and 4C). Moreover, as Grx2 entry into the 20S core particle increased, deglutathionylation and recovery of proteasomal activities were significantly improved (Fig. 6C and Table 2). Therefore, a question to be raised is whether these oxido-reductases undergo catalytic cycles during proteasomal deglutathionylation since they are degraded by the core. We do not have a definitive answer so far. Based on the results obtained by mass spectrometry analysis, a considerable proportion of Grx2 was not cleaved even after 2 h incubation (supplementary Fig. S3C). Furthermore, as noted above, it is possible that the 4898 kDa peptide detected after 30 min incubation that contains the conserved CXXC motif retains dethiolase activity. Nevertheless, the central point addressed here is that Grx2 is involved in redox regulation of the proteasome, either by an enzymatic or chemical reaction. The details of this process will be further investigated.

As already demonstrated in mammals, some proteins are able to enter the 20S core particle, whereas, for others, only partial structural loss or the existence of poorly structured domains allow free entry [36,37]. Crystallographic modeling shows that the molecular architecture of Grx2 consists of a four-stranded, mixed β-sheet and five α-helices. The β-sheet forms the central core of the protein, with helices 1 and 3 located on one side of the sheet and helices 2, 4 and 5 located on the other side [38] (Discola KF & Netto LES, unpublished results). Most probably, a specific interaction of particular domains of these oxido-reductases stimulates 20S PT opening to allow their entry. Additionally, glutaredoxins and thioredoxins share a common fold, the so-called thioredoxin fold [39], and isoforms of both oxido-reductase families (Grx2, Trx1 and Trx2) are able to deglutathionylate the 20S PT. The recognition of structural features in Grx2, Trx1 and Trx2 by 20S PT indicates that the deglutathionylase activity reported here represents a relevant signaling event. We are presently investigating whether that common feature is related to their easy entry into the latent 20S particle.

According to our data, Grx2 is ubiquitinated inside cells (Fig. 7). Although Grx2 degraded by the 20S PT *in vitro*, the present findings show that degradation of Grx2 might be controlled by ubiquitination at the physiological level. Reports in the literature raise the possibility that proteins that can freely enter the 20S PT can be degraded by both ubiquitin-dependent and -independent processes [37].

Experimental procedures

Materials

Anti-FLAG IgG, cardiolipin (CDL), dithionitrobenzoic acid, diethylenetriaminepentaacetic acid, dithiothreitol, *N*-ethylmaleimide, GSH, glutathione reductase (GR), $NaBH_4$ and Tris(2-carboxy-ethyl) phosphine hydrochloride were purchased from Sigma (St Louis, MO, USA). Anti-20S PT serum, cytochrome *c* from equine heart and the fluorogenic substrates carbobenzoxy-Leu-Leu-Glu-AMC (z-LLE-AMC), carbobenzoxy-Ala-Arg-Arg-AMC (z-ARR-AMC) and succinyl-Leu-Leu-Val-Tyr-AMC (s-LLVY-AMC) were obtained from Calbiochem (Darmstadt, Germany). Molecular mass markers for SDS–PAGE and Protein A–Sepharose 4B Fast Flow were obtained from Amersham Biosciences (Piscataway, NJ, USA). NBD and HED were purchased from Aldrich (St. Louis, MO, USA). AMC (7-amido-4-methylcoumarin) was purchased from Fluka (Buchs Switzerland). Anti-GSH serum was obtained from Invitrogen (Carlsbad, CA, USA). Anti-ubiquitin monoclonal serum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bradford protein assay reagent was purchased from Bio-Rad (Hercules, CA, USA). Sinapinic acid (matrix) and myoglobin (MS standard) were part of the ProteoMass kit (Sigma).

Yeast strain and growth

Saccharomyces cerevisiae RJD1144 (MATa his3Δ200 leu2-3,112 lys2-801 trp1Δ63 ura3-52 $PRE1^{FH}$::Ylplac211 URA3) derived from strain JD47-13C was kindly donated by R. Deshaies (Division of Biology, Caltech, Pasadena, CA, USA). In this strain, the 20S proteasome Pre1 subunit is tagged with the FLAG peptide sequence and a polyhistidine tail, which allows single-step purification [40]. Cells were cultured in glucose-enriched YPD medium (4% glucose, 1% yeast extract and 2% peptone) at 30 °C with reciprocal shaking, and harvested after 60 h incubation.

Extraction and purification of the 20S proteasome

The 20S PT was purified by nickel-affinity chromatography or by immunoprecipitation with anti-FLAG® M2 affinity gel freezer-safe (Sigma) as described previously [8].

Non-tagged 20S PT was purified by conventional multi-step chromatography as described previously [8]. 20S proteasome preparations obtained by affinity chromatography were utilized in all experiments. Preparations obtained by conventional chromatography were utilized as controls for the tagged 20S particle. The purity of 20S PT preparations was confirmed by SDS–PAGE and non-denaturing PAGE as described previously [8].

Proteasome activity determination by hydrolysis of fluorogenic peptides

Fluorogenic peptides (AMC, 7-amido-4-methylcoumarin as the fluorescent probe) were utilized for determination of proteasomal activity, as described elsewhere [41]. s-LLVY-AMC was utilized as a standard peptide to assess the chymotrypsin-like activity of the core, z-LLE-AMC for the post-acidic cleavage and z-ARR-AMC for the trypsin-like activity [41]. 20S PT (0.5–3 μg) was incubated at 37 °C in 20 mM Tris/HCl buffer, pH 7.5, herein referred to as standard buffer. Incubation was started by the addition of 10–50 μM of peptide. Fluorescence emission was recorded at 440 nm (excitation at 365 nm). The amount of AMC released from the substrates was calculated using a standard curve of free AMC.

Reduction, oxidation and S-glutathionylation of the 20S proteasome

Preparations of purified 20S PT (500–1000 μg) extracted from cells grown in glucose-containing medium were incubated overnight at 4 °C in 20 mM Tris buffer, pH 7.5, containing 300 mM dithiothreitol. Then, proteasome preparations were passed through a HiTrap desalting column to remove dithiothreitol, according to the manufacturer's protocol (Amersham Biosciences). Eluted protein fractions were tested for the presence of dithiothreitol by reaction with dithionitrobenzoic acid. Enriched protein fractions, identified by reactivity to Bradford reagent and for which dithiothreitol reactivity was decreased, were selected for further use. Combined fractions were filtered and concentrated through Microcon YM-100 filters (Millipore, Billerica, MA, USA). These preparations are referred to here as dithiothreitol-reduced 20S proteasome (PT-SH). Aliquots of these preparations were oxidized by incubation in standard buffer in the presence of 5 mM H_2O_2 and 100 μM diethylenetriaminepentaacetic acid for 30 min at room temperature. After incubation, excess H_2O_2 was removed by two cycles of centrifugation at 8000 ***g*** for 15 mins at room temperature, and re-dilution through Microcon YM-100 filters. S-glutathionylated 20S core (PT-SG) was obtained by incubation of oxidized 20S proteasome (PT-SOH) aliquots (100 μg) at room temperature for 20 min in the presence of 5–10 mM GSH. Afterwards, GSH was removed by four cycles of centrifugation at 8000 ***g*** for 15 mins at room temperature, and re-dilution through Microcon filters. The S-glutathionylated core used for the assays described here was either the natively S-glutathionylated proteasome(n-PT) purified from cells grown to stationary phase in glucose-enriched medium or the *in vitro* S-glutathionylated core (PT-SG), as described above. After determination of protein concentration, aliquots of PT-SH, n-PT or PT-SG preparations were used for further incubation, immunoblot analyses, SDS–PAGE and hydrolytic assays.

Cloning and expression of yeast *GRX2*

Cloning of the yeast *GRX2*, its expression in *E. coli*, and Grx2 purification have been described previously [20]. The recombinant protein is tagged with an N-terminal polyhistidine sequence. Purified Grx2 was analyzed by SDS–PAGE. Grx2 activity was determined spectrophotometrically by measuring the reduction of 0.5 mM HED in the presence of 0.5 mM GSH, 0.1 mM NADPH and 0.3 $U{\cdot}mL^{-1}$ GR at 37 °C, and following the disappearance of NADPH at 340 nm. All of the experiments with Grx2 were controlled by assaying non-tagged protein (thrombin-treated Grx2). No difference between tagged and non-tagged Grx2 was observed.

Cloning, expression and purification of yeast thioredoxin reductase 1 (Trr1)

Cloning of the yeast *TRR1*, its expression in *E. coli*, and Trr1 purification have been described previously [42]. The recombinant protein was tagged with an N-terminal polyhistidine sequence.

Cloning, expression and purification of yeast Trx1 and Trx2

The *trx1* and *trx2* genes were amplified by PCR from yeast genomic DNA (Research Genetics, Invitrogen), as described previously [43]. PCR products were cloned into the *Nde*I and *Spe*I restriction sites of pET17b expression vector (Novagen, Darmstadt, Germany). *E. coli* BL21 (DE3) cells were transformed with pET17b/*trx1* or pET17b/*trx2* vectors. Protein purification was performed as described previously [43].

Incubation of S-glutathionylated 20S proteasome with Grx2 and thioredoxins

S-glutathionylated 20S PT (20–50 μg), obtained either by growing cells to stationary phase in 4% glucose (n-PT) or by *in vitro* S-glutathionylation (PT-SG), was incubated at 37 °C for 15–120 min in the presence of Grx2 (5–15 μg) in

0.1 mL standard buffer containing 0.5 mM GSH, 2 mM NADPH and 0.3 $U \cdot mL^{-1}$ GR, herein referred to as the reductive system (RS). Incubation with thioredoxins was performed under the same conditions with standard buffer containing 2 mM NADPH and 0.5 μg Trr1 per 100 μL (final concentration). When specified, 1.75 μg CDL per μg 20S PT was added to the mixture. After incubation, samples were filtered by four cycles of centrifugation at 8000 ***g*** for 15 mins at room temperature, and re-dilution through YM-100 Microcon filters. Control samples were incubated in the presence of all reagents except Grx2, Trx1, Trx2 or CDL.

SDS–PAGE analysis of proteins

SDS–PAGE was performed as described previously [44]. Protein preparations, after incubation under the indicated conditions (described in figure legends), were mixed with gel loading buffer (60 mM Tris/HCl, pH 6.8, containing 25% glycerol, 2% SDS and 0.1% bromophenol blue) and frozen until applied to the gel. Gels were stained either by Coomassie brilliant blue or by the silver staining method, as described previously [44].

Immunoprecipitation with anti-Grx2 serum

Grx2 immunoprecipitation from yeast cell lysates was performed as follows: pellets (150–200 mg) of yeast cells were disrupted by vortexing cells mixed with 1 volume of glass beads and 2 volumes of standard buffer, containing 500 mM NaCl plus 1 μL protease inhibitor cocktail (Calbiochem) per 20 mg cellular pellet. After 10 cycles of 2 min vortexing and 1 min resting on ice, the cell lysate was centrifuged at 15 000 ***g*** for 40 min. The supernatant was used for immunoprecipitation. After pre-clearing the cell lysate (1 mg protein) in 200 μL of pre-treated Protein A–Sepharose, the supernatant obtained after centrifugation was used for incubation with anti-Grx2 serum (1 : 50 dilution). Incubation was performed for 1 h in a cold room at 8°C on a rotary shaker and transferred to 200 μL Protein A–Sepharose for a further 15 h incubation. Samples were then centrifuged at 10 000 ***g***, and the Protein A–Sepharose beads were washed five times with 500 μL Tris/NaCl buffer. After removing the final supernatant, 100 μL SDS–PAGE sample buffer was added to the immunoprecipitate, and samples were heated for 10 min at 100 °C. After centrifugation, at 15 000 ***g*** for 10 mins at room temperature samples were applied to 12.5% SDS–PAGE. Grx2 immunoprecipitates were immunoblotted with anti-Grx2 and anti-ubiquitin serum.

Immunoassays

Immunoblotting was performed using the ECL™ western blotting system (Amersham Biosciences) according to the manufacturer's protocol. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and protein signals were detected using enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences). Proteasome samples analyzed using anti-GSH serum were mixed with gel loading buffer containing 10 mM *N*-ethylmaleimide. As a control, samples of PT-SH were run in parallel to all experiments shown and no background signal was observed. The loading control was evaluated by anti-FLAG blotting, as follows: the same membranes utilized for anti-GSH blotting were incubated overnight with 10 mM Tris(2-carboxy-ethyl) phosphine hydrochloride in NaCl/P_i (100 mM Tris, pH 7.5 containing 200 mM NaCl). Next, membranes were washed five times with NaCl/P_i followed by the anti-FLAG blotting. Dilutions of antibodies were as follows: 1 : 200 (anti-ubiquitin), 1 : 1000 (anti-GSH and anti-20S PT) and 1 : 2000 (anti-Grx2 and anti-FLAG).

Optical density measurements

When specified, optical density measurements were performed using IMAGEQUANT software from Molecular Dynamics (Sunnyvale, CA, USA).

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References

1 Ellis HR & Poole LB (1997) Novel application of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole to identify cysteine sulfenic acid in the AhpC component of alkyl hydroperoxide reductase. *Biochemistry* **36**, 15013–15018.
2 Barrett WC, DeGnore JP, Keng YF, Zhang ZY, Yim MB & Chock PB (1999) Roles of superoxide radical anion in signal transduction mediated by reversible regulation of protein-tyrosine phosphatase 1B. *J Biol Chem* **274**, 34543–34546.
3 Claiborne A, Yeh JI, Mallett TC, Luba J, Crane EJ, Charrier V & Parsonage D (1999) Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry* **38**, 15407–15416.
4 Claiborne A, Mallett TC, Yeh JI, Luba J & Parsonage D (2001) Structural, redox, and mechanistic parameters for cysteine-sulfenic acid function in catalysis and regulation. *Adv Prot Chem* **58**, 215–276.
5 Yang KS, Kang SW, Woo HA, Hwang SC, Chae HZ, Kim K & Rhee SG (2001) Inactivation of human

peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. *J Biol Chem* **277**, 38029–38036.
6 Nulton-Persson AC & Szweda LI (2000) Modulation of mitochondrial function by hydrogen peroxide. *J Biol Chem* **276**, 23357–23361.
7 Nulton-Persson AC, Starke DW, Mieyal JJ & Szweda LI (2003) Reversible inactivation of alpha-ketoglutarate dehydrogenase in response to alterations in the mitochondrial glutathione status. *Biochemistry* **42**, 4235–4242.
8 Demasi M, Silva GM & Netto LE (2003) 20S proteasome from *saccharomyces cerevisiae* is responsive to redox modifications and is S-glutathionylated. *J Biol Chem* **278**, 679–685.
9 Fernandes AP & Holmgren A (2004) Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. *Antiox Redox Signal* **6**, 63–74.
10 Georgiou G & Masip L (2003) An overoxidation journey with a return ticket. *Science* **300**, 592–594.
11 Luikenhuis S, Perrone G, Dawes IW & Grant CM (1998) The yeast *Saccharomyces cerevisiae* contains two glutaredoxin genes that are required for protection against reactive oxygen species. *Mol Biol Cell* **9**, 1081–1091.
12 Shenton D, Perrone G, Quinn KA, Dawes IW & Grant CM (2002) Regulation of protein S-glutathionylation by glutaredoxin 5 in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* **277**, 16853–16859.
13 Garrido EO & Grant CM (2002) Role of thioredoxins in the response of *Saccharomyces cerevisiae* to oxidative stress induced by hydroperoxides. *Mol Microbiol* **43**, 993–1003.
14 Holmgren A (1976) Hydrogen donor system for *Escherichia coli* ribonucleoside-diphosphate reductase dependent upon glutathione. *Proc Natl Acad Sci USA* **73**, 2275–2279.
15 Jung CH & Thomas JA (1996) S-glutathiolated hepatocyte proteins and insulin disulfides as substrates for reduction by glutaredoxin, thioredoxin, protein disulfide isomerase, and glutathione. *Arch Biochem Biophys* **335**, 61–72.
16 Davis DA, Newcomb FM, Starke DW, Ott DE, Mieyal JJ & Yarchoan R (1997) Thioltransferase (glutaredoxin) is detected within HIV-1 and can regulate the activity of glutathionylated HIV-1 protease in vitro. *J Biol Chem* **272**, 25935–25940.
17 Bandyopadhyay S, Starke DW, Mieyal JJ & Gronostajski RM (1998) Thioltransferase (glutaredoxin) reactivates the DNA-binding activity of oxidation-inactivated nuclear factor I. *J Biol Chem* **273**, 392–397.
18 Rodríguez-Manzaneque MT, Ros J, Cabiscol E, Sorribas A & Herrero E (1999) Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in *Saccharomyces cerevisiae*. *Mol Cel Biol* **19**, 8180–8190.
19 Gan ZR (1992) Cloning and sequencing of a gene encoding yeast thioltransferase. *Biochem Biophys Res Commun* **187**, 949–955.
20 Pedrajas JR, Porras P, Martínez-Galisteo E, Padilla CA, Miranda-Vizuete A & Bárcena JA (2002) Two isoforms of *Saccharomyces cerevisiae* glutaredoxin 2 are expressed in vivo and localize to different subcellular compartments. *Biochem J* **364**, 617–623.
21 Lee JH, Kim K, Park EH, Ahn K & Lim CJ (2007) Expression, characterization and regulation of a *Saccharomyces cerevisiae* monothiol glutaredoxin (Grx6) gene in *Schizosaccharomyces pombe*. *Mol Cells* **24**, 316–322.
22 Mesecke N, Mittler S, Eckers E, Herrmann JM & Deponte M (2008) Two novel monothiol glutaredoxins from *Saccharomyces cerevisiae* provide further insight into iron–sulfur cluster binding, oligomerization, and enzymatic activity of glutaredoxins. *Biochemistry* **47**, 1452–1463.
23 Jamieson DJ (1998) Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast* **14**, 1511–1527.
24 Moradas-Ferreira P & Costa V (2000) Adaptive response of the yeast *Saccharomyces cerevisiae* to reactive oxygen species: defences, damage and death. *Redox Rep* **5**, 277–285.
25 Maris AF, Assumpcao AL, Bonatto D, Brendel M & Henriques JA (2001) Diauxic shift-induced stress resistance against hydroperoxides in *Saccharomyces cerevisiae* is not an adaptive stress response and does not depend on functional mitochondria. *Curr Genet* **39**, 137–149.
26 Reshetnyak YK, Kitson RP, Lu M & Goldfarb RH (2004) Conformational and enzymatic changes of 20S proteasome of rat natural killer cells induced by mono- and divalent cátions. *J Struct Biol* **145**, 263–271.
27 Huffman HA, Sadeghi M, Seemuller E, Baumeister W & Dunn MF (2003) Proteasome-cytochrome *c* interactions: a model system for investigation of proteasome host–guest interactions. *Biochemistry* **42**, 8679–8686.
28 Sharon M, Felderer WS, Rockel B, Baumeister W & Robinson CV (2006) 20S proteasomes have the potential to keep substrates in store for continual degradation. *J Biol Chem* **281**, 9569–9575.
29 Cussiol JR, Alves SV, de Oliveira MA & Netto LE (2003) Organic hydroperoxide resistance gene encodes a thiol-dependent peroxidase. *J Biol Chem* **278**, 11570–11578.
30 Oliveira MA, Guimarães BG, Cussiol JR, Medrano FJ, Gozzo FC & Netto LE (2006) Structural insights into enzyme–substrate interaction and characterization of enzymatic intermediates of organic hydroperoxide resistance protein from *Xylella fastidiosa*. *J Mol Biol* **359**, 433–445.

31 Cascio P, Hilton C, Kisselev AF, Rock KL & Goldberg AL (2001) 26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide. *EMBO J* **20**, 2357–2366.
32 Kisselev AF, Callard A & Goldberg AL (2006) Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J Biol Chem* **281**, 8582–8590.
33 Ruiz de Mena I, Mahillo E, Arribas J & Castaño PG (1993) Kinetic mechanism of activation by cardiolipin (diphosphatidylglycerol) of the rat liver multicatalytic proteinase. *Biochem J* **296**, 93–98.
34 Porras P, Padilla CA, Krayl M, Voos W & Bárcena JA (2006) One single in-frame AUG codon is responsible for a diversity of subcellular localizations of glutaredoxin 2 in *Saccharomyces cerevisiae*. *J Biol Chem* **281**, 16551–16562.
35 Netto LE, Oliveira MA, Monteiro G, Demasi AP, Cussiol JR, Discola KF, Demasi M, Silva GM, Alves SV, Faria VG *et al.* (2006) Reactive cysteine in proteins: protein folding, antioxidant defense, redox signaling and more. *Comp Biochem Physiol C Toxicol Pharmacol* **146**, 180–193.
36 Forster A & Hill CP (2003) Proteasome degradation: enter the substrate. *Trends Cell Biol* **13**, 550–553.
37 Orlowski M & Wilk S (2003) Ubiquitin-independent proteolytic functions of the proteasome. *Arch Biochem Biophys* **415**, 1–5.
38 Discola KF, Oliveira MA, Silva GM, Barcena JA, Porras P, Padilla A, Netto LE & Guimarães BG (2005) Crystallization and preliminary X-ray crystallographic studies of glutaredoxin 2 from *Saccharomyces cerevisiae* in different oxidation states. *Acta Crystallogr F Struct Biol Cryst Commun* **61**, 445–447.
39 Copley SD, Novak WR & Babbitt PC (2004) Divergence of function in the thioredoxin fold suprafamily: evidence for evolution of peroxiredoxins from a thioredoxin-like ancestor. *Biochemistry* **43**, 13981–13995.
40 Verma R, Chen S, Feldman R, Schieltz D, Yates J, Dohmen J & Deshaies RJ (2000) Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. *Mol Biol Cell* **11**, 3425–3439.
41 Gaczynska M & Osmulski PA (2005) Characterization of noncompetitive regulators of proteasome activity. *Methods Enzymol* **398**, 425–438.
42 Oliveira MA, Discola KF, Alves SV, Barbosa JA, Medrano FJ, Netto LE & Guimarães BG (2005) Crystallization and preliminary X-ray diffraction analysis of NADPH-dependent thioredoxin reductase I from *Saccharomyces cerevisiae*. *Acta Crystallogr F Struct Biol Cryst Commun* **61**, 387–390.
43 Jeong JS, Kwon SJ, Kang SW, Rhee SG & Kim K (1999) Purification and characterization of a second type thioredoxin peroxidase (type II TPx) from *Saccharomyces cerevisiae*. *Biochemistry* **38**, 776–783.
44 Bollag DM, Rozycki MD & Edelstein ST (1996) *Protein Methods*, 2nd edn. Wiley-Liss Publishers, New York.

Supplementary material

The following supplementary material is available online:

Fig. S1. SDS/PAGE profile of 20S PT preparations. 12.5% SDS–PAGE of 20S PT stained with Coomassie brilliant blue (lane 2). Lane 1 contains molecular mass markers.

Fig. S2. *In vitro* degradation of standard proteins by the 20S PT. Five micrograms of each protein, except casein (10 μg), were incubated for 2 h at 37 °C in standard buffer with 2.5 μg n-PT.

Fig. S3. MALDI-TOF analyses of Grx2 after incubation with natively S-glutathionylated 20S PT.

Fig. S4. Comparative 20S PT deglutathionylation by Grx2C30S and wild-type Grx2.

Doc. S1. MALDI-TOF analyses of Grx2.

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SUPPLEMENTAL DATA

FIGURE S1

12.5% SDS-PAGE pattern of 20S PT stained with Coomassie Brilliant Blue is shown (lane 2) and a molecular weight marker (lane 1).

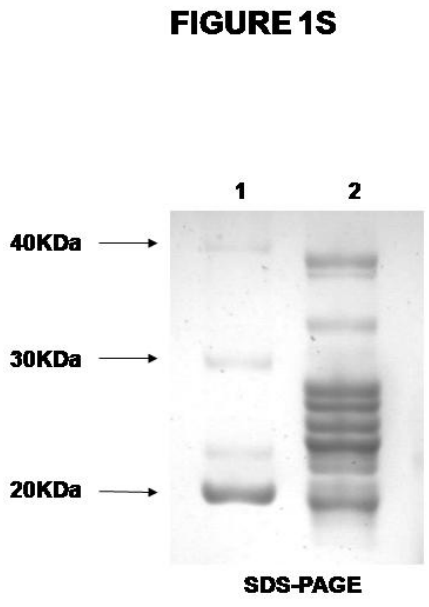


FIGURE S2

Five micrograms of each protein, except casein (10 μg), were incubated for 2 h at 37°C in standard buffer with 2.5 μg n-PT (+, even samples). The odd bands (-) correspond to standard protein samples incubated at the same conditions without n-PT. After incubation, n-PT was removed by filtration prior to application to a 15% SDS-PAGE gel. Protein samples were Grx2, Cytochrome C (*CytC*), Organic hydroperoxide resistance protein (*Ohr*), Ovalbumin (*Ova*) and Casein (*Cas*).

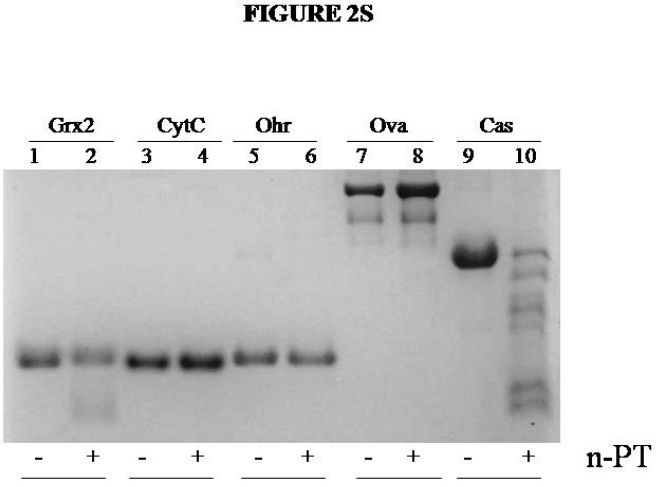


FIGURE S3

MALDI-TOF analyses of Grx2 after incubation with natively S-glutathionylated 20S PT.

All incubations were performed in standard buffer at 37°C. Molar ratio (n-PT:Grx2) was 1:20. After incubation, proteasome was removed by filtration, and Grx2 samples were utilized for MALDI-TOF analyses as described in Materials and Methods. **(A)** purified Grx2 incubated for 2h in the absence of proteasome and in the presence of n-PT **(B)** for 30 minutes or **(C)** 2 hours incubation. The fragments generated during incubation (ordered by letters) are shown in Table 1.

FIGURE 3S

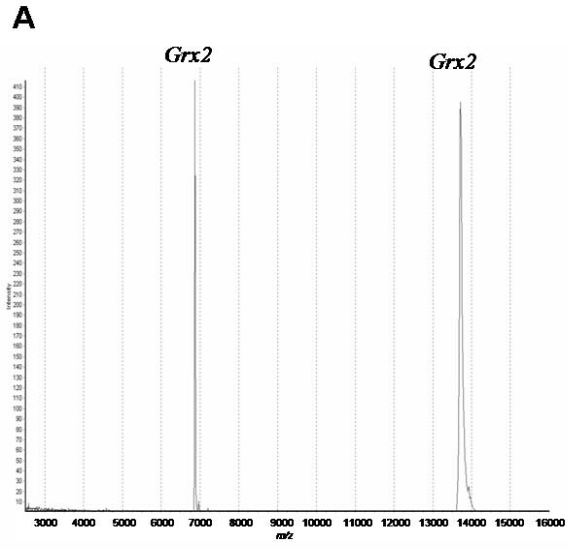
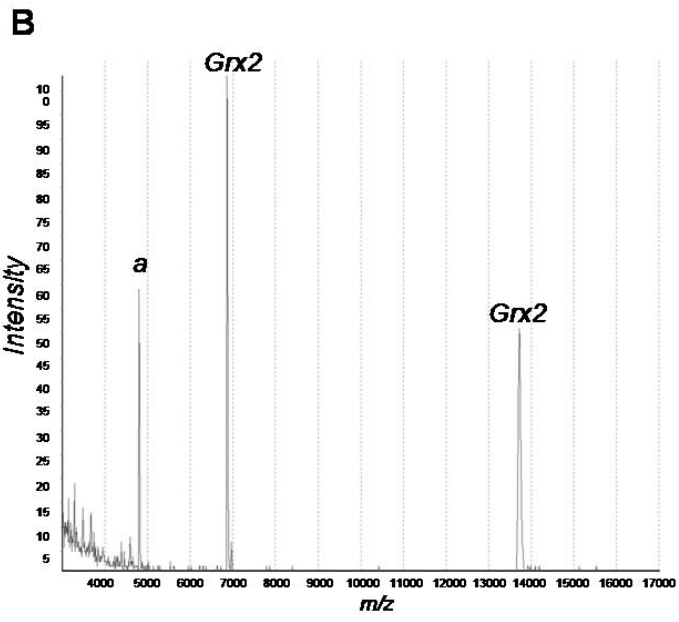


FIGURE 3S


B
Grx2
a
Grx2
Intensity
m/z
10
0
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90
85
80
75
70
65
60
55
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45
40
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20
15
10
5
4000
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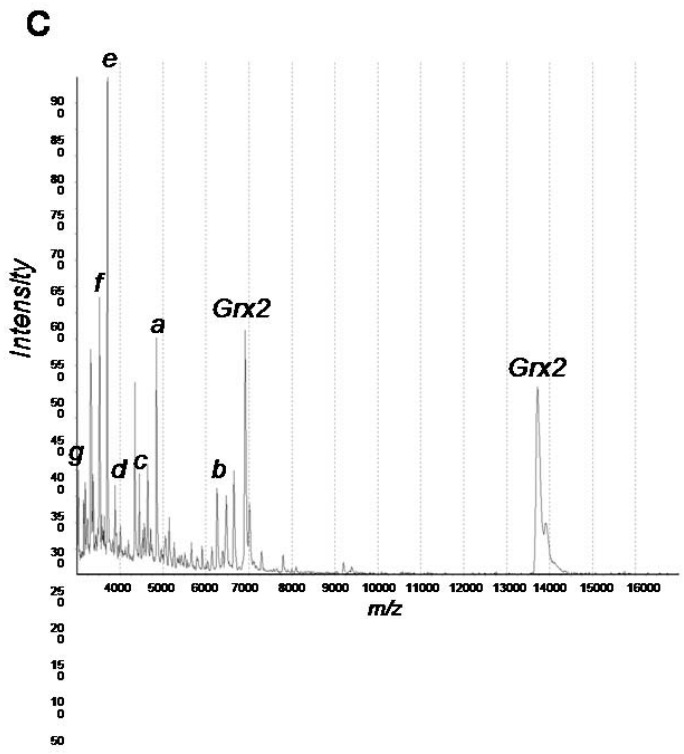
13000
14000
15000
16000
17000

FIGURE 3S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14000
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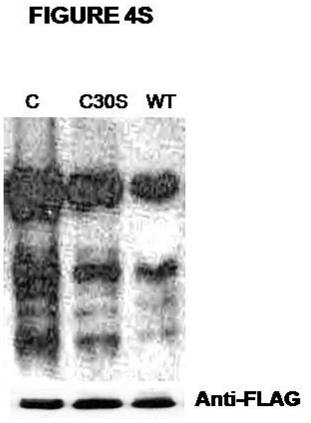
16000

FIGURE S4

Comparative 20S PT deglutathionylation by Grx2C30S and wild type Grx2. 20 μg n-PT was incubated (final volume of 40 μL standard buffer containing 2 mM NADPH, 0.3 U/ml GR and 0.5 mM GSH) in the presence of 10 μg Grx2C30S (*C30S*) or 10 μg wild type Grx2 (*WT*) for 30 minutes. *C*, n-PT samples incubated under the same conditions in the absence of the oxido-reductases. *Anti-FLAG*, loading control performed as described in Materials and Methods on the same membranes utilized for the anti-GSH blotting.



Doc. S1. MALDI-TOF analyses of Grx2

Prior to incubation with the 20S PT, Grx2 samples were filtered through YM-30 Microcon filters, and the protein recovered on the membrane was diluted into standard buffer. After incubation in the presence of the 20S core, the samples were filtered through 100 kDa cutoff microfilters to separate the proteasome from intact and fragmented Grx2. Total Grx2 obtained in the filtrate was used for MS determination. Samples were desalted through reverse-phase C_{18} Zip-Tips (Millipore), according to the manufacturer's protocol. MS analyses were performed on an Ettan MALDI/ToF-Pro instrument (Amersham Biosciences) under linear mode using sinapinic acid as the matrix. Briefly, 1 µl Zip-Tip processed samples were mixed with 1 µL saturated matrix solution (50% ACN containing 0.1% TFA) and 0.4 µl were deposited onto the sample slide and allowed to dry. Data acquisition and processing were performed using Ettan MALDI software. External calibration was performed with myoglobin

Cloning and expression of Grx2-C30S

Site-directed mutagenesis was employed to mutate the Grx2 cysteine 30 to a serine residue. The *GRX2* gene was PCR-amplified from *S. cerevisiae* genomic DNA (strain W303). Forward and reverse primers (mutated sequence is underlined), respectively, were 5'cgcgatccatatgatggtatcccaggaaacagttgctcacgtaaaggatctgattggccaaaaggaagtgtttgttgcagcaaagacatac tgcccttacagcaaagctactttg3', containing the NdeI restriction site, and 5'cgcaagcttggatccctattgaaataccggcttc3', containing the BamHI restriction site. The gene sequence was confirmed with an automated DNA sequencer. The *E. coli* BL21 (DE3) strain was transformed with the resulting pET15b/*grx2*-C30S construct. Expression was induced with 1 mM

isopropyl-beta-D-thiogalactopyranoside (IPTG) for 3 hours. The protein was purified by cobalt-affinity chromatography, and purity grade was confirmed by SDS-PAGE.

Anexo 3

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Crystallization and preliminary X-ray analysis of a decameric form of cytosolic thioredoxin peroxidase 1 (Tsa1), C47S mutant, from *Saccharomyces cerevisiae*

Marcos Antonio de Oliveira,[a]* Victor Genu,[a] Karen Fulan Discola,[a] Simone Vidigal Alves,[a] Luis Eduardo Soares Netto[a] and Beatriz Gomes Guimarães[b]*

[a]Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, 05508-900 São Paulo-SP, Brazil, and [b]Centro de Biologia Molecular Estrutural, Laboratório Nacional de Luz Síncrotron, 13084-971 Campinas-SP, Brazil

Correspondence e-mail: scaff@lnls.br, beatriz@lnls.br

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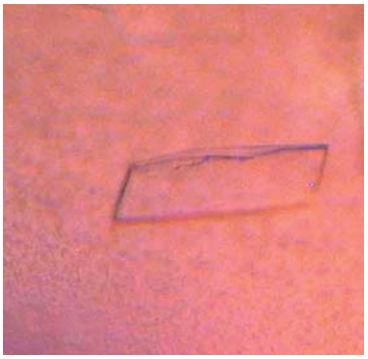
Saccharomyces cerevisiae cytosolic thioredoxin peroxidase 1 (cTPxI or Tsa1) is a bifunctional enzyme with protective roles in cellular defence against oxidative and thermal stress that exhibits both peroxidase and chaperone activities. Protein overoxidation and/or high temperatures induce great changes in its quaternary structure and lead to its assembly into large complexes that possess chaperone activity. A recombinant mutant of Tsa1 from *S. cerevisiae*, with Cys47 substituted by serine, was overexpressed in *Escherichia coli* as a His_6-tagged fusion protein and purified by nickel-affinity chromatography. Crystals were obtained from protein previously treated with 1,4-dithiothreitol by the hanging-drop vapour-diffusion method using PEG 3000 as precipitant and sodium fluoride as an additive. Diffraction data were collected to 2.8 Å resolution using a synchrotron-radiation source. The crystal structure was solved by molecular-replacement methods and structure refinement is currently in progress.

1. Introduction

Thioredoxin peroxidases (TPx) belong to a family of enzymes called peroxiredoxins that are able to reduce H_2O_2, peroxynitrites and a wide range of organic peroxides using a very reactive cysteinyl residue present in their active sites (Chae, Robison *et al.*, 1994; Chae, Chung *et al.*, 1994; Netto *et al.*, 1996; Park *et al.*, 2000; Nordberg & Arner, 2001; Bryk *et al.*, 2002). In this process, the catalytic cysteine residue of TPx become oxidized. Thioredoxins and in a few cases glutaredoxins are directly involved in the reduction of TPx. While glutaredoxins are reduced by glutathione, thioredoxins are reduced by systems involving thioredoxin reductase (Trr) and nicotinamide adenine dinucleotide phosphate (NADPH; reviewed by Rouhier *et al.*, 2001; Rouhier & Jacquot, 2005). In addition to their protective roles in oxidative or nitrosative stress, TPxs may regulate peroxide-mediated signalling cascades. Furthermore, TPx overexpression is observed in several cancers and is correlated with cellular resistance to apoptosis induced by radiation or chemotherapy-induced apoptosis (reviewed in Kang *et al.*, 2005).

TPxs are ubiquitous proteins found from archaea to man (Park *et al.*, 2000; Bryk *et al.*, 2002; Herbette *et al.*, 2002; Jung *et al.*, 2002). In eukaryotes, TPxs can be located in the cytosol as well as in organelles such as chloroplasts, mitochondria and even in nuclei (Park *et al.*, 2000; reviewed in Jang *et al.*, 2006). In *Saccharomyces cerevisiae*, five isoforms have been described: three are cytosolic (Tsa1, Tsa2 and Ahp1), one is mitochondrial (Prx1) and another is nuclear (Dot5). The distinct or overlapping subcellular localizations create a complex network and may reflect particular physiological roles of each isoform (Park *et al.*, 2000). In fact, yeast cells which have the *Tsa1* gene deleted are specifically sensitive to oxidative stress when the mitochondrial function is inhibited (Demasi *et al.*, 2006), accumulate aggregated proteins (Rand & Grant, 2006) and are more prone to accumulate mutations (Huang *et al.*, 2003; Huang & Kolodner, 2005).

All TPxs use a highly conserved cysteine residue called the peroxidatic cysteine (Cys_P) to reduce hydroperoxides. After nucleophilic attack, the Cys_P is oxidized to a cysteine sulfenic acid



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(Cys_P-SOH). When TPx is exposed to high doses of peroxides, this sulfenic acid can be further oxidized to a sulfinic acid (Cys_P-SO_2H). Initially, this overoxidized state was believed to be a dead end in the catalytic cycle, since this form is inactive and cannot be reduced by thioredoxin (Chae *et al.*, 1999). However, Biteau *et al.* (2003) identified an enzyme that is able to reduce yeast sulfinic acid-overoxidized Tsa1 and Tsa2 in an ATP-dependent process.

Tsa1 and Tsa2 were characterized as homodimers of approximately 43 kDa molecular weight. Nonetheless, exposure of yeast cells to heat shock or oxidative stress triggers an intense oligomerization of Tsa1 and Tsa2 dimers, resulting in the formation of $[(\alpha_2)_5]$ and high-molecular-weight complexes (Fig. 1). This structural change is correlated with a transition of the protein function from thiol peroxidase to molecular chaperone, but the molecular mechanisms by which TPx quaternary structures assemble and dissociate are not well understood (Jang *et al.*, 2004).

Tsa1 and Tsa2 are both cytosolic enzymes that present a high primary structure homology (86% identity and 96% similarity) and exhibit peroxidatic and chaperone activities. Despite their structural and functional similarities, Ogusucu *et al.* (2007) showed that the pK_a of the peroxidatic cysteines differ considerably between the two thioredoxin peroxidases (5.4 for Tsa1 and 6.3 for Tsa2). The difference in the peroxidatic reactivity probably relies on slight differences in the two protein structures, which may have consequences for their intrinsic peroxidatic and chaperone activities. Accordingly, human TPx cytosolic isoforms Prx1 and Prx2 (also named TPxA and TPxB, respectively), which share a very high degree of amino-acid sequence homology as in their yeast counterparts (78% identity and 91% similarity), present differences in their intrinsic chaperone and peroxidase activities. Prx1 is more efficient as a molecular chaperone, while Prx2 is a better peroxidase. A cysteine residue in Prx1 (Cys83) that is positioned at the decamer interface and is not found in Prx2 plays a critical role in these differences, since the $Prx1^{C83S}$ mutant presents chaperone and peroxidase activities that are very similar to those of Prx2 (Lee *et al.*, 2007). Sequence alignment of yeast Tsa1 and Tsa2 with human Prx1 and Prx2 shows that the yeast proteins do not possess an equivalent cysteine residue but that the amino-acid residues composing the putative dimer–dimer interface differ only slightly between yeast and human TPx (Fig. 2).

Here, we report preliminary X-ray diffraction analysis of Tsa1 from *S. cerevisiae* carrying the Cys47Ser substitution. The structure was solved by molecular-replacement methods using the atomic coordinates of human Prx2 (TPxB) as the search model. The comparison of the structure of Tsa1 with those of its human counterparts may help in comprehending the oligomerization process of TPx proteins and consequently in understanding the molecular mechanisms underlying their peroxidase and chaperone activities.

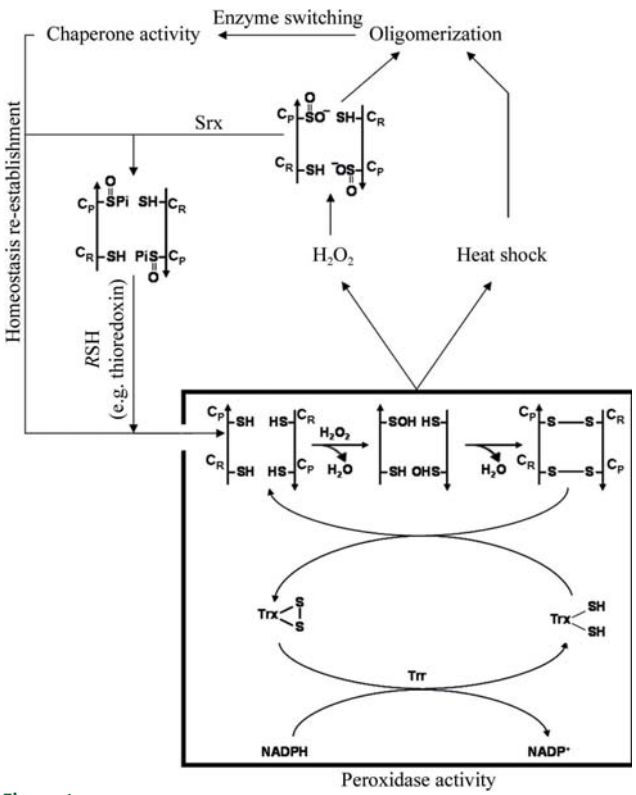

Figure 1
Molecular switching of thioredoxin peroxidases. At low H_2O_2 concentrations, Cys_P (Cys47 in yeast Tsa1) is oxidized to Cys_P-SOH, which reacts with the resolving cysteine (Cys_R; Cys170 in Tsa1) of the other subunit in the homodimer to form an intermolecular disulfide. Under physiological conditions, the enzyme is reduced by the thioredoxin system (represented by Trx, Trr and NADPH). The peroxidase inactivation is the result of heat shock or oxidative stress. In the case of oxidative stress the overoxidation occurs at Cys_P (Cys47 in yeast Tsa1), resulting in Cys_P-SO_2H. After re-establishment of homeostasis, peroxidase activity is restored by reduction of the Cys_P-SO_2H moiety in a reaction that requires ATP hydrolysis and is catalyzed by Srx, with reducing equivalents being provided by physiological thiols (*R*SH) such as Trx.

2. Methods

2.1. Tsa1 cloning and site-directed mutagenesis

The yeast *YML028C* gene encoding Tsa1 was amplified by PCR using forward primer 5′-Tsa1 (5′-CGA**GAATTC**ACAATGGTCGC-TCAA-3′) containing an *Eco*RI site (bold) and reverse primer 3′-Tsa1 (3′-GTTTATTCTGCGAAC**GGGCCC**AT-5′) containing an *Sma*I site (bold) from genomic DNA. The PCR product was cloned into the pGEM-T Easy vector (Promega), resulting in the pGEM/*tsa1* plasmid. *Escherichia coli* DH5α strain cells were transformed with pGEM/*tsa1* and white colonies were selected from LB/ampicillin/5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) medium. Plasmid extraction was performed using the Concert kit (Invitrogen). The plasmids were digested with *Eco*RI and *Sma*I, separated on a low-melting-point agarose gel, purified and introduced into pET15b expression vector. The resulting pET15b/*tsa1* was transferred to *E. coli* BL21 (DE3) cells. Mutation of the active cysteines ($Tsa1^{C47S}$ and $Tsa1^{C170S}$) was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene), plasmid pET15b/*tsa1* and the mutagenic primers TsaMut47 (5′-TTCACTTTCGTCTCTCCAACCGA-AATC-3′ and 5′-GATTTCGGTTGGACAGACGAAAGTGAA-3′) and TsaMut170 (5′-ACTGTCTTGCCACTGGACTCCA-3′ and 5′-TGGAGTCCAGTTAGATGGCAAGACAGT-3′). The plasmids pET15b/*tsa1*, pET15b/$tsa1^{C47S}$ and pET15b/$tsa1^{C170S}$ were sequenced using an Applied Biosystems ABI Prism 377 96 in order to confirm the constructions.

2.2. Expression and purification

E. coli BL21 (DE3) cells harbouring pET15b/$tsa1^{C47S}$ plasmid were grown overnight in 50 ml Luria–Bertani medium containing 50 µg ml^{-1} ampicillin (LB/amp) at 310 K, transferred to 1 l fresh LB/amp medium and cultured further until the OD_{600} reached 0.6–0.8. Expression was induced with 1 m*M* IPTG and the cells were harvested after 4 h incubation at 310 K. The cell pellet was resuspended in 20 m*M* sodium phosphate pH 7.4. Cells were lysed by sonication and the cell extract was kept on ice during streptomycin

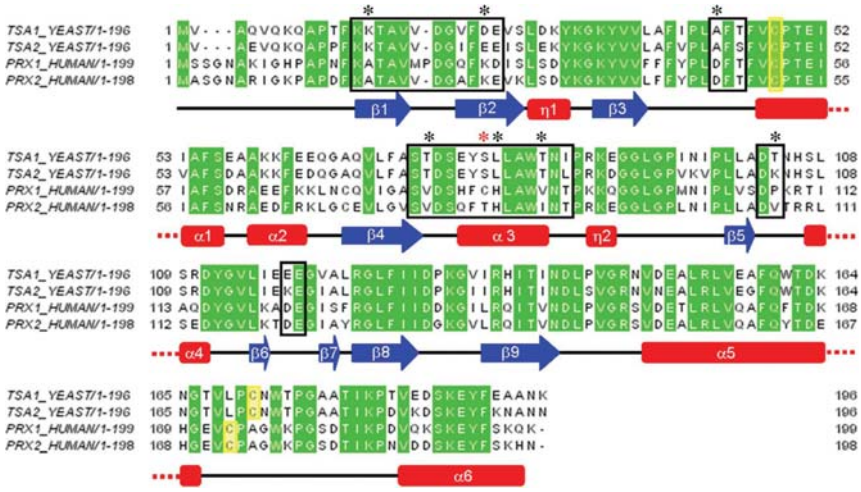

Figure 2
Alignment of the amino-acid sequences of yeast Tsa1 and Tsa2 and the human counterparts Prx1 and Prx2. The secondary-structure assignment corresponds to the human Prx2 structure. Sequence alignment was performed using *ClustalW* (Thompson *et al.*, 1994). Identical residues are highlighted in green; black boxes indicate residues involved in the dimer–dimer interface in the human Prx2 structure and their equivalents in Prx1 and yeast Tsa1 and Tsa2. Black asterisks show nonconserved residues in the dimer–dimer interface region and yellow boxes show the catalytic cysteines. The red asterisk denotes Cys83 of human Prx1.

sulfate (1%) treatment for 15 min. The suspension was centrifuged at 31 500*g* for 30 min at 277 K in order to remove nucleic acid precipitate. The resulting supernatant was filtered and applied onto a nickel affinity column (Hi-Trap, GE Healthcare). Bound protein was eluted with a linear imidazole gradient from 0 to 0.5 *M*. Protein purity was confirmed by SDS–PAGE. The purified protein was concentrated to 10 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5 for crystallization trials.

2.3. Crystallization and data collection

Protein samples were treated with H_2O_2, diamide or 1,4-dithiothreitol (DTT) (10 m*M*) at 310 K for 1 h and used directly in hanging-drop vapour-diffusion crystallization experiments. Initial screenings were performed at 293 K using Crystal Screen and Crystal Screen II from Hampton Research, and Wizard I and II from Jena Biosciences. Drops containing equal volumes (1.0 µl) of protein solution (10 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5) and reservoir solution were equilibrated against 0.3 ml reservoir solution. Several conditions from the initial screenings produced thin plate-shaped crystals. Promising crystals were identified in five conditions: conditions 16, 11 and 28 from Crystal Screen and conditions 16 and 31 from Crystal Screen II. All initial hits consisted of low-pH buffers with PEG as precipitant. Crystals suitable for diffraction experiments were obtained by slight variation of these conditions, such as the temperature of the assays (293 or 277 K). An additive search was also performed using Additive Screens 1, 2 and 3 from Hampton Research.

The best crystals were cryoprotected using reservoir solution supplemented with 25%(*v*/*v*) PEG 400 and cooled to 110 K in a nitrogen-gas stream. X-ray diffraction data were collected using synchrotron radiation at the D03B-MX1 protein crystallography beamline of Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil. D03B-MX1 is a monochromatic beamline with maximum photon flux between 1.3 and 1.6 Å. The wavelength of the incident X-rays was set to 1.431 Å and a MAR CCD detector was used to record the oscillation data with $\Delta\varphi = 1.0^\circ$. The data set was processed using the program *MOSFLM* (Leslie, 1992) and the resulting intensities were scaled and merged using the program *SCALA* (Evans, 1993) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Extensive attempts to determine the crystal structure of wild-type *S. cerevisiae* Tsa1 failed owing to the poor quality of the diffraction (maximum resolution ∼6–7 Å). We believe that partial oxidation of cysteines and/or nonspecific disulfide bridges may result in non-homogeneous samples that yield poorly diffracting crystals. In order to overcome this problem, two approaches were adopted. Firstly, enzyme samples were treated with H_2O_2, diamide or DTT prior to crystallization. However, crystals obtained after this protein treatment still presented poor diffraction quality. Subsequently, two Tsa1 mutants carrying cysteine-to-serine substitutions (Tsa1^{C47S} and Tsa1^{C170S}) were constructed. Microcrystals of both mutants were

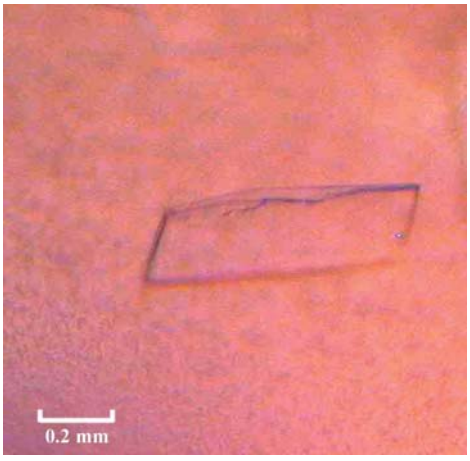

Figure 3
Crystal of yeast Tsa1^{C47S} obtained by hanging-drop vapour diffusion in the presence of sodium citrate pH 4.2, 10 m*M* sodium chloride and 10%(*w*/*v*) PEG 3000 with 100 m*M* sodium fluoride as an additive.

Table 1
Crystal parameters and crystallographic data statistics.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 239.98, b = 51.96, c = 192.35, β = 92.3
Resolution range (Å)	42.60–2.8 (3.2–2.8)
Total reflections	230659
Unique reflections	58102
Completeness (%)	97.7 (97.7)
Multiplicity	4.0 (3.8)
$R_{\rm sym}$† (%)	10.5 (42.4)
Average $I/\sigma(I)$	13.7 (3.2)

† $R_{\rm sym} = \sum_{\mathbf{h}} \sum_{l} |I_{\mathbf{h},l} - \langle I_{\mathbf{h}} \rangle| / \sum_{\mathbf{h}} \sum_{l} \langle I_{\mathbf{h}} \rangle$, where $I_{\mathbf{h}l}$ is the lth observation of reflection **h** and $\langle I_{\mathbf{h}} \rangle$ is the weighted average intensity for all observations l of reflection **h**.

obtained, but only Tsa1^{C47S} produced crystals suitable for X-ray diffraction experiments.

The best Tsa1^{C47S} crystals were obtained from a DTT-treated sample with a drop volume of 8.0 µl. 3.6 µl reservoir solution [sodium citrate pH 4.2, 10%(*w*/*v*) PEG 3000] was mixed with an equal volume of protein solution and 0.8 µl 0.1 *M* sodium fluoride was used as an additive. The best crystals of Tsa1^{C47S} reached dimensions of 0.5 × 0.3 × 0.05 mm after 72 h (Fig. 3) and diffracted to 2.8 Å resolution. Crystals belong to the monoclinic space group *C*2, with unit-cell parameters a = 239.98, b = 51.96, c = 192.35 Å, β = 92.3°. Table 1 summarizes the data-collection statistics.

The atomic coordinates of one monomer of the *Homo sapiens* Prx2 decamer (Schröder *et al.*, 2000) were used as the search model in molecular-replacement protocols (66% sequence identity; PDB code 1qmv). The orientations and positions of the molecules in the asymmetric unit were found using *MOLREP* (Vagin & Teplyakov, 1997). The molecular-replacement solution showed ten monomers in the asymmetric unit and preliminary analysis of the *S. cerevisiae* Tsa1^{C47S} structure revealed the quaternary organization to be similar to those observed in other previously reported crystal structures of decameric TPxs: a toroid-shaped pentamer of homodimers. Model completion and refinement are currently in progress. However, visual inspection of the initial Fourier difference maps shows that the C-terminal region of yeast Tsa1 (amino acids 167–196), which contains the resolving cysteine (Cys170), differs significantly from the human coordinates used in the molecular-replacement protocols, probably because the Cys_P of the human model is overoxidized to Cys_P-SO_2H.

4. Conclusions

Here, we report the production, crystallization and preliminary X-ray analysis of the decameric form of yeast Tsa1^{C47S} at 2.8 Å resolution. We expect that knowledge of the tertiary and quaternary structures of *S. cerevisiae* Tsa1 will contribute to the understanding of the molecular mechanism of peroxide decomposition and the process involved in the switch from peroxidase to chaperone activity, which may be involved in fundamental processes of cellular homeostasis. Additionally, the refined Tsa1 structure may be helpful in the construction of a good-quality theoretical model of Tsa2. It may shed light on the differences between the pK_a of the peroxidatic cysteines from the two TPs cytosolic isoforms and contribute to the understanding of the molecular basis of the functional switch among thioredoxin peroxidases from different organisms.

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References

Biteau, B., Labarre, J. & Toledano, M. B. (2003). *Nature (London)*, **425**, 980–984.
Bryk, R., Lima, C. D., Erdjument-Bromage, H., Tempst, P. & Nathan, C. (2002). *Science*, **295**, 1073–1077.
Chae, H. Z., Chung, S. J. & Rhee, S. G. (1994). *J. Biol. Chem.* **269**, 27670–27678.
Chae, H. Z., Kim, H. J., Kang, S. W. & Rhee, S. G. (1999). *Diabetes Res. Clin. Pract.* **45**, 101–112.
Chae, H. Z., Robison, K., Poole, L. B., Church, G., Storz, G. & Rhee, S. G. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 7017–7021.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Demasi, A. P. D., Pereira, G. A. G. & Netto, L. E. S (2006). *FEBS J.* **273**, 805–816.
Evans, P. R. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 114–122. Warrington: Daresbury Laboratory.
Herbette, S., Lenne, C., Leblanc, N., Julien, J. L., Drevet, J. R. & Roeckel-Drevet, P. (2002). *Eur. J. Biochem.* **269**, 2414–2420.
Huang, M. E., Rio, A. G., Nicolas, A. & Kolodner, R. D. (2003). *Proc. Natl Acad. Sci. USA*, **100**, 11529–11534.
Huang, M. E. & Kolodner, R. D. (2005). *Mol. Cell*, **17**, 709–720.
Jang, H. H. *et al.* (2004). *Cell*, **117**, 625–635.
Jang, H. H., Chi, Y. H., Park, S. K., Lee, S. S., Lee, J. R., Park, J. H., Moon, J. C., Lee, Y. M., Kim, S. Y., Lee, K. O. & Lee, S. Y. (2006). *Physiol. Plant.* **126**, 549–559.
Jung, B. G., Lee, K. O., Lee, S. S., Chi, Y. H., Jang, H. H., Kang, S. S., Lee, K., Lim, D., Yoon, S. C., Yun, D. J., Inoue, Y., Cho, M. J. & Lee, S. Y. (2002). *J. Biol. Chem.* **277**, 12572–12578.
Kang, S. W., Rhee, S. G., Chang, T. S., Jeong, W. & Choi, M. H. (2005). *Trends Mol. Med.* **11**, 571–578.
Lee, W., Choi, K. S., Riddell, J., Ip, C., Ghosh, D., Park, J. H. & Park, Y. M. (2007). In the press.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.
Netto, L. E. S., Chae, H. Z., Kang, S. W., Rhee, S. G. & Stadtman, E. R. (1996). *J. Biol. Chem.* **271**, 15315–15321.
Nordberg, J. & Arner, E. S. (2001). *Free Radic. Biol. Med.* **31**, 1287–1312.
Ogusucu, R., Rettori, D., Munhoz, D. C., Netto, L. E. & Augusto, O. (2007). *Free Radic. Biol. Med.* **42**, 326–334.
Park, S. G., Cha, M. K., Jeong, W. & Kim, I. H. (2000). *J. Biol. Chem.* **275**, 5723–5732.
Rand, J. D. & Grant, C. M. (2006). *Mol. Biol. Cell*, **17**, 387–401.
Rouhier, N., Gelhaye, E., Sautiere, P. E., Brun, A., Laurent, P., Tagu, D., Gerard, J., de Faÿ, E., Meyer, Y. & Jacquot, J. P. (2001). *Plant Physiol.* **127**, 1299–1309.
Rouhier, N. & Jacquot, J. P. (2005). *Free Radic. Biol. Med.* **38**, 1413–1421.
Schröder, E., Littlechild, J. A., Lebedev, A. A., Errington, N., Vagin, A. A. & Isupov, M. N. (2000). *Structure*, **8**, 605–615.
Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). *Comput. Appl. Biosci.* **10**, 19–29.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.

Anexo 4

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Review

Reactive cysteine in proteins: Protein folding, antioxidant defense, redox signaling and more☆

Luis Eduardo Soares Netto [a,*], Marcos Antonio de Oliveira [a], Gisele Monteiro [a], Ana Paula Dias Demasi [a], José Renato Rosa Cussiol [a], Karen Fulan Discola [a], Marilene Demasi [b], Gustavo Monteiro Silva [a], Simone Vidigal Alves [a], Victor Genu Faria [a], Bruno Brasil Horta [a]

[a] *Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo—SP, Brazil*
[b] *Laboratório de Bioquímica e Biofísica, Instituto Butantan, São Paulo-SP, Brazil*

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Abstract

Cysteine plays structural roles in proteins and can also participate in electron transfer reactions, when some structural folds provide appropriated environments for stabilization of its sulfhydryl group in the anionic form, called thiolate (RS^-). In contrast, sulfhydryl group of free cysteine has a relatively high pK_a (8,5) and as a consequence is relatively inert for redox reaction in physiological conditions. Thiolate is considerable more powerful as nucleophilic agent than its protonated form, therefore, reactive cysteine are present mainly in its anionic form in proteins. In this review, we describe several processes in which reactive cysteine in proteins take part, showing a high degree of redox chemistry versatility.

Keywords: Antioxidant; Cysteine; Disulfide; Peroxide; Electron transfer; Signaling; Thiols; Thiolate

Contents

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* Corresponding author. Tel.: +55 11 30917589; fax: +55 11 30917553.
E-mail address: nettoles@ib.usp.br (L.E.S. Netto).

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1. Introduction

Proteins are the final products of gene expression and are responsible to execute the biological information contained in the nucleotide sequences of their respective genes. A classical dogma in biology is that the genetic information presented in the nucleotide sequence of DNA is expressed in a two-stage

process: transcription and translation. Proteins need to adopt proper tertiary and quaternary structures to perform their biological functions. Because most proteins spontaneously fold into their native conformation under physiological conditions, the central dogma also implies that protein's primary structure dictates its tertiary structure.

Our interest is on proteins that have the ability to participate in electron transfer reactions. Most proteins rely on organic and on inorganic redox cofactors (NAD^+, FAD, heme, Cu, Fe and other transition metals) for redox activity. In contrast, for other proteins, amino acids, mainly cysteines, are responsible for this property. Free cysteine possesses low reactivity to undergo redox transitions (Wood et al., 2003b). However, protein folding can generate environments in which cysteine residues are reactive. The reactivity of a sulfhydryl group is related to its pK_a, since its deprotonated form (thiolate = RS^-) is more nucleophilic and reacts faster with oxidants than the protonated form (R-SH). The sulfhydryl groups of most cysteines (linked to a polypeptide backbone or free cysteine) possess low reactivity, which is related to the fact that their pK_a is around 8,5 (Benesch and Benesch, 1955). In contrast, some redox proteins possess a reactive cysteine that is stabilized in the thiolate form by a basic residue, in most cases a lysine or an arginine residue (Copley et al., 2004). In conclusion, reactive cysteines in proteins are kept in a reactive form (thiolate = RS^-) by structural interactions with other amino acids. These reactive cysteines residues are compounds with very versatile redox chemistry because its sulfur atom can undergo redox transitions into any oxidation state between +6 and −2 (Jacob et al., 2003). Several proteins took advantage of this versatility to perform various biological functions as will be discussed below.

2. Roles of reactive cysteine in biology

2.1. Reduction of disulfide bonds

Many proteins with reactive cysteines are involved in controlling thiol/disulfide exchange reactions (Fig. 1A), a central theme in biology. Thiol/disulfide-exchange reactions are nucleophilic substitutions. A thiol or thiolate (RSH or RS^-) acts as a nucleophilic agent on a disulfide bond (RS–SR). These reactions are, for example, used to form and reversibly destroy structural disulfides in proteins and peptides, to regulate enzyme activity and to maintain cellular redox balance. The rate of this reaction is dependent on the pK_a of the sulfhydryl compound that is the nucleophilic agent. The lower the pK_a, higher is the amount of deprotonated form of sulfhydryl group (thiolate) and faster are the reactions at physiological pH. The tri-peptide glutathione (γ-Glu–Cys–Gly, GSH) is the most abundant thiol in cells and is vital for the maintanance of the intracellular redox balance, among other functions (reviewed by Jacob et al., 2003). Glutathione is almost completely protonated at physiological pH because its pK_a is 9,2 (Jung et al., 1972) which disfavor its reaction with disulfides. However, it should be also considered that glutathione levels in cells are very high, which should favor disulfide reduction, since rate of a reaction depend also on the concentration of substrates.

Besides glutathione, thiol proteins such as thioredoxin, glutaredoxin (also known as thioltransferase) and protein disulfide isomerase are also involved in the regulation of the intracellular redox balance and, therefore, they are also known as thiol/disulfide oxido-reductases. Thioredoxin appears to be a very ancient protein since it is widespread among all the living organisms. These small proteins (12–13 kDa) possess disulfide reductase activity endowed by two vicinal cysteines present in a CXXC motif (typically CGPC), which are used to reduce target proteins that are recognized by other domains of thioredoxin polypeptide. The reduction of target proteins results in a disulfide bridge between the two cysteines from the thioredoxin CXXC motif, which is then reduced by thioredoxin reductase that utilizes reducing equivalents from NADPH. Some of the target proteins of thioredoxin include ribonucleotide reductase (important for DNA synthesis), methionine sulfoxide reductase, peroxiredoxins and transcription factors such as p53 and NF-kB (reviewed by Powis and Montfort, 2001).

Since thioredoxin plays multiple roles, it was surprising to observe that deletion of their genes in *Escherichia coli* resulted in a viable bacteria, capable to synthesize desoxyribonucleotides, among other processes. Holmgren (1976) showed that glutaredoxin was the backup for thioredoxin in the reduction of ribonucleotides. Like thioredoxin, glutaredoxin possess a CXXC motif in their active site (typically CPYC) and most of them are low molecular weight proteins (12–13 kDa). Glutaredoxin can also catalyze the reduction of disulfide bond in target proteins like thioredoxin through thiol/disulfide exchange reactions (Fig. 1A).

Furthermore, glutaredoxin also catalyzes the reduction of mixed disulfides with glutathione in a process that only the N-terminal cysteine thiolate participates (reviewed by Fernandes and Holmgren, 2004). Interestingly, some glutaredoxin isoforms possess only the N-terminal cysteine and are only capable to reduce mixed disulfides with glutathione. Thioredoxins reduce a wider range of disulfides in proteins than glutaredoxins, but cannot reduce mixed disulfides with glutathione. The disulfide form of glutaredoxin is reduced by glutathione, which is then reduced by NADPH in a reaction catalyzed by glutathione reductase.

The thioredoxin system (NAPDH + thioredoxin reductase + thioredoxin) and the glutathione system (NADPH + glutathione reductase + glutathione) are the major thiol dependent redox pathways present in the cells. Glutathione systems may or may not contain glutaredoxin, depending on the process considered. Several enzymes and other effectors can be reduced by both systems but many processes are reduced by either thioredoxin or by glutathione system. Interestingly, in platyhelminths, the thioredoxin and glutathione systems are linked in only one pathway. This worm possesses a seleno-cysteine containing enzyme named thioredoxin-glutathione reductase, which possess thioredoxin reductase, glutathione reductase and glutaredoxin activities (Sun et al., 2001).

The two electron redox potential of the cysteine/cystine couple in thiol/disulfide oxido-reductases is influenced by several factors. Thioredoxin and glutaredoxin are strong reducing agents and therefore possess a very negative redox

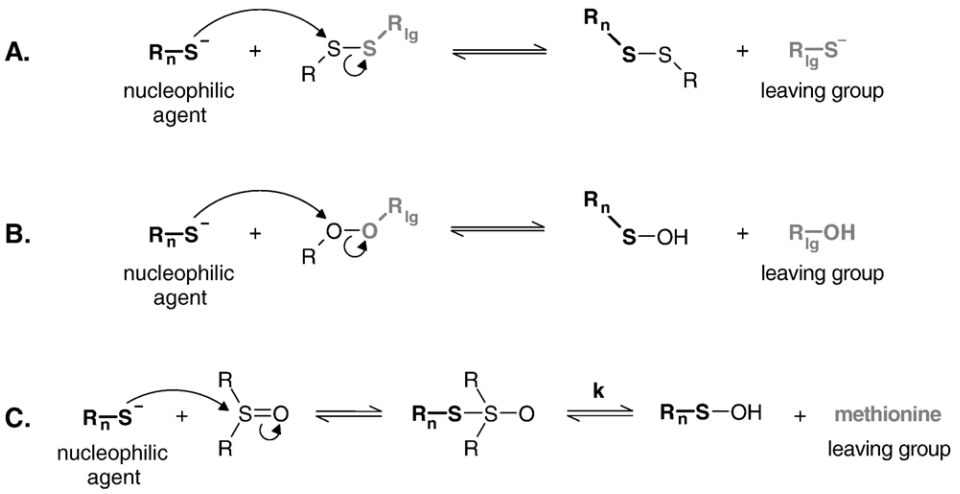

Fig. 1. Nucleophilic substitutions and reactive cysteines. (A) Thiol-disulfide exchange reaction. This reaction is faster when the sulfhydryl group is deprotonated (thiolate). Therefore, rate of this reaction is given by $V = k[R_n - S^-]\,[RSSR_{lg}]$. The same rationale applies for the other reactions depicted here. (B) Peroxide reduction, resulting in a sulfenic acid derivative (Cys-SOH) and an alcohol corresponding to the peroxide. The sulfenic acid derivative can have different outcomes depending on the kind of peroxiredoxin considered (see Fig. 4) and on the environment where it is located. (C) Sulfoxide reduction, resulting in methionine regeneration and sulfenic acid formation in methionine sulfoxide reductase, which is reduced back to its sulfhydryl from by thioredoxin (reviewed by Weissbach et al., 2002). Abbreviations: lg = leaving group; n = nucleophilic agent.

potential. In contrast, protein disulfide isomerases (PDI) and DbsAs (bacterial PDI counterparts) possess much lower negative potentials (Table 1). Therefore reduction potential of the best oxidant (DsbA) is 146 mV higher than the best reductant (thioredoxin), corresponding to a ratio of 105 in thermodynamic stability of the dithiol/disulfide equilibrium (Xiao et al., 2005). This diversity in potentials reflects the biological roles of these thiol proteins. Thioredoxins and glutaredoxins preferentially reduce disulfide bridges, whereas protein disulfide isomerase and DbsAs preferentially generate disulfide bonds in proteins (Jacob et al., 2003).

It is interestingly to observe that in spite of these great differences in redox properties all these oxido-reductases share several similarities: (1) Sγ atom is mostly deprotonated in N-terminal cysteine residue of the CXXC motif at physiological conditions. Therefore, the most N-terminal cysteine is more nucleophilic and more exposed than the second cysteine. The most C-terminal cysteine is usually protonated and more buried in the polypeptide chain; (2) global fold of five stranded β-sheet flanked by four helices, the so-called thioredoxin fold (Fig. 2A); (3) the active site that contains the CXXC motif is located on a surface loop at the end of strand β2 and followed by a long α-helix (Fig. 2A).

Several reasons have been raised to explain this variation of the reducing capabilities, such as the composition of the amino acids in the CXXC motif (Table 1), network of charged amino acids and structural factors, such as the dipole property of the α-helix where the buried cysteine is located (reviewed by Carvalho et al., 2006). These different redox properties among thiol/disulfide oxido-reductases appear not to be related with the stability of the disulfide bonds, since their lengths are very similar among these proteins (reviewed by Carvalho et al., 2006). In any case, for all of these enzymes, the pK_a of the reactive cysteine is considerably lower than the pK_a of free cysteine (Table 1), but the mechanism by which the thiolate is stabilized varies.

The stabilization of the thiolate anion in thioredoxin is relatively well characterized and was taken as an example for thiol/disulfide oxido-reductases. It depends on: (i) a network of charged residues, especially on specific aspartate and lysine residues (Fig. 2B); (ii) dipole character of the α-helix where the C-terminal cysteine is located and (iii) hydrogen bonding between the reactive and the C-terminal cysteine residues (reviewed by Carvalho et al., 2006).

Interestingly, mutations of Asp26 and Lys57 of thioredoxin affect only the pK_a of the active site thiol, but not the structure of the protein (Dyson et al., 1997). For the other oxido-reductases the network of charged residues is different and involves Glu 30 for PDI (PDB ID = 1MEK), Glu 24 for DsbA (PDB ID = 1A23) and Glu30, Asg26 and Lys27 for glutaredoxin (PDB ID = 1KTE).

Table 1
Properties of Thiol/disulfide oxido-reductases

Oxido-reductase	Motif in active site	Redox Potential (E^o, mV)	pK_a
Thioredoxin	Cys–Gly–Pro–Cys	−270[a]	6.3–7.5[f]
Glutaredoxin	Cys–Pro–Tyr–Cys	−198 to −233[b]	3.5–3.8[g]
Tryparedoxin	Cys–Pro–Pro–Cys	−249[c]	7.2[h]
Protein Disulfide Isomerase (PDI)	Cys–Gly–His–Cys	−127[d]	3.5–6.7[i]
DbsA	Cys–Pro–His–Cys	−125[e]	3.5[j]

[a] — Miranda-Vizuete et al. (1997), Nishinaka et al. (2001). [b] — Aslund et al. (1997). [c] — Reckenfelderbaumer et al. (2002). [d] — Lundstrom and Holmgren (1993). [e] — Collet and Bardwell (2002). [f] — Holmgren (1972), Kallis and Holmgren (1980), Reutimann et al. (1981), Dyson et al. (1997), Li et al. (1993), Chivers et al. (1997), Dillet et al. (1998), Vohnik et al. (1998). [g] — Gan et al. (1990), Yang and Wells (1991), Mieyal et al. (1991), Jao et al. (2006). [h] — Reckenfelderbaumer and Krauth-Siegel (2002); [i] — Darby and Creighton (1995), Hawkins and Freedman (1991); [j] — Nelson and Creighton (1994), Grauschopf et al. (1995).

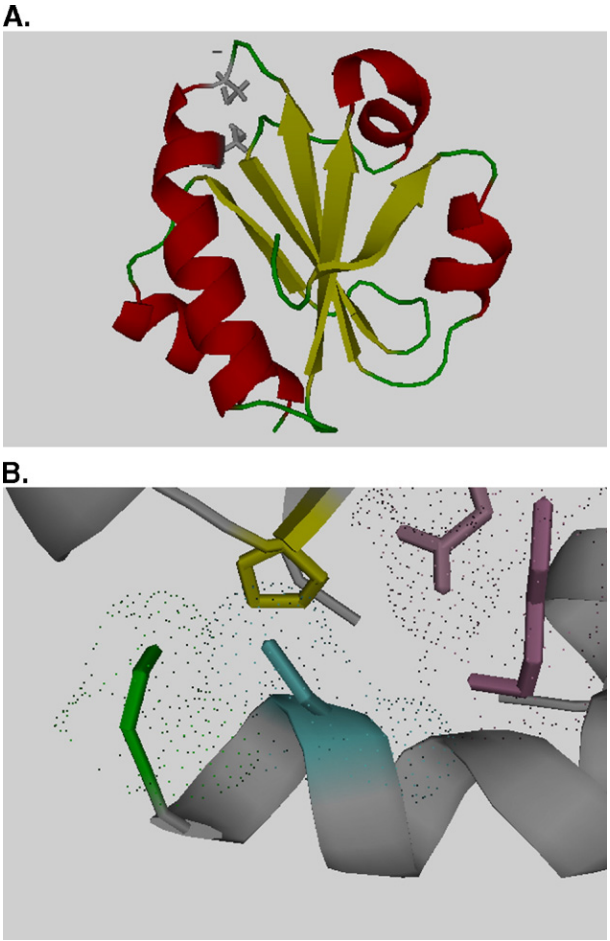

Fig. 2. Structural characteristics of thiol/disulfide oxido-reductases. Thioredoxin from *Escherichia coli* (PDB ID = 1XOB) was chosen as a model to describe several features common to thiol/disulfide oxido-reductases. (A) General view of thioredoxin fold: β-sheet composed of five strands (yellow) flanked by four α-helixes (red). Both main and side chains of the two cysteine residues belonging to the CXXC motif are showed (gray). The reactive cysteine (Cys 32) is the most exposed one. (B) View of the active site, showing the network of amino acids involved in the stabilization of reactive cysteine in the thiolate form. Residues involved in the network of charged amino acids are represented with colors and with dots representing their electronic densities (Asp 26 — magenta, Cys 32 — green, Cys 35 — cyan, Lys 57 — magenta). Pro76 (yellow) is not involved in the network of charged residues that stabilize the thiolate form of reactive cysteine, but its main and side chains are shown here because this residue plays a central role in the recognition of thioredoxin substrates. Figures were generated by the Pymol software (www.pymol.org). (For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of this article.)

We are interested in the functional and structural characterization of proteins belonging to thioredoxin and glutathione systems form the yeast *Saccharomyces cerevisiae*. In this regard, we solved the structure of thioredoxin reductase I (Oliveira et al., 2005) and preliminary data indicated that the two thioredoxin systems are not completely redundant.

2.2. Formation of disulfide bonds

The formation of disulfide bonds stabilizes the most active conformation of proteins that will be secreted. One would expect that PDI and DsbA would be more suitable to catalyze the formation of disulfide bond given their reduction potential (Table 1). In fact, the search for the enzymatic catalyst of oxidative folding led to isolation of PDI (Goldberger et al., 1963). PDI can catalyze the formation, reduction or isomerization of disulfide bonds depending on the redox conditions of the assay and on the nature of the substrate protein. However, the environment in which PDI is found (endoplasmic reticulum), favors only the formation and isomerization of disulfide bonds. Both, the formation of disulfide bond and isomerase activities occur by thiol/disulfide exchange reactions (Fig. 1A). Like thioredoxins and glutaredoxins, the ability of PDI to catalyze thiol/disulfide exchange reactions is given by a CXXC motif (typically CGHC) among other factors (Aslund et al., 1997). When the cysteines in the active site are present in the disulfide form, PDI can directly oxidize thiol groups of target proteins into disulfide bridges (dithiol oxidase activity). In contrast, the isomerase activity of PDI relies on the dithiol (reduced) configuration state of the active site cysteines, suitable for disulfide reshuffling (reviewed by Frand et al., 2000).

In spite of the different redox properties among all these oxido-reductases, they can catalyze both reduction and formation of disulfide bonds *in vitro*, depending on the experimental conditions. In fact, Grx1 from *E. coli* is even more efficient than PDI to catalyze disulfide bond formation (Xiao et al., 2005), indicating that kinetic parameters should also to be taken into account. Therefore, it is important to consider the environment in which the thiol oxido-reductase is located to analyze its function. In eukaryotic cells, protein disulfide bond formation takes place within the lumen of the endoplasmic reticulum. Proteins that will be secreted to the extracellular space are processed inside this organelle. The redox state of the endoplasmic reticulum is more oxidizing than that of cytosol, a difference that favors the formation of disulfide bonds, which is important to maintain the structure of the exported protein in the harsh extracellular environment. The major redox buffer in the cytosol as well as in the lumen of ER is the couple GSH/GSSG. However, GSH/GSSG ratios are quite different: 1:1 to 3:1 for the lumen of endoplasmic reticulum and 30:1 to 100:1 for the cytosol and mitochondrial matrix. Therefore, the ability of thioredoxin and glutaredoxin to catalyze reduction of disulfide bond in protein and of PDI to catalyze the reverse process is consequence of several factors such as redox potentials of vicinal sulfhydryl groups in these proteins and redox balance of the environment.

2.3. Protein S-glutathionylation

Another thiol/disulfide exchange process that deserves special consideration here is *S*-glutathionylanion of cysteine residues in proteins. In resting state, levels of *S*-glutathionylated proteins in cells are around 1%, but upon oxidative stress a significant increase is observed. Therefore, initially, the meaning of the *S*-glutathionylation was thought to be the protection of cysteine residues against overoxidation to sulfinic (RSO_2H) or sulfonic (RSO_3H) acids, which can lead to protein inactivation

(Thomas et al., 1995). Later, it was shown that for some enzymes, protein *S*-glutathionylation affects enzyme activities, suggesting a regulatory role for this process (Chrestensen et al., 2000; Davis et al., 1997; Demasi et al., 2003). If *S*-glutathionylation is in fact a regulatory event, it is expected the occurrence of proteins capable to catalyze the addition and removal of glutathione from target proteins. Glutaredoxins, especially those containing only one cysteine in their active site, have been most frequently implied as dethiolases (Molina et al., 2004). The yeast *S. cerevisiae* has five glutaredoxins, three monothiolic and two dithiolic, distributed in different compartments and performing similar, but not completely redundant roles (Wheeler and Grant, 2004). We have recently solved the crystal structure of glutaredoxin 2 (Discola et al., 2005) and unpublished results have demonstrated its role on the removal of GSH from *S*-glutathionylated 20S proteasome extracted from yeast cells. We hope that with the elucidation of glutaredoxin 2 structure it will be possible to obtain insights into the mechanisms by which this thiol/disulfide oxido-reductase act as a dethiolase in the yeast *S. cerevisiae*.

2.4. Antioxidant defense

Proteins with reactive cysteine considered so far, catalyze thiol/disulfide exchange reactions. In contrast, thiol-dependent peroxidases have evolved the ability to cleave a peroxide bond that is a more difficult process than the reduction of a disulfide bond (Fig. 1B). Copley et al. (2004) elegantly hypothesized that peroxiredoxins, a class of thiol-dependent peroxidases, present several amino acids substitutions from the more ancient thiol/disulfide oxido-reductases, which make them capable to reduce OO bonds through a reactive cysteine.

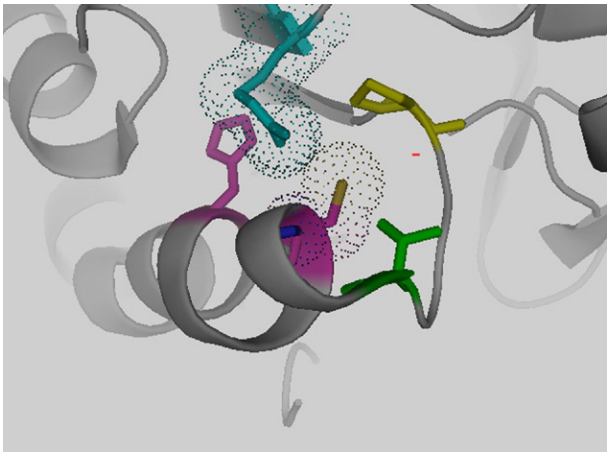

Fig. 3. Structure of peroxiredoxin active site. Reactive cysteine in peroxiredoxins corresponds to the C-terminal cysteine of CXXC motifs in thioredoxins (Copley et al., 2004). Therefore, they are located in an α-helix as the C-terminal cysteine of thioredoxin is. Structure of human peroxiredoxin 5 (PDB ID = 1HD2) is shown as an example. Electronic density of arginine residue (Arg127) involved in the stabilization of the thiolate is represented with dots, as well as reactive cysteine (Cys47). Thiolate function (RS^-) of Cys 47 is brown. Main and side chains of a threonine residue (Thr44 that corresponds to the N-terminal cysteine in thioredoxin) that plays a role in stabilization of thiolate is shown in green, as well as the chains of Pro 40 (yellow) that is involved in protection of peroxiredoxin from overoxidation. Finally, main and side chains of histidine 51 (purple), forming a salt bridge with Arg 127 (cyan) is also shown here. Figure was generated by the Pymol software (www.pymol.org). (For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of this article.)

Both hydrogen and organic hydroperoxides can be decomposed by peroxiredoxins and in most of cases they utilize reductive equivalents from thioredoxins (Netto et al., 1996). Therefore, the majority of peroxiredoxins are also called thioredoxin peroxidases. Recently, it was shown that some peroxiredoxins can also decompose peroxynitrite (Bryk et al., 2000; Dubuisson et al., 2004; Trujillo et al., 2004; Wong et al., 2002). These reactions catalyzed by peroxiredoxins have been implied in both peroxide detoxification and cellular signaling as will be discussed below. Like other thiol/disulfide oxido-reductases, peroxiredoxins are widespread in nature and are found in several cell compartments such as cytosol, mitochondria, nucleus and chloroplast (Rhee et al., 2005a).

As described for the thiol/disulfide oxido-reductases, the high reactivity of the active site cysteine in peroxiredoxins is related to the fact that the thiol group of this residue possesses very low pK_a. In the case of peroxiredoxins, the presence of a guanidine group from a fully conserved arginine residue (Wood et al., 2003b) is a key factor for the stabilization of the thiolate. Interestingly, the reactive cysteine from peroxiredoxins is homologous to the C-terminal cysteine of the CXXC motif in oxido-reductases, which is not the most nucleophilic. The reactive cysteine (the most N-terminal and most solvent exposed seen in Fig. 2) in oxido-reductases was replaced by a threonine residue in peroxiredoxins and the other cysteine acquired high nucleophilicity due to several structural features and amino acids interactions, such as the hydrogen bonding with an arginine residue mentioned above (Fig. 3).

Other peroxide-removing enzymes evolved other strategies to decompose peroxides. Catalase and mammalian glutathione peroxidase utilize heme or seleno-cysteine to decompose peroxides, whereas peroxiredoxins have a very reactive cysteine in their active site. Initially, these differences in the active sites was thought to reflect the fact that peroxiredoxins would have moderate catalytic efficiency ($\sim 10^5\ M^{-1}\ s^{-1}$), (Hofmann et al., 2002) when compared with catalases ($\sim 10^6\ M^{-1}\ s^{-1}$) (Hillar et al., 2000) and glutathione peroxidases ($\sim 10^8\ M^{-1}\ s^{-1}$) (Hofmann et al., 2002). Recently, however, some reports have described higher rate constants (10^6–$10^7\ M^{-1}\ s^{-1}$) for the reaction of reduced peroxiredoxins with different kinds of peroxides (Akerman and Muller, 2005; Baker and Poole, 2003; Dubuisson et al., 2004; Parsonage et al., 2005). In any case, it is important to emphasize that peroxiredoxins are abundant in aerobic cells. For example: (i) peroxiredoxins are among the ten most abundant proteins in *E. coli* (Link et al., 1997); (ii) peroxiredoxins are the second or third most abundant protein in erythrocytes (Moore et al., 1991) and (iii) compose 0.1–0.8% of the soluble proteins in other mammalian cells (Chae et al., 1999). Furthermore, it was demonstrated that peroxiredoxin, but not catalase, was responsible for protection of bacteria against endogenously generated hydrogen peroxide (Costa Seaver and Imlay, 2001).

There are several kinds of peroxiredoxins and several classifications were proposed based on different criteria.

Generally, every aerobic cell possesses several different kinds of peroxiredoxins. The most frequently used criteria for classification is the presence or absence of additional conserved cysteines (Wood et al., 2003b). Peroxiredoxins that contain two conserved cysteines are called 2-Cys Prx, whereas those that possess only one conserved cysteine are referred as 1-Cys Prx. In both cases, the reactive cysteine attacks the hydroperoxide and is oxidized to sulfenic acid (Cys-SOH), while the corresponding alcohol is released (Fig. 4). Because the reactive cysteine is the one that directly interact with peroxides it is called peroxidatic cysteine and is located at the N-terminal part of the protein. Three peroxiredoxin classes can be recognized based on the next step of the catalytic cycle (1-Cys Prx; typical 2-Cys Prx and atypical 2-Cys Prx). The 1-Cys Prx presents the simplest mechanism: they are oxidized to a stable sulfenic acid and then reduced back by a reductant. The biological electron donors of most 1-Cys Prx are still unknown. One exception is the 1-Cys Prx from yeast, whose electron donor is mitochondrial thioredoxin (Pedrajas et al., 2000). Furthermore, mammalian 1-Cys Prx can form heterodimer complexes with Glutathione *S*-transferase π, being capable to accept electrons from glutathione (Ralat et al., 2006).

The enzymatic mechanism of 2-Cys Prx differs from the 1-Cys Prx's mechanism because these proteins have a second conserved cysteine, also called resolving cysteine, which is also involved in the catalytic cycle. The sulfenic acid formed in the peroxidatic cysteine reacts with the resolving cysteine of other protein, generating an intermolecular disulfide bridge. In the case of atypical 2-Cys Prx, the resolving cysteine belongs to the same polypeptide backbone of the peroxidatic cysteine, therefore an intramolecular disulfide bond is generated. For the majority of the typical and atypical 2-Cys Prx proteins, disulfide bonds are reduced by thioredoxins (Fig. 4).

Alternatively, peroxiredoxins can be classified according to their amino acid sequence, which is very variable among five different groups (Trivelli et al., 2003). In spite of the fact that peroxiredoxins groups share very low amino acid sequence similarity, they have residues that are very conserved among all members (Wood et al., 2003b): (1) A proline that limits solvent and peroxide access in the active site and therefore probably

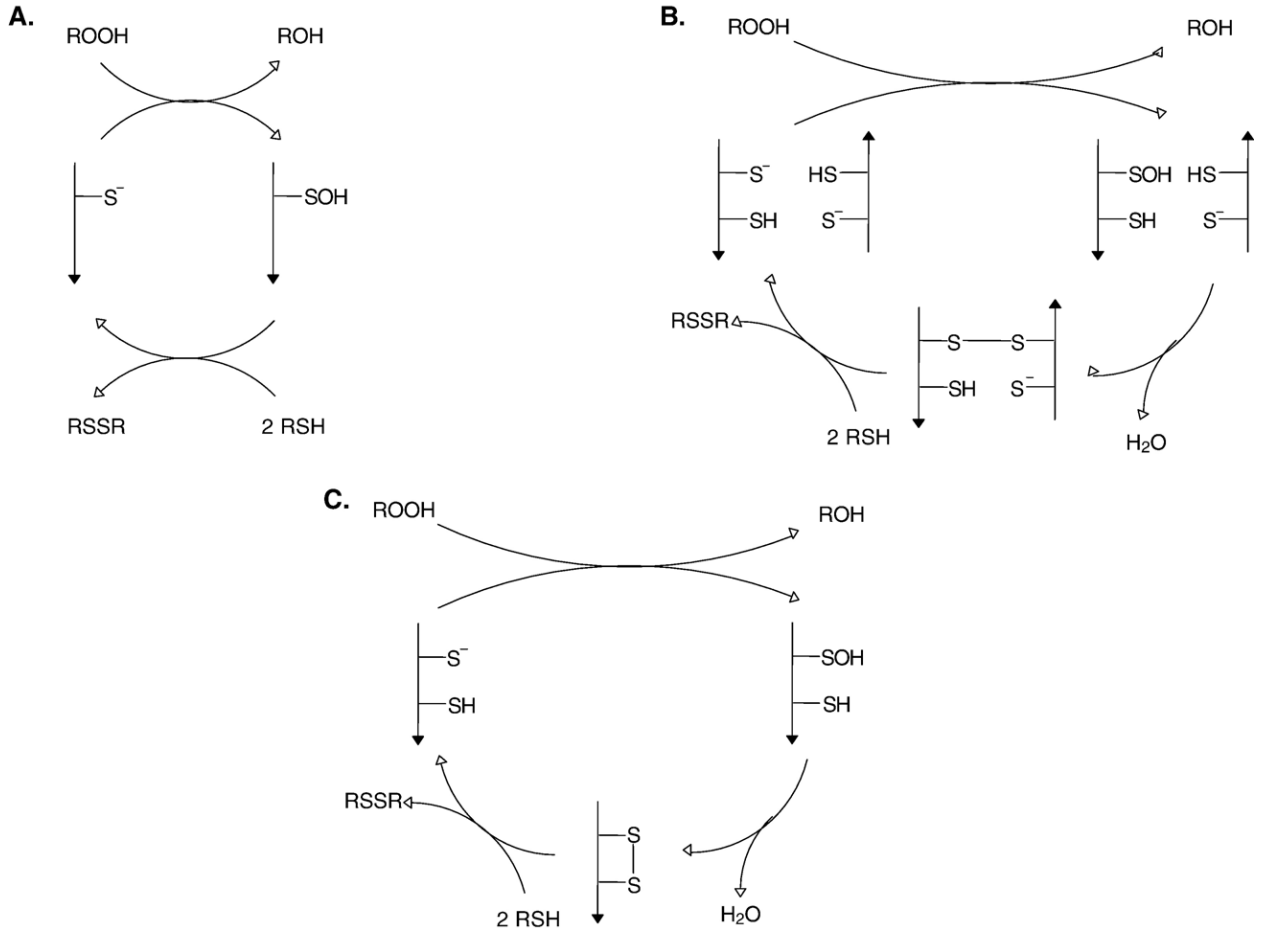

Fig. 4. Catalytic mechanism of Prxs. As described in Fig. 1B reduction of peroxides by reactive cysteines generated a sulfenic acid derivative in all kinds of peroxiredoxins. (A) In 1-Cys peroxiredoxins the sulfenic acid derivative is stabilized by the polypeptide backbone and is directly reduced by a thiol reductant. (B) In typical 2-Cys peroxiredoxins, the sulfenic acid interacts with another thiol group from other subunit, generating an intermolecular disulfide bond, which is then reduced by a biological substrate, in most cases thioredoxin. (C) In atypical 2-Cys peroxiredoxins, the catalytical mechanism is very similar to 2-Cys typical, with the exception that an intramolecular disulfide bond is formed.

shields the cysteine sulfenic acid from overoxidation; (2) an arginine residue that is involved in the stabilization of peroxidatic cysteine in the thiolate form and (3) an threonine residue that also interacts with the sulfur atom of peroxidatic cysteine (Fig. 3). Besides these similarities, all peroxiredoxins possess a common structural feature: the thioredoxin fold, which was described before. Interestingly, all other thiol/disulfide oxido-reductases presented here (thioredoxin, glutaredoxin and protein disulfide isomerase) also possess the thioredoxin fold (Fig. 2A). Differently than the thiol/disulfide oxido-reductases, peroxiredoxins contain central insertions, N-terminal and C-terminal expansions to the thioredoxin fold that are different for different groups of these thiol dependent peroxidases. Due to these structural similarities and through a motif analysis, it was proposed that all these thiol proteins might have a common ancestor (Copley et al., 2004).

The yeast *S. cerevisiae,* which has been used as model for higher eukaryotes, possesses five peroxiredoxins belonging to four different sub-groups (Park et al., 2000). Our studies have demonstrated that although all five yeast peroxiredoxins have the same biochemical activity (thioredoxin dependent peroxidase); their cellular functions are not completely redundant. For example, cytosolic thioredoxin peroxidase I (Tsa1/YML028W) is specifically important for the defense of yeast with dysfunctional mitochondria (Demasi et al., 2001; Demasi et al., 2006), whereas mitochondrial thioredoxin peroxidase I (PrxI/YBL064C) is more important in conditions where yeast obtain ATP preferentially by respiration (Monteiro et al., 2002; Monteiro and Netto, 2004). Finally, cytosolic thioredoxin peroxidase II (cTPxII/Tsa2/YDR453C) appears to be an important backup for cTPxI for the defense against organic peroxides, independently of the functional state of mitochondria (Munhoz and Netto, 2004). Interestingly, mitochondria are protected not only by the mitochondrial isoform (PrxI/YBL064C) but also by cytosolic isoforms and in cooperation with mitochondrial pool of glutathione against Ca^{2+} induced stress (Monteiro et al., 2004). This partial redundancy observed among yeast peroxiredoxins probably parallels the roles that these peroxidases play in mammalian cells.

Recently, a new kind of peroxidase that also operates through a reactive cysteine was described (Lesniak et al., 2002; Cussiol et al., 2003). Initially, it was demonstrated that the deletion of genes encoding these peroxidases rendered *Xanthomonas campestris* specifically sensitive to organic peroxides, but not to hydrogen peroxide (Mongkolsuk et al., 1998). Therefore, this gene was named organic hydroperoxide resistance (Ohr) and was later shown to be exclusively present in bacteria, most of them pathogenic. Interestingly, only dithiols support the peroxidase activity of Ohr and it is considerably more efficient in the removal of organic peroxides than in the decomposition of hydrogen peroxide (Cussiol et al., 2003). It was noteworthy to observe that differently than other thiol-dependent peroxidases (glutathione peroxidases and peroxiredoxins), Ohr does not possess the thioredoxin fold. Instead, Ohr is a dimer composed of two six-strand β-sheet and two central α-helixes (Lesniak et al., 2002; Meunier-Jamin et al., 2004; Oliveira et al., 2006). Contrary to the other thiol/disulfide oxido-reductases and peroxidases described so far, the reactive cysteine is located in a very hydrophobic environment (Fig. 5). Due to these differences and because Ohr are exclusively present in bacteria, these peroxidases might represent interesting targets for drug design.

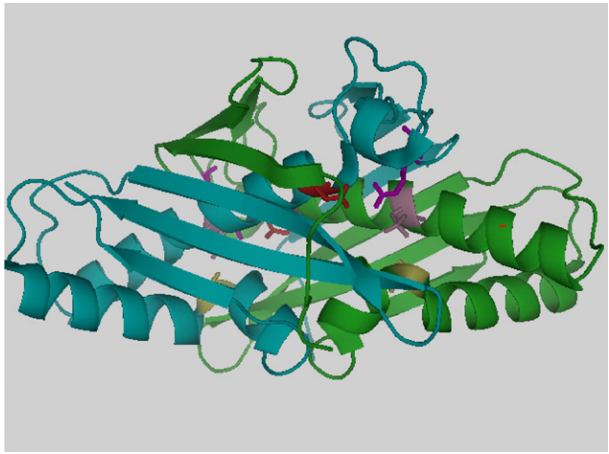

Fig. 5. Ohr structure with hidden cysteines residues. Overall view of *Xylella fastidiosa* quartenary structure (PDB = 1ZB9). Contrary to peroxiredoxins and thiol/disulfide oxido-reductases, reactive cysteine (Cys61 in pink) is buried in the polypeptide backbone (two β-sheet composed of six strands). The side chain of Arg 19 (magenta) that is involved in the stabilization of thiolate form of Cys 61 and Glu51 (red) that forms a salt bridge with Arg19 are also shown in dark color. Cys 125 (in yellow), involved in the formation of an intramolecular disulfide bond, is also represented with black color. Figure was generated by the Pymol software (www.pymol.org). (For interpretation of the reference to colour in this figure legend, the reader is referred to the web version of this article.)

Finally, antioxidant proteins also make use of reactive cysteine to repair oxidative damage. Methionine sulfoxide reductase has a reactive cysteine capable to cleave an S═O bond, also by a nucleophilic substitution mechanism (Weissbach et al., 2002), (Fig. 1C).

2.5. Redox signalling

Since reactive cysteines can decompose peroxides yielding products that can be reduced back to the sulfhydryl form, several proteins containing this kind of residues are in principle adapted to participate in redox signaling mediated by hydrogen peroxide. Although hydrogen peroxide has been classically associated with oxidative stress, there is a growing amount of evidences about the role of this mild oxidant as a cell messenger (Rhee et al., 2005b). Hydrogen peroxide can cross membranes and is relatively stable, two features suitable for a cell messenger in analogy to nitric oxide (Stone, 2004). This idea was strengthened by the discovery that non-phagocytic cells also possess NADPH oxidase, a source for hydrogen peroxide (Bokoch and Knaus, 2003). In fact, there are numerous reports about the effect of hydrogen peroxide in terms of both cellular responses and signaling pathways activated (reviewed by Stone, 2004).

The best characterized mediator of peroxide induced stress is OxyR, a transcription activator found only in bacteria. Genes regulated by OxyR includes enzymes involved in peroxide

decomposition (catalase, peroxiredoxin, thioredoxin, glutathione reductase and glutaredoxin) and cell signaling (small RNA molecule). The mechanism by which OxyR senses H_2O_2 involves a reactive cysteine that once again is stabilized in the thiolate form by a conserved arginine among other amino acids (Choi et al., 2001). The oxidation of this cysteine generates a disulfide bond that causes a conformational change in the protein. Both the oxidized and reduced forms of OxyR can bind DNA, but only the oxidized form is capable to recognize specific elements in the promoters of target genes and activate their transcription (Fig. 6A). The reactive cysteine of OxyR possesses a relatively high rate constant (2×10^5 M^{-1} s^{-1}, see Aslund et al., 1999) and can activate transcription when intracellular concentrations of hydrogen peroxide are as little as 100 nM (Costa Seaver and Imlay, 2001). The activation of OxyR is reversed by reduction of reactive cysteine by GSH and glutaredoxin 1 (Fig. 6).

Response of bacteria to oxidative stress is mediated by other transcriptional regulators besides OxyR. OhrR is a transcriptional repressor that is also capable to sense peroxides through a reactive cysteine (Mongkolsuk and Helmann, 2002). The only one known target of OhrR so far described is Ohr that is specifically induced by organic peroxides, the preferable substrate of this dithiol-dependent peroxidase. Therefore, Ohr/OhrR is a pathway specifically involved in the oxidative stress response to organic, but not to hydrogen peroxide (Klomsiri et al., 2005). In vitro, reduced OhrR binds tightly to its target DNA and therefore blocks the transcription of ohr (Fuangthong et al., 2001). Oxidation of a conserved and reactive cysteine in OhrR by peroxides leads to derepression of ohr transcription, which is reversed by a reducing agent such as DTT. Differently than OxyR, OhrR is oxidized to a sulfenic acid (CysSOH) instead of a disulfide (Fuangthong and Helmann, 2002).

Another transcriptional repressor of bacteria that was implied in peroxide sensing in bacteria through reactive cysteines is PerR (Mongkolsuk and Helmann, 2002). PerR belongs to a family of transcriptional regulators that are dimeric proteins and that contain two metal sites per monomer. One binds zinc and appears to play mainly structural roles, whereas the second site can bind both iron and manganese and has a regulatory role. PerR complexed with either Mn^{+2} or Fe^{+2} can bind DNA and repress transcription of its target genes such as catalase and peroxiredoxin. However, only when PerR is complexed with Fe^{+2} there is derepression of gene expression and lack of DNA binding ability (Herbig and Helmann, 2001). Because DNA binding of PerR is restored by thiol reductants and because PerR has a CXXC motif, it was proposed that peroxide sensing might involve a reactive cysteine being oxidized to a disulfide bond. Very recently, however, the same group has shown that PerR senses hydrogen peroxide by a Fenton-like reaction mediated by Fe^{+2} complexed with histidines. This process provokes oxidation of histidine residues (His37 and His91) to 2-oxo-histidines. This is the first description of a metal catalyzed protein oxidation process involved with redox signaling (Lee and Helmann, 2006).

Besides transcriptional regulators, bacteria also possess a chaperone (Hsp33), whose activity is redox regulated through reduction/oxidation cycles that involve a reactive cysteine (Janda et al., 2004). In this case, cysteines residues in the reduced state can bind zinc but after oxidation to disulfide bonds, Hsp33 loses this ability but acquires high affinity for unfolded proteins (chaperone holdase activity). Thioredoxin (or glutaredoxin) can then reduce the reactive cysteine of Hsp33, restoring its ability to bind zinc. This ensures that proteins with transient exposed hydrophobic surfaces do not form insoluble aggregates. Upon return to non stress conditions other chaperone systems are available to interact with the partially unfolded proteins released by Hsp33 (reviewed by Winter and Jakob, 2004). Interestingly, Hsp33 appears to be active in severe oxidative stress, condition in which other chaperones are inactive (Winter et al., 2005).

Another level of regulation was possible in eukaryotes with the appearance of cellular compartments. In fact, the control of a transcriptional regulator's activity by regulated nuclear

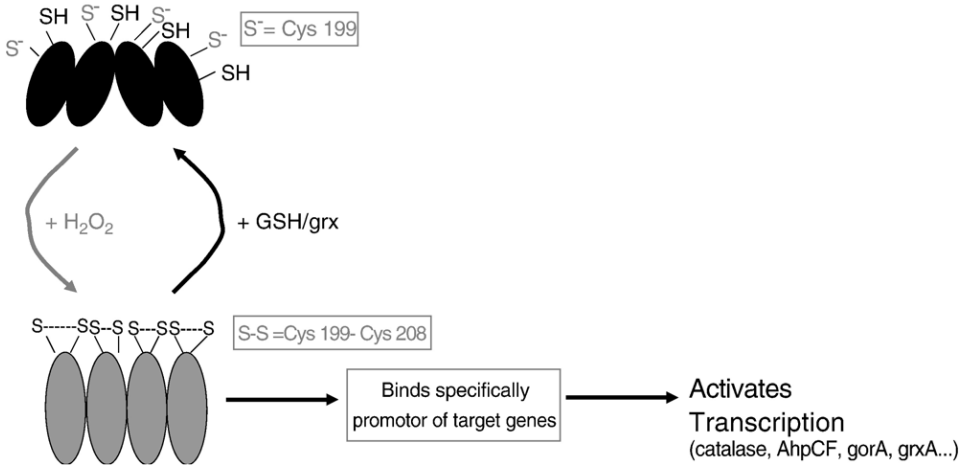

Fig. 6. Redox regulation by OxyR. Each OxyR subunit is represented here by an elliptical symbol. The darker symbols represent the reduced tetramer and the lighter the oxidized (disulfide) tetramer that assumes different conformations. Only the oxidized formed is capable to recognize specific sequences (elements) repeated four times in the promoters of targets genes and as a consequence stimulate their transcription.

accumulation is a common theme in biology. Therefore, the higher the amount of a transcriptional regulator in the nucleus, the higher is its activity (repression or induction of gene expression). The best characterized mechanism of a redox signaling process in an eukaryotic cell through a reactive cysteine is that mediated by Yap1 (reviewed by Paget and Buttner, 2003).Yap1 belongs to the AP-1 family of proteins that includes the proto-oncogenes Jun and Fos, all of them possessing a Leu zipper involved in the dimerization of these proteins (Fig. 7A). Yap1 also possesses a nuclear export signal (NES) that in basal conditions is recognized by Cmr1 that then transport this transcriptional activator from the nucleus to the cytosol (Fig. 7B i). Therefore, in basal conditions Yap1 is preferentially located in the cytosol, does not interact with target promoters and consequently does not induce gene expression. Upon oxidation, Yap1 cysteine residues are oxidized and NES adopt a different conformation, not recognizable by Crm1. Therefore, Yap1 accumulates in the nucleus, being capable to physically interact with target promoters.

Yap1 can be oxidized into two products: (1) a disulfide between cysteines residues of the C-terminal cysteine rich domain (Fig. 7B ii) or (2) a disulfide between one cysteine of the N-terminal and the other of the C-terminal rich domain (Fig. 7B v). Mode (1) of Yap1 oxidation is the simplest and is mediated by thiol oxidizing agents such as diamide (Fig. 7B ii). The mode (2) is a pathway that involves other proteins besides Yap1. In this case, the oxidant is a peroxide molecule that is sensed by a protein, homologous to the selenium-dependent glutathione peroxidase (Gpx3/Orp1) from mammalian cells (Delaunay et al., 2002). Gpx3/Orp1 is oxidized to a sulfenic acid derivative (Fig. 7B iii), which condenses with a reactive cysteine of Yap1, generating a mixed disulfide bond (Fig. 7B iv). Finally a thiolate group from the N-terminal cysteine rich domain attacks the mixed disulfide, generating an intra-molecular disulfide bond in Yap1, which is not recognized by Crm1 and accumulates in the nucleus (Fig. 7B v). Besides Yap1, other transcriptional regulators are involved in the response of yeast to oxidative stress which is a very complex phenomenon. As an example, the regulation of mitochondrial thioredoxin peroxidase I involves Hap1 (YLR256W), Msn2/4 (YMR037C/YKL062W) and Yap1 among other regulators (Monteiro et al., 2002; Monteiro and Netto, 2004).

The mechanisms by which hydrogen peroxide is sensed in mammalian cells are much more controversial. Much attention

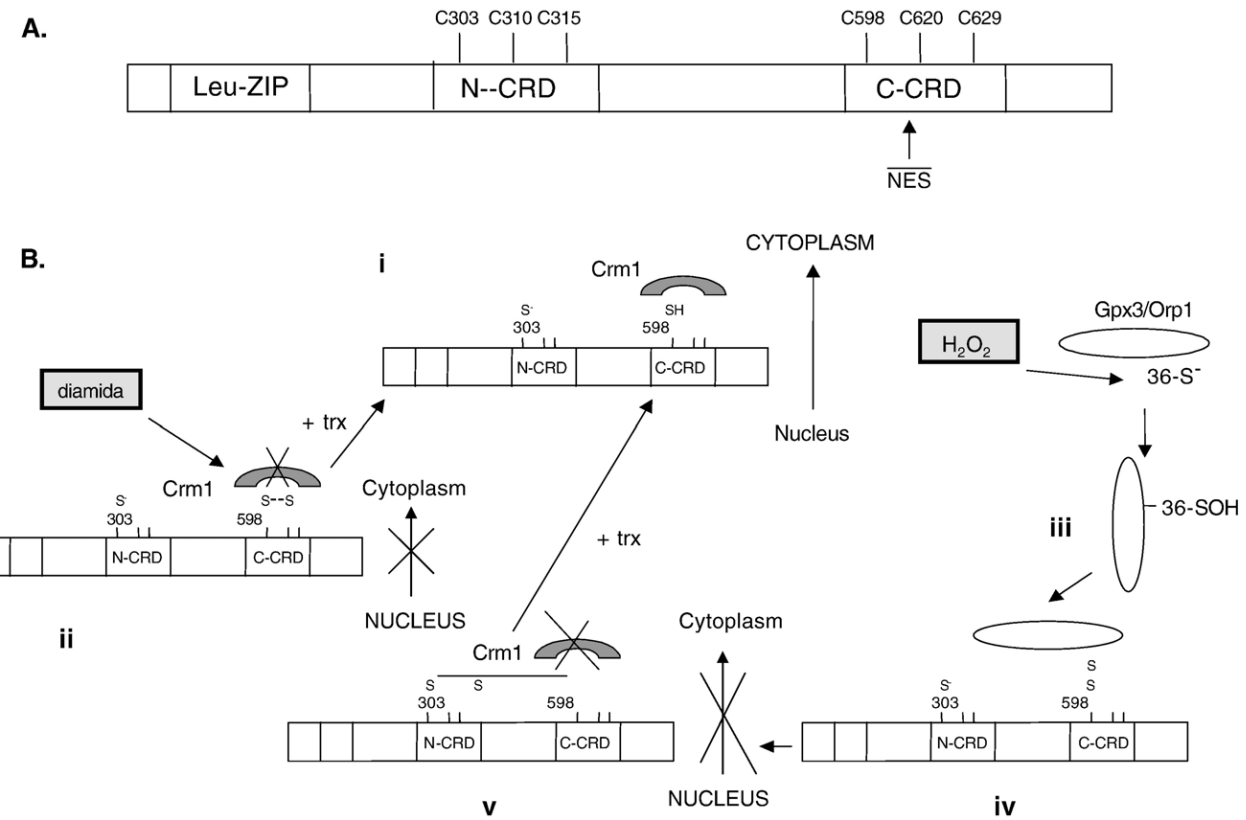

Fig. 7. Yap1 activation by nuclear accumulation dependent on oxidation. (A) Yap1 domains. Leu-ZIP = leucine rich domain; N-CRD = N-terminal cysteine rich domain; C-CRD = C-terminal cysteine rich domain. NES= Nuclear Export Signal. (B) The names of cellular compartments with capitol letters indicate the location where Yap1 accumulates. The arrow represents exportation of Yap1 out of the nucleus and the symbols of arrows with an axis represent inhibition of this process by oxidation of Yap1 cysteines. (i) Yap1 in the ground state is reduced and, therefore, its NES is recognized by Cmr1, leading to its exportation out of the nucleus. (ii) Thiols oxidizing agents, such as diamide, oxidize thiolate groups of the C-CRD, provoking inhibition of its exportation. After consumption of the oxidant, thioredoxin can reduce Yap1 back to the reduced state (i). (iii) Gpx3/Orp1 is oxidized by peroxide, generating a sulfenic acid derivative. (iv) Sulfenic acid form of Gpx3/Orp1 condenses with a thiolate group from C-CRD generating a mixed disulfide bridge, which is attacked by a thiolate from N-CRD, generating an intra-molecular disulfide bridge between cysteines of different domains (v). After consumption of the peroxide this disulfide can be reduced back to the ground state (i).

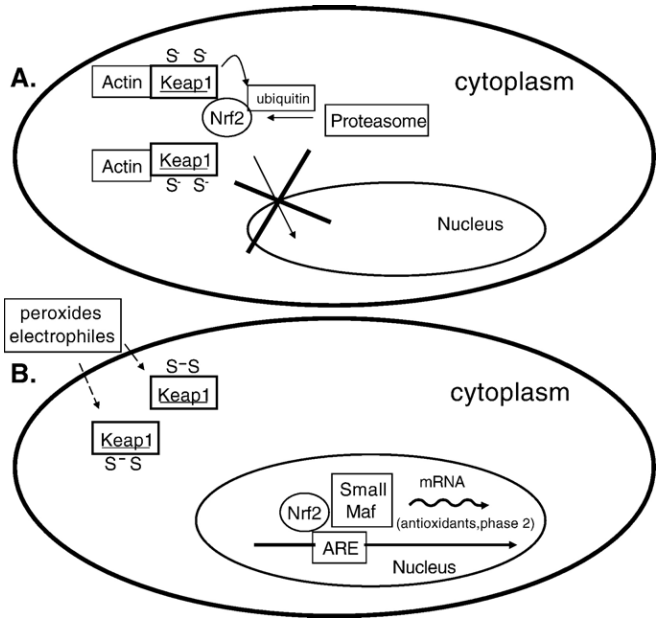

Fig. 8. Nrf2 activation by nuclear accumulation dependent on oxidation of Keap1. (A) Under basal conditions, Keap1 is in the reduced state and sequester Nrf2 in the cytoplasm. Keap1 is also connected to the cell cytoskeleton. In this condition, Keap1 also induces ubiquitination of Nfr2 that is then degraded by proteasome. (B) Under oxidative stress, thiolate groups of keap1 are oxidized, leading to Nrf2 release that can then accumulate in the nucleus and activate transcription in the target genes.

is given to protein tyrosine phosphatases (PTP) as biological sensors of hydrogen peroxide. Hydrogen peroxide can react with cysteines from the active site of PTP, generating sulfenic acids, which was proposed to be a redox regulatory event (Lee et al., 1998). Later, two groups have shown independently that sulfenic acids in PTP are converted to sulfenyl-amide by reaction of sulfenic acids with backbone amide of a serine residue (Salmeen et al., 2003; Van Montfort et al., 2003). The sulfenyl-amide form of PTP is inactive; therefore this process should provoke an increase in the levels of tyrosine phosphorylation. As a consequence, PTP targets such as MAP kinases should be phosphorylated in higher levels. Besides sulfenyl-amides, reactive cysteines were also found in the sulfinate (RSO_2^-) and sulfonate (RSO_3^-) forms in the crystal structure of PTP, when these proteins were treated with large excess of hydrogen peroxide (Van Montfort et al., 2003). Contrary to the sulfinate and sulfonate forms, sulfenyl-amides can be reduced back by classical reductants such as DTT and thioredoxin (Salmeen et al., 2003; Van Montfort et al., 2003). Therefore, because their formation is reversible, sulfenyl-amides were proposed as an important step in the redox signaling by PTPs.

However, redox regulation by PTP is controversial, mainly because the reaction of these phosphatases with hydrogen peroxide is slow (reaction constant is around 10 M^{-1} s^{-1}), (Stone, 2004). Considering that intracellular concentration of hydrogen peroxide is in between 1 to 700 nM and that the levels of glutathione are around 1–10 mM, a target for redox regulation should react faster with this mild oxidant than PTP does. In fact, as mentioned before, one biological sensor of hydrogen peroxide in bacteria, the transcriptional factor OxyR possesses a reaction constant of 2×10^5 M^{-1} s^{-1} (Aslund et al., 1999). OxyR, like other thiol proteins mentioned here, possesses a very reactive cysteine, which is deprotonated at physiological pH. Therefore, the hydrogen peroxide sensor in mammalian cells should be in principle a protein that possesses a reaction constant with hydrogen peroxide in this range.

Peroxiredoxins are good candidates as biological redox sensors in mammalian cell, since their reaction constants with hydrogen peroxide are around 10^5 M^{-1} s^{-1} or even higher (Akerman and Muller, 2005; Baker and Poole, 2003; Parsonage et al., 2005). In fact, there are many suggestions that peroxiredoxins could be the biological sensors of hydrogen peroxide (Wood et al., 2003a,b). In this regard, it was shown that bacterial 2-Cys Prx are one hundred times more resistant to hydrogen peroxide inactivation than some of their counterparts in eukaryotic cells (Wood et al., 2003a). In both cases, the inactivation by hydrogen peroxide occurs due to oxidation of sulfenic acid (Cys-SOH) in the reactive cysteine to sulfinic acid (Cys-SO_2H). Interestingly, the all 2-Cys Prx that are sensitive to inactivation possess two common motifs: GGLG and YF (Wood et al., 2003a). Therefore, it seems very probable that the high sensitivity of these peroxiredoxins to peroxide inactivation it is not a limitation in the mechanism of catalysis, but instead a property that was selected during evolution of eukaryotes (Wood et al., 2003a).

In support to this hypothesis, it was shown that sulfinic acids in 2-Cys Prx are reduced *in vivo* in sensitive 2-Cys Prx (Woo et al., 2003). This was a quite surprising result, since it is well established that sulfinic acids in peroxiredoxins and in any other protein are not reducible in vitro by classical reducing agents, such as DTT and thioredoxin. The enzymatic system responsible to regenerate sulfhydryl groups from sulfinic acids in 2-Cys Prx was first identified in the yeast *S. cerevisiae* and was named sulfiredoxin (Biteau et al., 2003). Sulfiredoxin is a low molecular weight protein (13 kDa) that possesses homologues in higher eukaryotes including human, but its physiological role was unknown. The proposed mechanism of catalysis involves phosphotransferase and thiol transferase activities through a reactive cysteine and it is dependent on ATP. The basis of this mechanism was confirmed by biochemical and crystallographic studies (Jonsson et al., 2005). Biteau et al. (2003) suggested that sulfinic acid formation in 2-Cys Prx could represent an additional level of redox regulation for peroxiredoxins. The sulfiredoxin homologue in mammalian cells was also identified and in this case it was shown that sulfinic acid regeneration is exclusive for 2-Cys Prx (Chang et al., 2004; Woo et al., 2005). Recently, Budanov et al. (2004) have shown that another class of proteins can also reduce sulfinic acids specifically of mammalian peroxiredoxins. Like sulfiredoxins, sestrin possesses a conserved cysteine that is responsible for the catalytic mechanism. Moreover, the reduction is also dependent on ATP. However, sestrins do not share homology with sulfiredoxins. Sestrin expression is regulated by p53 indicating that this process possesses high physiological relevance.

The importance of sulfinic acids generated in the peroxidatic cysteines of 2-Cys Prx was further strengthened by the observation that peroxiredoxins from yeast also possess

chaperone activity. Interestingly, the chaperone activity is independent of peroxidatic and resolving cysteines. Both peroxides and high temperatures induce chaperone activity, which is dependent on the oligomerization of peroxiredoxin polypeptides. Remarkably, under oxidative and thermal stresses these protein form very high molecular weight complexes that can be visualized by electron microscopy. Therefore, peroxidatic cysteines of these yeast peroxiredoxins are important not only for the decomposition of peroxides but also to induce protein oligomerization and consequently chaperone activity. In fact, sulfinic acid formation was suggested as a trigger event for the formation of a superchaperone that possesses a molecular weight of more than 1000 kDa (Jang et al., 2004). This dual chaperone/peroxidase activities of yeast 2-Cys Prx was implied with the observation that it specifically protects cells with dysfunctional mitochondria from peroxide insult (Demasi et al., 2006). Recently, the chaperone activity was also described for peroxiredoxins from mammals and bacteria (Moon et al., 2005; Chuang et al., 2006).

The versatility of peroxiredoxin function can be further demonstrated by the observation that addition of single amino acid (Phe) close to the reactive cysteine converts a bacterial peroxiredoxin into a disulfide reductase (Ritz et al., 2001). This appears to be a relevant phenomenon, since bacteria lacking both thioredoxin reductase and glutathione reductase are viable only if cells possess peroxiredoxin with disulfide reductase activity (Ritz et al., 2001).

Recently, a novel redox mechanism for regulation of gene expression was demonstrated in mammals (Venugopal and Jaiswal, 1996; Itoh et al., 1997). As the redox regulation of Yap1 activity, the regulation of Nfr2, also a leucine zipper transcriptional activator, involves control of its nuclear localization. However, differently than Yap1, Nfr2 is not directly redox regulated, but instead reactive cysteines of a cytoplasmatic anchor (Keap1) are susceptible to oxidation by peroxides and electrophiles. Under basal conditions, Nfr2 is sequestered from the nuclei by Keap1 through non-covalent interactions (Itoh et al., 1999). Because Keap1 is bound to actin, Nrf2 is also connected to the cell cytoskeleton (reviewed by Motohashi and Yamamoto, 2004). These protein–protein interactions also induced ubiquitination of Nfr2 and consequently proteolytic digestion by proteasome (Fig. 8A). When mammalian cells are exposed to peroxides and electrophiles, reactive cysteines of Keap1 are oxidized to disulfide bonds, it suffers a conformational change and consequently Nfr2 is released and accumulates in the nucleus, being capable to recognize its target promoters (Fig. 8B).

3. Conclusions

The majority of the cysteine residues in proteins play no role in electron transfer reaction, because their pK_a make them appear mainly in the protonated form in physiological conditions. In contrast, some protein foldings create environments in which the deprotonated form of cysteine (RS^- = thiolate) is stabilized, being susceptible to oxidation. Thiol/disulfide reactions are the most frequently considered, but thiol/sulfenic acid reactions have also been implicated in some biological processes a long time ago. Recently, thiol/sulfinic redox chemistry has received attention in terms of redox signaling. Therefore, the versatile redox chemistry of thiolate in proteins has served to various biological roles as described in this review and should be a promising research field.

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References

Akerman, S.E., Muller, S., 2005. Peroxiredoxin-linked detoxification of hydroperoxides in *Toxoplasma gondii*. J. Biol. Chem. 280, 564–570.

Aslund, F., Berndt, K.D., Holmgren, A., 1997. Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein–protein redox equilibria. J. Biol. Chem. 272, 30780–30786.

Aslund, F., Zheng, M., Beckwith, J., Storz, G., 1999. Regulation of the OxyR transcription factor by hydrogen peroxide and the cellular thiol-disulfide status. Proc. Natl. Acad. Sci. U. S. A. 96, 6161–6165.

Baker, L.M., Poole, L.B., 2003. Catalytic mechanism of thiol peroxidase from *Escherichia coli*. Sulfenic acid formation and overoxidation of essential Cys61. J. Biol. Chem. 278, 9203–9211.

Benesch, R.E., Benesch, R., 1955. The acid strength of the-SH group in cysteine and related compounds. J. Am. Chem. Soc. 77, 5877–5881.

Biteau, B., Labarre, J., Toledano, M.B., 2003. ATP-dependent reduction of cysteine–sulphinic acid by *Saccharomyces cerevisiae* sulphiredoxin. Nature 425, 980–984.

Bokoch, G.M., Knaus, U.G., 2003. NADPH oxidases: not just for leukocytes anymore! Trends Biochem. Sci. 28, 502–508.

Bryk, R., Griffin, P., Nathan, C., 2000. Peroxinytrite reductase activity of bacterial peroxiredoxins. Nature 407, 211–215.

Budanov, A.V., Sablina, A.A., Feinstein, E., Koonin, E.V., Chumakov, P.M., 2004. Regeneration of peroxiredoxins by p53 — regulated sestrins, homologs of bacterial AhpD. Science 304, 596–600.

Carvalho, A.P., Fernandes, P.A., Ramos, M.J., 2006. Similarities and differences in the thioredoxin superfamily. Prog. Biophys. Mol. Biol. 91, 229–248.

Chae, H.Z., Kim, H.J., Kang, S.W., Rhee, S.G., 1999. Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. Diabetes Res. Clin. Pract. 45, 101–112.

Chang, T.-S., Jeong, W., Woo, H.A., Lee, S.M., Park, S., Rhee, S.G., 2004. Characterization of mammalian sulfiredoxin and its reactivation of hyperoxiidized peroxiredoxin through reduction of cysteine sulfinic acid in the active site cysteine. J. Biol. Chem. 279, 50994–51001.

Chivers, P.T., Prehoda, K.E., Volkman, B.F., Kim, B.M., Markley, J.L., Raines, R.T., 1997. Microscopic pK_a values of *Escherichia coli* thioredoxin. Biochemistry 36, 14985–14991.

Chrestensen, C.A., Starke, D.W., Mieyal, J.J., 2000. Acute Cadmium exposure inactivate thioltransferase (glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis. J. Biol. Chem. 295, 26556–26565.

Choi, H., Kim, S., Mukhopadhyay, P., Cho, S., Woo, J., Storz, G., Ryu, S., 2001. Structural basis of the redox switch in the OxyR transcription factor. Cell 105, 103–113.

Chuang, M.H., Wu, M.S., Lo, W.L., Lin, J.T., Wong, C.H., Chiou, S.H., 2006. The antioxidant protein alkylhydroperoxide reductase of *Helicobacter pylori* switches from a peroxide reductase to a molecular chaperone function. Proc. Natl. Acad. Sci. U. S. A. 103, 2552–2557.

Collet, J.F., Bardwell, J.C., 2002. Oxidative protein folding in bacteria. Mol. Microbiol. 44, 1–8.

Copley, S.D., Novak, W.R.P., Babbitt, P.C., 2004. Divergence of function in the thioredoxin fold suprafamily: evidence for evolution of peroxiredoxins from a thioredoxin-like ancestor. Biochemistry 43, 13981–13995.

Costa Seaver, L., Imlay, J.A., 2001. Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. J. Bacteriol. 183, 7173–7181.

Cussiol, J.R., Alves, S.V., Oliveira, M.A., Netto, L.E.S., 2003. Organic hydroperoxide resistance gene encodes a thiol-dependent peroxidase. J. Biol. Chem. 180, 2636–2643.

Darby, N.J., Creighton, T.E., 1995. Characterization of the active site cysteine residues of the thioredoxin-like domains of protein disulfide isomerase. Biochemistry 34, 16770–16780.

Davis, D.A., Newcomb, F.M., Strke, D.W., Ott, D.E., Mieyal, J.J., Yarchoan, R., 1997. Thioltransferase (glutaredoxin) is detected within HIV-1 and can regulate the activity of glutathionylated HIV-1 protease in vitro. J. Biol. Chem. 272, 25935–25940.

Delaunay, A., Pflieger, D., Barrault, M.B., Vinh, J., Toledano, M.B., 2002. A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell 111, 471–481.

Demasi, A.P.D., Pereira, G.A.G., Netto, L.E.S., 2001. Cytosolic thioredoxin peroxidase I is essential for the antioxidant defense of yeast with dysfunctional mitochondria. FEBS Lett. 509, 430–434.

Demasi, M., Silva, G.M., Netto, L.E.S., 2003. 20S proteasome from *Saccharomyces cerevisiae* is responsive to redox modifications and is *S*-glutathionylated. J. Biol. Chem. 278, 679–685.

Demasi, A.P.D., Pereira, G.A.G., Netto, L.E.S., 2006. Yeast oxidative stress response: influences of cytosolic thioredoxin peroxidase I and of the mitochondrial functional state. FEBS J. 273, 805–816.

Dillet, V., Dyson, H.J., Bashford, D., 1998. Calculations of electrostatic interactions and pKas in the active site of *Escherichia coli* thioredoxin. Biochemistry 37, 10298–10306.

Discola, K.F., Oliveira, M.A., Silva, G.M., Barcena, J.A., Porras, P., Padilla, A., Netto, L.E.S., Guimarães, B.G., 2005. Crystallization and preliminary X-ray diffraction analysis of glutaredoxin 2 from *Saccharomyces cerevisiae* in different oxidation states. Acta Crystallogr., F F61, 445–447.

Dubuisson, M., Vander-Stricht, D., Clippe, A., Etienne, F., Nauser, T., Kissner, R., Koppenol, W.H., Rees, J.F., Knoops, B., 2004. Human peroxiredoxin 5 is a peroxynitrite reductase. FEBS Lett. 571, 161–165.

Dyson, H.J., Jeng, M.F., Tennant, L.L., Slaby, I., Lindell, M., Cui, D.S., Kuprin, S., Holmgren, A., 1997. Effects of buried charged groups on cysteine thiol ionization and reactivity in *Escherichia coli* thioredoxin: structural and functional characterization of mutants of Asp 26 and Lys 57. Biochemistry 36, 2622–2636.

Fernandes, A.P., Holmgren, A., 2004. Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxid. Redox Signal. 6, 63–74.

Frand, A.R., Cuozzo, J.W., Kaiser, C.A., 2000. Pathways for protein disulphide bond formation. Trends Cell Biol. 10, 203–210.

Fuangthong, M., Helmann, J.D., 2002. The OhrR repressor senses organic hydroperoxides by reversible formation of a cysteine–sulfenic acid derivative. Proc. Natl. Acad. Sci. U. S. A. 99, 6690–6695.

Fuangthong, M., Atichartpongkul, S., Mongkolsuk, S., Helmann, J.D., 2001. OhrR is a repressor of ohrA, a key organic hydroperoxide resistance determinant in *Bacillus subtilis*. J. Bacteriol. 183, 4134–4141.

Gan, Z.R., Sardana, M.K., Jacobs, J.W., Polokoff, M.A., 1990. Yeast thioltransferase — the active site cysteines display differential reactivity. Arch. Biochem. Biophys. 282, 110–115.

Goldberger, R.F., Epstein, C.J., Anfinsen, C.B., 1963. Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. J. Biol. Chem. 238, 628–635.

Grauschopf, U., Winther, J.R., Korber, P., Zander, T., Dallinger, P., Bardwell, J.C.A., 1995. Why is DsbA such an oxidizing disulfide catalyst? Cell 83, 947–955.

Hawkins, H.C., Freedman, R.B., 1991. The reactivities and ionization properties of the active-site dithiol groups of mammalian protein disulphide–isomerase. Biochem. J. 275 (Pt 2), 335–339.

Herbig, A.F., Helmann, J.D., 2001. Roles of metal ions and hydrogen peroxide in modulating the interaction of the Bacillus subtilis PerR peroxide regulon repressor with operator DNA. Mol. Microbiol. 41, 849–859.

Hillar, A., Peters, B., Pauls, R., Loboda, A., Zhang, H., Mauk, A.G., Loewen, P.C., 2000. Modulation of the activities of catalase–peroxidase HPI of *Escherichia coli* by site-directed mutagenesis. Biochemistry 39, 5868–5875.

Hofmann, B., Hecht, H.J., Flohe, L., 2002. Peroxiredoxins. Biol. Chem. 383, 347–364.

Holmgren, A., 1972. Tryptophan fluorescence study of conformational transitions of the oxidized and reduced form of thioredoxin. J. Biol. Chem. 247, 1992–1998.

Holmgren, A., 1976. Hydrogen donor system for *Escherichia coli* ribonucleoside–diphosphate reductase dependent upon glutathione. Proc. Natl. Acad. Sci. U. S. A. 73, 2275–2279.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., Nabeshima, Y., 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun. 236, 313–322.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J.D., Yamamoto, M., 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev. 13, 76–86.

Jacob, C., Giles, G.I., Giles, N.M., Sies, H., 2003. Sulfur and selenium: the role of oxidation state in protein structure and function. Angew. Chem., Int. Ed. 42, 4742–4758.

Janda, I., Devedjiev, Y., Derewenda, U., Dauter, Z., Bielnicki, J., Cooper, D.R., Graf, P.C., Joachimiak, A., Jakob, U., Derewenda, Z.S., 2004. The crystal structure of the reduced, Zn2+-bound form of the B. subtilis Hsp33 chaperone and its implications for the activation mechanism. Structure 12, 1901–1907.

Jang, H.H., Lee, K.O., Chi, Y.H., Jung, B.G., Park, S.W., Park, J.H., Lee, J.R., Lee, S.S., Moon, J.C., Yun, J.W., Choi, Y.K., Kim, W.Y., Kang, J.S., Cheong, G.-W., Yun, D.-J., Rhee, S.G., Cho, M.J., Lee, S.Y., 2004. Two enzymes in one: two yeast peroxiedoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone. Cell 117, 625–635.

Jao, S.C., English Ospina, S.M., Berdis, A.J., Starke, D.W., Post, C.B., Mieyal, J.J., 2006. Computational and mutational analysis of human glutaredoxin (thioltransferase): probing the molecular basis of the low pK_a of cysteine 22 and its role in catalysis. Biochemistry 45, 4785–4796.

Jonsson, T.J., Murray, M.S., Johnson, L.C., Poole, L.B., Lowther, W.T., 2005. Structural basis for the retroreduction of inactivated peroxiredoxins by human sulfiredoxin. Biochemistry 44, 8634–8642.

Jung, G., Breitmaier, E., Voelter, W., 1972. Dissociation equilibrium of glutathione. A Fourier transform-13C-NMR spectroscopic study of pH-dependence and of charge densities. Eur. J. Biochem. 24, 438–445.

Kallis, G.B., Holmgren, A., 1980. Differential reactivity of the functional sulfhydryl groups of cysteine-32 and cysteine-35 present in the reduced form of thioredoxin from *Escherichia coli*. J. Biol. Chem. 255, 10261–10265.

Klomsiri, C., Panmanee, W., Dharmsthiti, S., Vattanaviboon, P., Mongkolsuk, S., 2005. Novel roles of ohrR-ohr in Xanthomonas sensing, metabolism, and physiological adaptive response to lipid hydroperoxide. J. Bacteriol. 187, 3277–3281.

Lee, J.W., Helmann, J.D., 2006. The PerR transcription factor senses H2O2 by metal-catalysed histidine oxidation. Nature 440, 363–367.

Lee, S.R., Kwon, K.S., Kim, S.R., Rhee, S.G., 1998. Reversible inactivation of protein tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J. Biol. Chem. 273, 15366–15372.

Lesniak, J., Barton, W.A., Nikolov, D.B., 2002. Structural and functional characterization of the Pseudomonas hydroperoxide resistance protein Ohr. EMBO J. 21, 6649–6659.

Li, H., Hanson, C., Fuchs, J.A., Woodward, C., Thomas Jr., G.J., 1993. Determination of the pK_a values of active-center cysteines, cysteines-32 and -35, in *Escherichia coli* thioredoxin by Raman spectroscopy. Biochemistry 32, 5800–5808.

Link, A.J., Robison, K., Church, G.M., 1997. Comparing the predicted and observed properties of proteins encoded in the genome of *Escherichia coli* K-12. Electrophoresis 18, 1259–1313.

Lundstrom, J., Holmgren, A., 1993. Determination of the reduction-oxidation potential of the thioredoxin-like domains of protein disulfide–isomerase from the equilibrium with glutathione and thioredoxin. Biochemistry 32, 6649–6655.

Meunier-Jamin, C., Kapp, U., Leonard, G.A., McSweeney, S., 2004. The structure of organic hydroperoxide resistance protein from *Deinococcus radiodurans*: do conformational changes facilitate recycling of the redox disulphide? J. Biol. Chem. 279, 25830–25837.

Mieyal, J.J., Starke, D.W., Gravina, S.A., Hocevar, B.A., 1991. Thioltransferase in human red blood cells: kinetics and equilibrium. Biochemistry 30, 8883–8891.

Miranda-Vizuete, A., Damdimopoulos, A.E., Gustafsson, J., Spyrou, G., 1997. Cloning, expression, and characterization of a novel *Escherichia coli* thioredoxin. J. Biol. Chem. 272, 30841–30847.

Molina, M.M., Belli, G., Torre, M.A., Rodriguez-Manzaneque, M.T., Herrero, E., 2004. Nuclear monothiol glutaredoxins of *Saccharomyces cerevisiae* can function as mitochondrial glutaredoxins. J. Biol. Chem. 279, 51923–51930.

Mongkolsuk, S., Helmann, J.D., 2002. Regulation of inducible peroxide stress responses. Mol. Microbiol. 45, 9–15.

Mongkolsuk, S., Praituan, W., Loprasert, S., Fuangthong, M., Chamnongpol, S., 1998. Identification and characterization of a new organic hydroperoxide resistance (ohr) gene with a novel pattern of oxidative stress regulation from Xanthomonas campestris pv. phaseoli. J Bacteriol. 180, 2636–2643.

Monteiro, G., Netto, L.E.S., 2004. Glucose repression of PRX1 expression is mediated by Tor1p and Ras2p through inhibition of Msn2/4p in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 241, 221–228.

Monteiro, G., Pereira, G.A.G., Netto, L.E.S., 2002. Regulation of mitochondrial thioredoxin peroxidase I expression by two different pathways: one dependent on cAMP and the other on heme. Free Radic. Biol. Med. 32, 278–288.

Monteiro, G., Kowaltowski, A.J., Barros, M.H., Netto, L.E.S., 2004. Glutathione and thioredoxin peroxidases mediate susceptibility of yeast mitochondria to Ca(2+)-induced damage. Arch. Biochem. Biophys. 425, 14–24.

Moon, J.C., Hah, Y.S., Kim, W.Y., Jung, B.G., Jang, H.H., Lee, J.R., Kim, S.Y., Lee, Y.M., Jeon, M.G., Kim, C.W., Cho, M.J., Lee, S.Y., 2005. Oxidative stress-dependent structural and functional switching of a human 2-Cys peroxiredoxin isotype II that enhances HeLa cell resistance to H2O2-induced cell death. J. Biol. Chem. 280, 28775–28784.

Moore, R.B., Mankad, M.V., Shriver, S.K., Mankad, V.N., Plishker, G.A., 1991. Reconstitution of Ca(2+)-dependent K+ transport in erythrocyte membrane vesicles requires a cytoplasmatic protein. J. Biol. Chem. 266, 18964–18968.

Motohashi, H., Yamamoto, M., 2004. Nrf2-Keap1 defines a physiologically important stress response mechanism. Trends Mol. Med. 10, 549–557.

Munhoz, D.C., Netto, L.E.S., 2004. Cytosolic thioredoxin peroxidase I and II are important defenses of yeast against organic hydroperoxide insult: catalases and peroxiredoxins cooperate in the decomposition of H_2O_2 by yeast. J. Biol. Chem. 279, 35219–35227.

Nelson, J.W., Creighton, T.E., 1994. Reactivity and ionization of the active site cysteine residues of DsbA, a protein required for disulfide bond formation in vivo. Biochemistry 33, 5974–5983.

Netto, L.E.S., Chae, H.Z., Kang, S.W., Rhee, S.G., Stadtman, E.R., 1996. Removal of hydrogen peroxide by thiol-specific antioxidant enzyme (TSA) is involved with its antioxidant properties. TSA possesses thiol peroxidase activity. J. Biol. Chem. 271, 15315–15321.

Nishinaka, Y., Masutani, H., Nakamura, H., Yodoi, J., 2001. Regulatory roles of thioredoxin in oxidative stress-induced cellular responses. Redox Rep. 6, 289–295.

Oliveira, M.A., Discola, K.F., Alves, S.V., Barbosa, J.A.R.G., Medrano, F.J., Netto, L.E.S., Guimarães, B.G., 2005. Crystallization and preliminary X-ray diffraction analysis of NADPH-dependent thioredoxin reductase from *Saccharomyces cerevisiae*. Acta Crystallogr., Sect. F Struct. Biol. Cryst. Commun. 61, 387–390.

Oliveira, M.A., Guimarães, B.G., Cussiol, J.R.R., Medrano, F.J., Gozzo, F.C., Netto, L.E.S., 2006. Strutural insights into enzyme-substrate interaction and characterization of enzymatic intermediates of organic hydroperoxide resistence protein from *Xylella fastidiosa*. J. Mol. Biol. 359, 433–445.

Paget, M.S., Buttner, M.J., 2003. Thiol-based regulatory switches. Annu. Rev. Genet. 37, 91–121.

Park, S.G., Cha, M.K., Jeong, W., Kim, I.H., 2000. Distinct physiological functions of thiol peroxidase isoenzymes in *Saccharomyces cerevisiae*. J. Biol. Chem. 275, 5723–5732.

Parsonage, D., Youngblood, D.S., Sarma, G.N., Wood, Z.A., Karplus, P.A., Poole, L.B., 2005. Analysis of the link between enzymatic activity and oligomeric state in AhpC, a bacterial peroxiredoxin. Biochemistry 44, 10583–10592.

Pedrajas, J.R., Miranda-Vizuete, A., Javanmardy, N., Gustfsson, J.A., Spyrou, G., 2000. Mitochondrial of *Saccharomyces cerevisiae* contain one-conserved cysteine type peroxiredoxin with thioredoxin peroxidase activity. J. Biol. Chem. 26, 16296–16301.

Powis, G., Montfort, W.R., 2001. Properties and biological activities of thioredoxins. Annu. Rev. Biophys. Biomol. Struct. 30, 421–455.

Ralat, L.A., Manevich, Y., Fisher, A.B., Colman, R.F., 2006. Direct evidence for the formation of a complex between 1-cysteine peroxiredoxin and glutathione S-transferase pi with activity changes in both enzymes. Biochemistry 45, 360–372.

Reckenfelderbaumer, N., Krauth-Siegel, R.L., 2002. Catalytic properties, thiol pK value, and redox potential of *Trypanosoma brucei* tryparedoxin. J. Biol. Chem. 277, 17548–17555.

Reutimann, H., Straub, B., Luisi, P.L., Holmgren, A., 1981. A conformational study of thioredoxin and its tryptic fragments. J. Biol. Chem. 256, 6796–6803.

Rhee, S.G., Chae, H.Z., Kim, K., 2005a. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free Radic. Biol. Med. 38, 1543–1552.

Rhee, S.G., Kang, S.W., Jeong, W., Chang, T.S., Yang, K.S., Woo, H.A., 2005b. Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. Curr. Opin. Cell Biol. 17, 183–189.

Ritz, D., Lim, J., Reynolds, C.M., Poole, L.B., Beckwith, J., 2001. Conversion of a peroxiredoxin into a disulfide reductase by a triplet repeat expansion. Science 294, 158–160.

Salmeen, A., Andersen, J.N., Myers, M.P., Meng, T.C., Hinks, J.A., Tonks, N.K., Barford, D., 2003. Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. Nature 423, 769–773.

Stone, J.R., 2004. An assessment of proposed mechanisms for sensing hydrogen peroxide in mammalian systems. Arch. Biochem. Biophys. 422, 119–124.

Sun, Q.A., Kirnarsky, L., Sherman, S., Gladyshev, V.N., 2001. Selenoprotein oxidoreductase with specificity for thioredoxin and glutathione systems. Proc. Natl. Acad. Sci. U. S. A. 98, 3673–3678.

Thomas, J.A., Poland, B., Honzatko, R., 1995. Protein sulfhydryls and their role in the antioxidant function of protein *S*-thiolation. Arch. Biochem. Biophys. 319, 1–9.

Trivelli, X., Krimm, I., Ebel, C., Verdoucq, L., Prouzet-Mauleon, V., Chartier, Y., Tsan, P., Lauquin, G., Meyer, Y., Lancelin, J.M., 2003. Characterization of yeast peroxiredoxin ahp1 in its reduced and overoxidized inactive forms using NMR. Biochemistry 42, 14139–14149.

Trujillo, M., Budde, H., Pineyro, M.D., Stehr, M., Robello, C., Flohe, L., Radi, R., 2004. Trypanosoma brucei and *Trypanosoma cruzi* tryparedoxin peroxidases catalytically detoxify peroxynitrite via oxidation of fast reacting thiols. J. Biol. Chem. 279, 34175–34182.

Van Montfort, R.L., Congreve, M., Tisi, D., Carr, R., Jhoti, H., 2003. Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. Nature 423, 773–777.

Venugopal, R., Jaiswal, A.K., 1996. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc. Natl. Acad. Sci. U. S. A. 93, 14960–14965.

Vohnik, S., Hanson, C., Tuma, R., Fuchs, J.A., Woodward, C., Thomas Jr., G.J., 1998. Conformation, stability, and active-site cysteine titrations of *Escherichia coli* D26A thioredoxin probed by Raman spectroscopy. Protein Sci. 7, 193–200.

Weissbach, H., Etienne, F., Hoshi, T., Heinemann, S.H., Lowther, W.T., Matthews, B., St John, G., Nathan, C., Brot, N., 2002. Peptide methionine sulfoxide reductase: structure, mechanism of action, and biological function. Arch. Biochem. Biophys. 397, 172–178.

Wheeler, G.L., Grant, C.M., 2004. Regulation of redox homeostasis in the yeast *Saccharomyces cerevisiae*. Physiol. Plant. 120, 12–20.

Winter, J., Jakob, U., 2004. Beyond transcription—new mechanisms for the regulation of molecular chaperones. Crit. Rev. Biochem Mol Biol. 39, 297–317.

Winter, J., Linke, K., Jatzek, A., Jakob, U., 2005. Severe oxidative stress causes inactivation of DnaK and activation of the redox-regulated chaperone Hsp33. Mol. Cell 17, 381–392.

Wong, C.M., Zhou, Y., Ng, R.W., Kung, H.F., Jin, D.Y., 2002. Cooperation of yeast peroxiredoxins Tsa1p and Tsa2p in the cellular defense against oxidative and nitrosative stress. J. Biol. Chem. 277, 5385–5394.

Woo, H.A., Chae, H.Z., Hwang, S.C., Yang, K.S., Kang, S.W., Kim, K., Rhee, S.G., 2003. Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. Science 300, 653–656.

Woo, H.A., Jeong, W., Chang, T.-S., Park, K.J., Park, S.J., Yang, J.S., Rhee, S.G., 2005. Reduction of cysteine sulfinicacid by sulfiredoxin is specific to 2-Cys peroxiredoxins. J. Biol. Chem. 280, 325–328.

Wood, Z.A., Poole, L.B., Karplus, P.A., 2003a. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science 300, 650–653.

Wood, Z.A., Schroder, E., Harris, J.R., Poole, L.B., 2003b. Structure, mechanism and regulation of peroxiredoxins. Trends Biochem. Sci. 28, 32–40.

Xiao, R., Lundstrom-Ljung, J., Holmgren, A., Gilbert, H.F., 2005. Catalysis of thiol/disulfide exchange. Glutaredoxin 1 and protein-disulfide isomerase use different mechanisms to enhance oxidase and reductase activities. J. Biol. Chem. 280, 21099–21106.

Yang, Y.F., Wells, W.W., 1991. Identification and characterization of the functional amino acids at the active center of pig liver thioltransferase by site-directed mutagenesis. J. Biol. Chem. 266, 12759–12765.

Anexo 5

Acta Crystallographica Section F
Structural Biology and Crystallization Communications
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Crystallization and preliminary X-ray crystallographic studies of glutaredoxin 2 from *Saccharomyces cerevisiae* in different oxidation states

Karen Fulan Discola,[a] Marcos Antonio de Oliveira,[a] Gustavo Monteiro Silva,[a] José Antonio Barcena,[b] Pablo Porras,[b] Alicia Padilla,[b] Luis Eduardo Soares Netto[a] and Beatriz Gomes Guimarães[c]*

[a]Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, São Paulo, SP, Brazil, [b]Departamento de Bioquímica y Biología Molecular, Campus de Rabanales, Edificio 'Severo Ochoa', Universidad de Córdoba, 14071 Córdoba, Spain, and [c]Laboratório Nacional de Luz Síncrotron, CP 6192, CEP 13084-971, Campinas, SP, Brazil

Correspondence e-mail: beatriz@lnls.br

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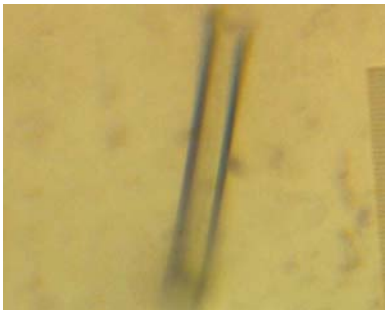

Glutaredoxins are small (9–12 kDa) heat-stable proteins that are highly conserved throughout evolution; the glutaredoxin active site (Cys-Pro-Tyr-Cys) is conserved in most species. Five glutaredoxin genes have been identified in *Saccharomyces cerevisiae*; however, Grx2 is responsible for the majority of oxidoreductase activity in the cell, suggesting that its primary function may be the detoxification of mixed disulfides generated by reactive oxygen species (ROS). Recombinant Grx2 was expressed in *Escherichia coli* as a 6×His-tagged fusion protein and purified by nickel-affinity chromatography. Prior to crystallization trials, the enzyme was submitted to various treatments with reducing agents and peroxides. Crystals suitable for X-ray diffraction experiments were obtained from untreated protein and protein oxidized with *t*-butyl hydroperoxide (10 m*M*). Complete data sets were collected to resolutions 2.15 and 2.05 Å for untreated and oxidized Grx2, respectively, using a synchrotron-radiation source. The crystals belong to space group $P4_12_12$, with similar unit-cell parameters.

1. Introduction

Glutaredoxins (Grx) are small (9–12 kDa) heat-stable proteins with at least one cysteine in their active site that are highly conserved throughout evolution (reviewed by Holmgren, 1989). The ubiquitous distribution of glutaredoxins is probably related to the fact that these proteins are involved in many cellular processes, including protein folding, regulation of protein activity, reduction of dehydroascorbate, repair of oxidatively damaged proteins and sulfur metabolism (Holmgren, 1989; Rietsch & Beckwith, 1998).

There are five glutaredoxin genes (*GRX1–5*) in the yeast *Saccharomyces cerevisiae*. The Grx1 and Grx2 isoforms are dithiol proteins with a Cys-Pro-Tyr-Cys motif in their active site, whereas Grx3, Grx4 and Grx5 are monothiol proteins that contain Cys-Gly-Phe-Ser in their active site (Rodríguez-Manzaneque *et al.*, 1999; Bellí *et al.*, 2002). The importance of glutaredoxin is related to the fact that these proteins possess the ability to reduce disulfide bonds (Holmgren, 1989). Changes in the thiol–disulfide redox status of proteins are important not only for the reactivation of enzymes and for protein folding and stability, but also for the control of protein function (Ritz & Beckwith, 2001). *In vitro* studies have shown that both Grx1 and Grx2 can reduce mixed disulfides. However, despite the homology between Grx2 and Grx1 (64% identity and 85% similarity), Grx2 is responsible for the majority of oxidoreductase activity in the cell (Luikenhuis *et al.*, 1998; Grant, 2001). Grx2 performs the reversible disulfide-bond exchange reaction between its active site and the active site of the substrate protein *via* a monothiol mechanism, initiated by the nucleophilic N-terminal cysteine residue (Luikenhuis *et al.*, 1998; Collinson & Grant, 2003; Ritz & Beckwith, 2001).

It was recently proposed that Grx2 also possesses peroxidase and glutathione *S*-transferase activities (Collinson *et al.*, 2002; Collinson & Grant, 2003). Accordingly, the resistance of yeast to oxidative stress induced by hydroperoxides is affected by changes in the levels of glutaredoxins among other factors (Collinson *et al.*, 2002). Yeast strains with deletions in either the *GRX1* or *GRX2* genes are sensitive to hydroperoxides; however, the *GRX2* null mutant has higher hydrogen peroxide sensitivity, showing that Grx2 is probably the

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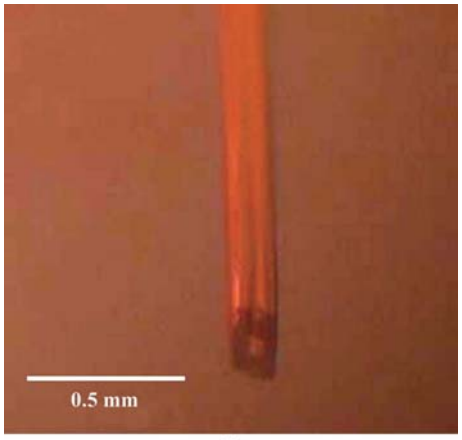
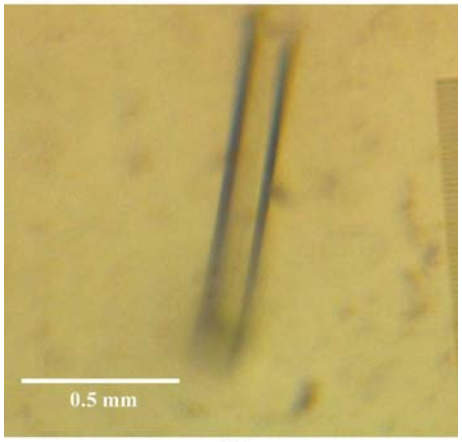
main glutaredoxin involved in the detoxification of this oxidant in the cell (Collinson *et al.*, 2002; Wheeler & Grant, 2004). Both dithiolic glutaredoxins are capable of reducing hydroperoxides directly in a catalytic manner: their active sites act as an electron-transfer site and undergo a reversible oxidation of two vicinal protein thiol groups to a disulfide bridge (Collinson *et al.*, 2002). The resulting oxidized glutaredoxin is reduced by two GSH molecules and the resulting GSSG is reduced back by NADPH in a reaction catalyzed by glutathione reductase (Jordan *et al.*, 1997). Preliminary results from our group have indicated that Grx2 is also involved in another process: the deglutathionylation of the 20S proteasome. The converse process of deglutathionylation, glutathionylation, occurs avoiding the irreversible oxidation of the proteasome (Demasi *et al.*, 2003).

Recently, two different isoforms of Grx2 have been identified, one with a molecular weight of 15.9 kDa and the other of 11.9 kDa. Both isoforms are localized in the mitochondria, but the shorter isoform is also detected in the cytosolic fraction (Pedrajas *et al.*, 2002). The multitude of Grx2 functions can probably be related to the fact that this protein possesses these two isoforms.

At present, three-dimensional structures of glutaredoxins from only four sources have been determined: *Escherichia coli* Grx1 (Sodano *et al.*, 1991), Grx2 (Xia *et al.*, 2001) and Grx3 (Nordstrand *et al.*, 1999), bacteriophage T4 Grx (Eklund *et al.*, 1992), *Sus scrofa* Grx (Katti *et al.*, 1995) and *Homo sapiens* Grx (Sun *et al.*, 1998). These proteins share the same thioredoxin overall fold, despite variations in amino-acid sequences (Stefankova *et al.*, 2005). The glutaredoxin 2 from *E. coli*, the only Grx2 of known three-dimensional structure, is an atypical glutaredoxin with a molecular weight of 24 kDa, compared with the typical molecular weight of 9–12 kDa for other known glutaredoxins (Xia *et al.*, 2001). In this work, we present preliminary X-ray crystal analysis aiming towards the three-dimensional structure determination of the shorter *S. cerevisiae* Grx2 isoform, which may be helpful in comprehension of the molecular mechanisms by which Grx2 operates.



(*a*)



(*b*)

Figure 1
Crystals of Grx2 from *S. cerevisiae*. (*a*) Untreated protein, (*b*) oxidized protein. Both Grx2 crystals were grown using 30% PEG 4000, 0.1 *M* sodium acetate pH 4.6 and 0.2 *M* ammonium acetate.

2. Methods

2.1. Cloning

The 432 bp *GRX2* gene was PCR amplified from *S. cerevisiae* genomic DNA of strain W303 using primers which, in addition to the open reading frame, contained cloning adaptors (*Nde*I and *Bam*HI restriction sites) to favour the gene transfer to expression plasmids. The PCR product and the expression vector pET15b were first digested with *Nde*I and then with *Bam*HI. The fragments generated by *Nde*I/*Bam*HI digestion containing the *GRX2* gene and pET15b were extracted from agarose gel by the Rapid Gel Extraction Concert kit (Invitrogen). After purification, the *GRX2* gene was ligated to the digested pET15b expression vector. The cloned gene sequence was confirmed by automated DNA sequencing and the resulting pET15b/*GRX2* was used to transform *E. coli* BL21 (DE3).

2.2. Expression and purification

E. coli BL21 (DE3) strain harbouring the pET15b/*GRX2* plasmid was grown (50 ml) overnight in LB medium containing 100 µg ml^{-1} ampicillin at 310 K and transferred to 1 l fresh LB/amp medium and cultured further at 310 K until the OD_{600} reached 0.6–0.8. Expression was induced with 0.5 m*M* isopropyl-β-D-thiogalactopyranoside and the cells were harvested after 4 h incubation at 310 K.

After cell lysis, the protein was purified by cobalt-affinity chromatography with an imidazole gradient (Talon metal-affinity resin from Clontech). We have obtained approximately 50 mg pure protein from 1 l cell culture. Protein purity was confirmed by SDS–PAGE and the purified protein was concentrated to 10 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5.

2.3. Crystallization

Crystallization trials were executed using the hanging-drop vapour-diffusion method. Prior to crystallization, enzyme samples were submitted to treatment for 1 h at 310 K with hydrogen peroxide, *t*-butyl hydroperoxide, diamide and DTT. Initial screening was performed at 293 K using the commercially available Crystal Screen and Crystal Screen II kits from Hampton Research. The drops were prepared by mixing equal volumes (2 µl) of protein solution and reservoir solution. Promising crystals were identified in four conditions of the Crystal Screen kit: condition Nos. 10, 28, 32 and 47. The conditions were optimized by variation of precipitant or salt concentrations and pH. Crystals suitable for X-ray diffraction experiments were obtained from the untreated protein and from protein after oxidation with 10 m*M* *t*-butyl hydroperoxide. In both cases, the optimal condition was obtained with a reservoir solution consisting of 30% PEG 4000, 0.1 *M* sodium acetate pH 4.6 and 0.2 *M* ammonium acetate. The crystals grown from the untreated sample reached dimensions of ~0.10 × 0.15 × 0.9 mm after one week (Fig. 1*a*), while crystals grown from the oxidized protein reached ~0.10 × 0.15 × 0.8 mm in about the same time (Fig. 1*b*).

Table 1
Data-collection parameters and crystallographic data statistics.

Values in parentheses refer to the highest resolution shell.

	Untreated protein	Oxidized protein
Wavelength (Å)	1.431	1.431
Temperature (K)	110	110
Space group	$P4_12_12$	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 47.66$, $c = 95.00$	$a = b = 47.63$, $c = 94.59$
Resolution range (Å)	42.60–2.15 (2.27–2.15)	42.56–2.05 (2.16–2.05)
Total reflections	68434	90136
Unique reflections	6439	7295
Completeness (%)	100.0 (100.0)	99.8 (99.3)
Multiplicity	10.6 (10.7)	12.3 (11.2)
$R_{\rm sym}$† (%)	10.3 (31.2)	8.0 (29.4)
Average $I/\sigma(I)$	23.5 (7.3)	28.9 (7.8)

† $R_{\rm sym} = \sum_{hkl}\sum_i |I_{hkl,i} - \langle I\rangle_{hkl}|/\sum|\langle I\rangle_{hkl}|$.

2.4. Data collection and processing

The crystals, cryoprotected by a solution consisting of 30% PEG 4000, 0.1 *M* sodium acetate pH 4.6, 0.2 *M* ammonium acetate and 20% glycerol, were cooled to 110 K in a nitrogen-gas stream and X-ray diffraction data were collected using synchrotron radiation at the protein crystallography beamline D03B of the Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil. LNLS D03B is a monochromatic beamline with maximum photon flux between 1.3 and 1.6 Å. The wavelength of the incident X-ray was set to 1.431 Å. A MAR CCD detector was used to record the oscillation data with $\Delta\varphi = 1^\circ$. Data sets were processed using the programs *MOSFLM* (Leslie, 1992) and *SCALA* (Evans, 1993) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Glutaredoxin 2 from *S. cerevisiae* was submitted to crystallization trials after treatment with hydrogen peroxide, *t*-butyl hydroperoxide, diamide and DTT. Microcrystals were obtained from all samples, but to date crystals suitable for X-ray diffraction experiments only grew from the untreated protein and the sample after oxidation with 10 m*M* *t*-butyl hydroperoxide.

A crystal of untreated Grx2 diffracted to 2.15 Å resolution and belonged to space group $P4_12_12$, with unit-cell parameters $a = b = 47.66$, $c = 95.00$ Å. The oxidized Grx2 crystal diffracted to 2.05 Å resolution and also belongs to space group $P4_12_12$, with similar unit-cell parameters. Table 1 summarizes the data-collection statistics. In order to estimate the number of molecules in the asymmetric unit, the Matthews coefficient $V_{\rm M}$ was calculated (Matthews, 1968). Both crystals presented one molecule per asymmetric unit with $V_{\rm M} = 2.3$ Å^3 Da^{-1} and solvent contents of 45.8 and 45.0% for the native and oxidized protein, respectively.

Application of the molecular-replacement method using the program *AMoRe* (Navaza, 2001) indicated a probable solution when a polyalanine theoretical model constructed with the program *MODELLER* (Claude *et al.*, 2004) was used as the search model. The theoretical model was built based on the coordinates of a thiol-transferase from *S. scrofa* (Katti *et al.*, 1995; PDB code 1kte; 36% sequence identity). Previous attempts to solve the structure using the 1kte coordinates as the search model for molecular replacement had failed. Currently, structure refinement is in progress.

We expect that knowledge of the three-dimensional structure of Grx2 in different redox states will contribute to the understanding of the catalytic mechanism of the enzyme, which is involved in several biological processes.

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References

Bellí, G., Polaina, J., Tamarit, J., Torre, M. A., Rodríguez-Manzaneque, M. T., Ros, J. & Herrero, E. (2002). *J. Biol. Chem.* **277**, 37590–37596.
Claude, J. B., Suhre, K., Notredame, C., Claverie, J. M. & Abergel, C. (2004). *Nucleic Acid Res.* **32**, W606–W609.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Collinson, E. J. & Grant, C. M. (2003). *J. Biol. Chem.* **278**, 22492–22497.
Collinson, E. J., Wheeler, G. L., Garrido, E. O., Avery, A. M., Avery, S. V. & Grant, C. M. (2002). *J. Biol. Chem.* **277**, 16712–16717.
Demasi, M., Silva, G. M. & Netto, L. E. S. (2003). *J. Biol. Chem.* **278**, 679–685.
Eklund, H., Ingelman, M., Söderberg, B. O., Uhlin, T., Nordlund, P., Nikkola, M., Sonnerstam, U., Joelson, T. & Petratos, K. (1992). *J. Mol. Biol.* **228**, 596–618.
Evans, P. R. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by N. Isaacs, L. Sawyer & S. Bailey, pp. 114–122. Warrington: Daresbury Laboratory.
Grant, C. M. (2001). *Mol. Microbiol.* **39**, 533–541.
Holmgren, A. (1989). *J. Biol. Chem.* **264**, 13963–13966.
Jordan, A., Aslund, F., Pontis, E., Reichard, P. & Holmgren, A. (1997). *J. Biol. Chem.* **272**, 18044–18050.
Katti, S. K., Robbins, A. H., Yang, Y. & Wells, W. W. (1995). *Protein Sci.* **4**, 1998–2005.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.
Luikenhuis, S., Perrone, G., Dawes, I. W. & Grant, C. M. (1998). *Mol. Biol. Cell*, **9**, 1081–1091.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Navaza, J. (2001). *Acta Cryst.* D**57**, 1367–1372.
Nordstrand, K., Aslund, F., Holmgren, A., Otting, G. & Berndt, K. D. (1999). *J. Mol. Biol.* **286**, 541–552.
Pedrajas, J. R., Porras, P., Martinez-Galisteo, E., Padilla, C. A., Miranda-Vizuete, A. & Bárcena, J. A. (2002). *Biochem. J.* **364**, 617–623.
Rietsch, A. & Beckwith, J. (1998). *Annu. Rev. Genet.* **32**, 163–184.
Ritz, D. & Beckwith, J. (2001). *Annu. Rev. Microbiol.* **55**, 21–48.
Rodríguez-Manzaneque, M. T., Ros, J., Cabiscol, E., Sorribas, A. & Herrero, E. (1999). *Mol. Cell. Biol.* **19**, 8180–8190.
Sodano, P., Xia, T. H., Bushweller, J. H., Bjornberg, O., Holmgren, A., Billeter, M. & Wulthrich, K. (1991). *J. Mol. Biol.* **221**, 1311–1324.
Stefankova, P., Maderova, J., Barak, I., Kollarova, M. & Otwinowski, Z. (2005). *Acta Cryst.* F**61**, 164–168.
Sun, C., Berardi, M. J. & Bushweller, J. H. (1998). *J. Mol. Biol.* **280**, 687–701.
Wheeler, G. L. & Grant, C. M. (2004). *Physiol. Plant.* **120**, 12–20.
Xia, B., Vlamis-Gardikas, A., Holmgren, A., Wright, P. E. & Dyson, H. J. (2001). *J. Mol. Biol.* **310**, 907–918.

Anexo 6

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Crystallization and preliminary X-ray diffraction analysis of NADPH-dependent thioredoxin reductase I from *Saccharomyces cerevisiae*

Marcos Antonio de Oliveira,[a] Karen Fulan Discola,[a] Simone Vidigal Alves,[a] João Alexandre Ribeiro Gonçalves Barbosa,[b] Francisco Javier Medrano,[b] Luis Eduardo Soares Netto[a] and Beatriz Gomes Guimarães[b]*

[a]Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, São Paulo SP, Brazil, and [b]Laboratório Nacional de Luz Síncrotron, CP 6192, CEP 13084-971, Campinas SP, Brazil

Correspondence e-mail: beatriz@lnls.br

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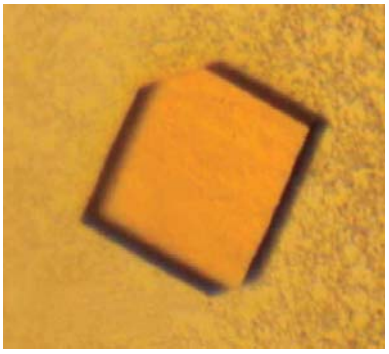

Thioredoxin reductase 1 (Trr1) from *Saccharomyces cerevisiae* is a member of the family of pyridine nucleotide-disulfide oxidoreductases capable of reducing the redox-active disulfide bond of the cytosolic thioredoxin 1 (Trx1) and thioredoxin 2 (Trx2). NADPH, Trr1 and Trx1 (or Trx2) comprise the thioredoxin system, which is involved in several biological processes, including the reduction of disulfide bonds and response to oxidative stress. Recombinant Trr1 was expressed in *Escherichia coli* as a His_6-tagged fusion protein and purified by nickel-affinity chromatography. The protein was crystallized using the hanging-drop vapour-diffusion method in the presence of PEG 3000 as precipitant after treatment with hydrogen peroxide. X-ray diffraction data were collected to a maximum resolution of 2.4 Å using a synchrotron-radiation source. The crystal belongs to the centred monoclinic space group $C2$, with unit-cell parameters $a = 127.97$, $b = 135.41$, $c = 75.81$ Å, $\beta = 89.95^\circ$. The crystal structure was solved by molecular-replacement methods and structure refinement is in progress.

1. Introduction

In the cytoplasm of the majority of cell types, proteins are in a reduced state, in contrast to extracellular proteins where disulfide bonds are commonly found and help to maintain their structure in a very harsh environment. Thioredoxins and glutaredoxins are heat-stable proteins capable of reducing disulfide bonds in target proteins. These oxidoreductases contain two vicinal cysteines at their active sites that after reduction of the target disulfide become oxidized to a disulfide bond. The thioredoxin disulfide is reduced by nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction catalyzed by thioredoxin reductase (Ritz & Beckwith, 2001).

Thioredoxin reductases are important flavoenzymes that belong to a family of proteins that includes lipoamide dehydrogenase and glutathione reductase. These proteins play a critical role in maintaining the redox status of cytoplasm. All these flavoenzymes contain two redox centres: a flavin adenine dinucleotide (FAD) and a dithiol/disulfide group (reviewed by Williams *et al.*, 2000).

Because the reduction of disulfide bonds is a biochemical event involved in several processes, the thioredoxin system (comprised of NADPH, thioredoxin reductase and thioredoxin) is implicated in several phenomena, such as synthesis of deoxyribonucleotides, activation of transcription factors, regulation of the cell cycle, reduction of methionine sulfoxide, assimilation of sulfur and photosynthesis (Mustacich & Powis, 2000; Williams *et al.*, 2000) (Fig. 1).

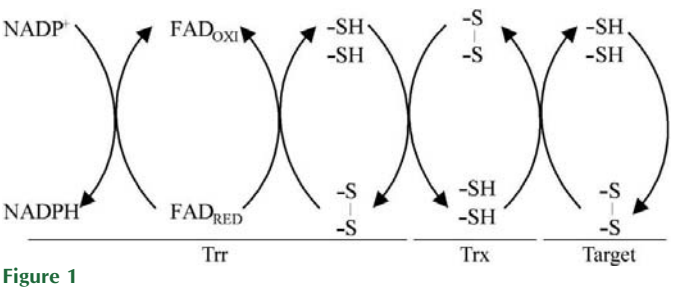

Figure 1
Electron transfer between Trr, Trx and protein targets. Reducing equivalents from NADPH are transferred to the Trr FAD, then to enzyme disulfide. These equivalents are transferred to oxidized Trx, which finally reduces the target proteins.

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Another important aspect of the thioredoxin system is its capacity to regenerate the reduced form of thiol-dependent peroxidases belonging to the peroxiredoxin family. Because of their enzymatic activity, these enzymes were named thioredoxin peroxidases (Chae *et al.*, 1994). Thioredoxin peroxidases are able to reduce H_2O_2 or organic peroxides to water and alcohols, respectively (Chae, Chung *et al.*, 1994; Chae, Robison *et al.*, 1994; Park *et al.*, 2000; Nordberg & Arner, 2001; Yoshida *et al.*, 2003). This antioxidant system is conserved from prokaryotes to mammals and plants. In eukaryotes there are several counterparts in the cytosol and in organelles such as chloroplasts, mitochondria and even in the nucleus (Waksman *et al.*, 1994; Dai *et al.*, 1996; Park *et al.*, 2000; Hofmann *et al.*, 2002). The fact that the *Saccharomyces cerevisiae trr1* null mutant is not viable (Giaever *et al.*, 2002) probably reflects the fact that thioredoxin reductase 1 is implicated in several processes.

Two types of thioredoxin reductases appeared during evolution (Mustacich & Powis, 2000; Williams *et al.*, 2000). Both are homodimeric proteins that catalyze the transfer of electrons through their FAD and a redox-active disulfide. In prokaryotes, lower eukaryotes and plants, each thioredoxin reductase subunit has a molecular weight of about 35 kDa. Thioredoxin reductases from higher eukaryotes possess a molecular weight of about 55 kDa and the active site contains a selenocysteine (Luthman & Holmgren, 1982; Buettner *et al.*, 1999; Zhong & Holmgren, 2000).

Crystallographic studies have revealed that the location of the NADPH-binding and FAD-binding domains differ significantly in these two enzyme types. In higher eukaryotes, the distance between these domains and their orientation allows electron transport from NADPH to the disulfide of Trx without the need for a large alteration in protein conformation (Sandalova *et al.*, 2001). On the other hand, Waksman *et al.* (1994) showed that in *Escherichia coli* Trr, the NADPH-binding and FAD-binding domains are located on distinct sides of the molecule, with the nicotinamide ring at a distance of more than 17 Å from the flavin ring. Moreover, the reactive cysteines of thioredoxin reductase are inaccessible for interaction with thioredoxin.

Lennon *et al.* (2000) explained the catalytic mechanism of this enzyme by describing the structure of a complex formed by *E. coli* Trr, Trx and $AADP^+$ (3-aminopyridine dinucleotide phosphate), which is an analogue of NADPH. These authors showed that a large conformational change takes place to permit electron transport. The complex possesses a disulfide connecting Cys32 of Trx to Cys138 of Trr. In order to stabilize the complex, Cys35 of Trx and Cys135 of Trr were replaced by serines. Under these conditions, NADPH and FAD domains undergo a large rotation of 67° with respect to each other, which is necessary for electron transfer. This rotation places the nicotinamide ring of $AADP^+$ and the disulfide bond close to the flavin ring and exposes the cysteine residues to reaction with thioredoxin. This twist of the two domains permits FAD reduction by NADPH and oxidation of the enzyme dithiol by thioredoxin (Lennon *et al.*, 2000).

To date, *Arabidopsis thaliana* Trr (Dai *et al.*, 1996) and *E. coli* Trr are the only low-molecular-weight thioredoxin reductases to have had their three-dimensional structures reported. Here, we report the preliminary X-ray diffraction analysis of Trr1 from *S. cerevisiae* in an oxidized state that represents the first Trr structure described in yeasts. The yeast Trr1 shares a sequence identity of 50 and 63% with its counterparts in *E. coli* and *A. thaliana*, respectively.

The structure was solved by molecular-replacement methods using the atomic coordinates of *A. thaliana* Trr as the search model. Analysis of the Trr1 structure should provide insights into the evolution and enzymatic mechanism of this protein and may also provide reasons for the specificity of the protein towards cytosolic thioredoxins.

2. Methods

2.1. Cloning

The 960-base-pair *trr1* gene (YDR353W) was amplified by PCR from genomic DNA of *S. cerevisiae* (Invitrogen, catalogue No. 40802) and cloned in pPROEX-1 vector (Invitrogen) using *Bam*HI-*Nde*I restriction sites. The resulting pPROEX/*trr1* was sequenced in an Applied Biosystems ABI Prism 377 96 to confirm that the construction was correct.

2.2. Expression and purification

E. coli DH5α strain harbouring the pPROEX/*trr1* plasmid was grown (50 ml) overnight in Luria–Bertani (LB) medium containing 50 µg ml^{-1} ampicillin at 310 K and transferred to 1 l of fresh LB/amp medium and cultured further at 310 K until the OD_{600} reached 0.6–0.8. Expression was induced with 1 m*M* IPTG and the cells were harvested after 4 h incubation. The cell pellet was resuspended in the starting buffer (20 m*M* sodium phosphate pH 7.4). The cells were lysed by sonication and the cell extract was kept on ice during streptomycin sulfate (1%) treatment for 15 min. The suspension was centrifuged at 31 500*g* for 30 min at 277 K to remove nucleic acid precipitate. Finally, the resulting supernatant was applied onto a nickel-affinity column (Hi-Trap from GE Healthcare). Bound protein was eluted with a linear gradient of 0–0.5 *M* imidazole. Protein purity was confirmed by SDS–PAGE. The purified protein was concentrated to 10 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5 for crystallization trials.

2.3. Crystallization and data collection

After H_2O_2 treatment (1 m*M*) at 310 K for 1 h, the samples were used to perform crystallization experiments using the hanging-drop vapour-diffusion method. Initial screenings were performed at 293 K using Crystal Screen and Crystal Screen 2 from Hampton Research. The drops, containing equal volumes (2.0 µl) of protein solution (10 mg ml^{-1} in 5 m*M* Tris–HCl pH 7.5) and reservoir solution, were equilibrated against 0.3 ml reservoir solution. From the initial screenings, several conditions produced thin plate-shaped crystals. Promising crystals were identified in five conditions: condition Nos. 10, 20 and 37 from Crystal Screen and condition Nos. 12 and 47 from Crystal Screen 2. All initial hits contained low-pH buffers and PEG as precipitant. Crystals suitable for diffraction experiments were obtained by slight variation of these conditions, such as the temperature of the assays (293 or 277 K). An additive search was also performed using Additive Screens 1, 2 and 3 from Hampton Research.

The best crystals, cryoprotected with the reservoir solution supplemented with 25% glycerol, were cooled to 110 K in a nitrogen-gas stream and X-ray diffraction data were collected using synchrotron radiation at the protein crystallography beamline D03B of the Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil. LNLS D03B is a monochromatic beamline with a maximum photon flux between 1.3 and 1.6 Å. The wavelength of the incident X-ray was set to 1.431 Å and a MAR CCD detector was used to record the oscillation data with $\Delta\varphi = 1.0°$, covering a total oscillation range of 240°. The data set was processed using the program *MOSFLM* (Leslie, 1992) and the resulting intensities were scaled and merged using the program *SCALA* (Evans, 1993) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The optimal yeast Trr1 crystallization condition was obtained with a drop volume of 6.0 µl. 2.7 µl reservoir solution (sodium citrate pH 4.0, 12% PEG 3000) was mixed with an equal volume of protein solution and 0.6 µl 0.1 *M* trimethylamine hydrochloride was used as an additive. The best Trr1 crystals reached dimensions of 0.3 × 0.3 × 0.05 mm after four weeks (Fig. 2). Despite the presence of ice rings in the diffraction pattern (Fig. 3), data processing yielded a good quality data set to 2.4 Å resolution. A total of 297 954 measured reflections were merged into 48 181 unique reflections with an R_{sym} of 9.9%. The crystal belongs to the monoclinic space group *C*2, with unit-cell parameters a = 127.97, b = 135.41, c = 75.81 Å, β = 89.95°. Table 1 summarizes the data-collection statistics.

The protein structure was solved by molecular-replacement methods with the program *AMoRe* (Navaza, 2001) using the atomic coordinates of *A. thaliana* Trr (Dai *et al.*, 1996) as the search model

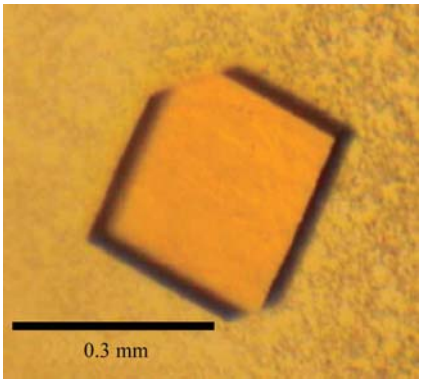

Figure 2
Trr1 crystal from *S. cerevisiae*. Crystals were obtained by vapour-diffusion equilibration against a reservoir consisting of sodium citrate pH 4.0, 12% PEG 3000 and 0.1 *M* trimethylamine hydrochloride.

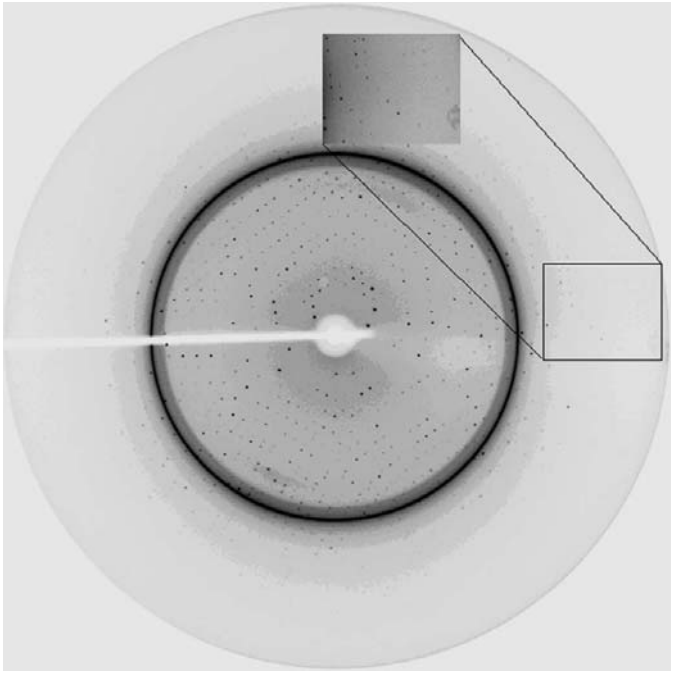

Figure 3
X-ray diffraction pattern obtained from a Trr1 crystal on beamline D03B at LNLS. The exposure time was 45 s and the oscillation range per frame was 1°. Despite the presence of ice rings, data processing yielded a good-quality data set to 2.4 Å resolution.

Table 1
Data-collection parameters and crystallographic data statistics.

Values in parentheses refer to the highest resolution shell.

Temperature (K)	110
Wavelength (Å)	1.431
Space group	*C*2
Unit-cell parameters (Å, °)	a = 127.97, b = 135.41, c = 75.81, β = 89.95
Resolution limits (Å)	46.6–2.4 (2.53–2.4)
Total No. of reflections	297954
No. of unique reflections	48181
Completeness (%)	99.6 (99.7)
Multiplicity	6.1 (6.0)
R_{sym} (%)	9.9 (32.5)
$\langle I/\sigma(I)\rangle$	17.6 (4.3)

(63% sequence identity; PDB code 1vdc). The molecular-replacement solution shows four monomers in the asymmetric unit, in agreement with the Matthews coefficient calculation (Matthews, 1968), the best result giving V_M = 2.3 Å^3 Da^{-1} and a solvent content of 45.5%. The molecular-replacement protocol resulted in an *R* factor and correlation factor of 51.0 and 33.3%, respectively, for the first solution and 53.1 and 27.7%, respectively, for the second solution. Initial rigid-body refinement was carried out using *AMoRe*, yielding an *R* factor of 0.459. Model completion and refinement are currently in progress.

Here, we report the X-ray diffraction data collection from yeast thioredoxin reductase crystals. Trr enzymes play important roles in cellular redox homeostasis and are present from archaea to mammals. Because the protein structure and mechanism of catalysis of Trr from higher eukaryotes differ substantially from those of its counterparts in plants and lower eukaryotes, an evolutionary divergence is suggested (Sandalova *et al.*, 2001; Waksman *et al.*, 1994). At present, only two Trr1 structures from lower organisms have been described: *E. coli* Trr and *A. thaliana* Trr (Waksman *et al.*, 1994; Dai *et al.*, 1996). The comparison of the yeast Trr1 structure with the bacterial and plant homologues should provide valuable information concerning the evolution of Trr and its enzymatic mechanism.

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References

Buettner, C., Harney, J. W. & Barry, M. J. (1999). *J. Biol. Chem.* **274**, 21598–21602.
Chae, H. Z., Chung, S. J. & Rhee, S. G. (1994). *J. Biol. Chem.* **269**, 27670–27678.
Chae, H. Z., Robison, K., Poole, L. B., Church, G., Storz, G. & Rhee, S. G. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 7017–7021.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Dai, S., Saarinen, M., Ramaswamy, S., Meyer, Y., Jacquot, J. P. & Eklund. H. (1996). *J. Mol. Biol.* **264**, 1044–1057.
Evans, P. R. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 114–122. Warrington: Daresbury Laboratory.
Giaever, G. *et al.* (2002). *Nature (London)*, **418**, 387–391.
Hofmann, B., Hecht, H.-J. & Flohe, L. (2002). *Biol. Chem.* **383**, 347–364.
Lennon, B. W., Williams, C. H. Jr & Ludwig, M. L. (2000). *Science*, **289**, 1190–1194.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACBM Newsl. Protein Crystallogr.* **26**.
Luthman, M. & Holmgren A. (1982). *Biochemistry*, **26**, 6628–6633.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Mustacich, D. & Powis, G. (2000). *Biochem. J.* **346**, 1–8.
Navaza, J. (2001). *Acta Cryst.* D**57**, 1367–1372.
Nordberg, J. & Arner, E. S. (2001). *Free Radic. Biol. Med.* **31**, 1287–1312.
Park, S. G., Cha, M. K., Jeong, W. & Kim, I. H. (2000). *J. Biol. Chem.* **275**, 5723–5732.
Ritz, D. & Beckwith, J. (2001). *Annu. Rev. Microbiol.* **55**, 21–48.
Sandalova, T., Zhong, L., Lindqvist, Y., Holmgren, A. & Schneider, G. (2001). *Proc. Natl Acad. Sci. USA*, **98**, 9533–9538.
Waksman, G., Krishna, T. S. R., Williams, C. H. Jr & Kuriyan, J. (1994). *J. Mol. Biol.* **236**, 800–816.
Williams, C. H., Arscott, L. D., Muller, S., Lennon, B. W., Ludwig, M. L., Wang, P. F., Veine, D. M., Becker, K. & Schirmer, R. H. (2000). *Eur. J. Biochem.* **267**, 6110–6117.
Yoshida, T., Oka, S., Masutani, H., Nakamura, H. & Yodoi, J. (2003). *Antioxid. Redox Signal.* **5**, 563–570.
Zhong, L. & Holmgren, A. (2000). *J. Mol. Biol.* **275**, 18121–18128.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/tese de Mestrado/Doutorado intitulada "Caracterização funcional e estrutural da glutarredoxinas ditiólicas de *Saccharomyces cerevisiae*":

( ) não se enquadra no § 3° do Artigo 1° da Informação CCPG 01/08, referente a bioética e biossegurança.

( ) está inserido no **Projeto CIBio/IB/UNICAMP** (Protocolo n°_____), intitulado _____;

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(**X**) tem autorização de comissão de bioética ou biossegurança externa à UNICAMP. Especificar: O trabalho não envolve estudos com animais ou seres humanos. O certificado de qualidade de biossegurança é do **Instituto de Biociências – USP (n° 0044-98)**, de 31 de março de 1998.

Karen Fulan Discola
Aluno: Karen Fulan Discola

Luis E.S. Netto
Orientador: Prof. Dr. Luis Eduardo Soares Netto

Para uso da Comissão ou Comitê pertinente:
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Informo a V.Sa. que a Comissão Interna de Biossegurança deste Instituto, reunida em 19/03 p.p., autorizou o desenvolvimento do projeto de pesquisa "Aspectos biológicos de tióis: estrutura protéica, defesa antioxidante, sinalização e estados redox" e os sub-projetos: "Peroxirredoxinas de *Saccharomyces cerevisiae*", "Sistemas glutarredoxina de *Saccharomyces cerevisiae*" e "Regulação redox de proteassomo", envolvendo organismos geneticamente modificados da classe de risco I, sob sua responsabilidade (Resolução Normativa da CTNBio nº 2).

Esclareço que deverá ser mencionada no relatório anual a situação de momento de cada projeto de pesquisa (concluído, andamento, novo), bem como o encaminhamento de uma cópia do resumo do respectivo projeto.

Atenciosamente,

MARIA APARECIDA VISCONTI
Presidente da Comissão Interna de Biossegurança

Ilmo. Sr.
Prof. Dr. LUIS EDUARDO SOARES NETTO
Responsável pelo Laboratório de Genética e Biologia Molecular de Oxidantes e Radicais Livres

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