GRAZIELA MILANI NAREZZI

Este exemplar corresponde à versão final da Tese de Doutorado apresentada ao Curso de Pós-Graduação Ciências Médicas da Faculdade de Ciências Médicas da UNICAMP, para obtenção do título de Doutor em Ciências Médicas, Área Ciências Biomédicas da aluna **Graziela Milani Narezzi.**

Campinas, 18 de fevereiro de 2002.

Prof. Dr. Anibal Eugenio Vercesi
Orientador

BIOENERGÉTICA MITOCONDRIAL E HOMEOSTASE INTRACELULAR DE CA^{2+} EM CANDIDA PARAPSILOSIS

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GRAZIELA MILANI NAREZZI

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Banca examinadora da tese de Doutorado

Orientador: Prof. Dr. Anibal Eugenio Vercesi

Membros:

1. **Prof. Dr. José Roberto M. Fernandes**
2. **Prof. Dr. Luiz Travassos**
3. **Profa. Dra. Fernanda R. Gadelha**
4. **Prof. Dr. Gonçalo A . G. Ferreira**
5. **Prof. Dr. Anibal E. Vercesi**

Curso de pós-graduação em Ciências Médicas da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

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PÁG.

RESUMO ..... *xii*

1. INTRODUÇÃO GERAL 15

1.1. Homeostase intracelular de cálcio 17

1.1.1. Transporte de cálcio pela mitocôndria 22

1.2. A cadeia respiratória mitocondrial 23

1.3. A cadeia respiratória mitocondrial em *C. Parapsilosis* 26

1.4. As proteínas desacopladoras – UCPs 27

1.4.1. Características estruturais da UCP 28

1.4.2. Mecanismos de desacoplamento 29

1.4.3. A proteína desacopladora de plantas 32

1.5. A oxidase alternativa – AOX 33

2. OBJETIVOS 34

3. RESULTADOS 36

3.1. **1° Trabalho** – Milani, G., Schreiber, A.Z and Vercesi, A.E. (2001) Characterization of Ca^{2+} uptake by an intracellular acidic compartment in Candida parapsilosis **FEBS Letters, 500: 80-84** 37

3.2. **2° Trabalho** – Jarmuszkiewicz, W.; Milani,G.; Fortes, F.; Schreiber, A.Z.; Sluse, F.E. and Vercesi, A.E. (2000) First evidence and characterization of an uncoupling protein in fungi kingdom: CpUCP of Candida parapsilosis. **FEBS Letters, 467: 145-149** 43

3.3. **3° Trabalho** – Milani, G., Jarmuszkiewicz, W., Schreiber, A.Z., Vercesi, A.E. and Sluse, F.E. (2001) Respiratory chain network of Candida parapsilosis: ADP/O ratio appraisal of the multiple electron pathways. **FEBS Letters, 508: 231-235**..... 49

4. CONCLUSÕES..... 55

4.1. Conclusões do 1° trabalho..... 56

4.2. Conclusões do 2° trabalho..... 57

4.3. Conclusões do 3° trabalho..... 59

5. SUMMARY..... 62

6. REFERÊNCIAS BIBLIOGRÁFICAS..... 64

7. ANEXOS..... 78

ATP	Adenosina-5'-trifosfato
ADP	Adenosina-5'-difosfato
AOX	Oxidase Alternativa
BSA	Bovine Serum Albumine
BHT	3,5-dibutil-4-hidroxitolueno
CAT	Carboxiatractilosídeo
CRC	Cadeia Respiratória dos citocromos
EGTA	Ácido etileno glycol-bis (β-aminoetil éter) N,N,N',N' tetra acético
FCCP	carbonil cianide p-trifluoromethoxiphenilhidrazone
FFA	Ácidos Graxos Livres
LA	Ácido Linoléico
$NAD(P)^+$	Nicotinamida adenina dinucleotídeo (fosfato), forma oxidada
NAD(P)H	Nicotinamida adenina dinucleotídeo (fosfato), forma reduzida
NAD^+	Nicotinamida adenina dinucleotídeo, forma oxidada
NADH	Nicotinamida adenina dinucleotídeo, forma reduzida
PAR	Cadeia Paralela
P_i	Fosfato inorgânico
$\Delta\psi$	Potencial de Membrana Mitocondrial
SIDA	Síndrome da Imunodeficiência Adquirida
TPM	Transição de permeabilidade mitocondrial
UQ	Forma oxidada da coenzima Q
$UQH^{\cdot}$	Forma semiquinona da coenzima Q
UQH_2	Forma reduzida da coenzima Q

PÁG.

Figura 1: Esquema sumarizando os mecanismos de transporte de Ca^{2+} localizados nas membranas plasmática, nuclear, mitocondrial e do retículo endoplasmático em uma célula eucariótica típica..... 19

Figura 2: Modelo de um acidocalcisoma. (DOCAMPO & MORENO, 1999)..... 21

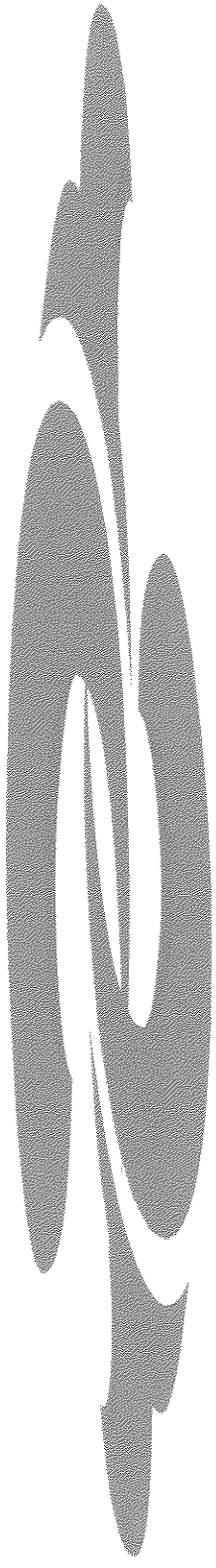
Figura 3: Modelo topológico da UCP. As α-hélices estão representadas por retângulos vermelhos e numeradas de I a VI..... 29

Figura 4: Modelos propostos de mecanismo de ação da UCP1. A UCP está representada como um retângulo laranja inserida na membrana mitocondrial interna (retângulo cinza). A orientação do potencial de membrana está indicada. (A) UCP funciona como um transportador de H^+. O grupo carboxil do ácido graxo funciona como cofator no transporte de H^+. (B) Hipótese do ciclo de FFA. O ácido graxo protonado se movimenta livremente na fase lipídica da membrana em direção a matriz mitocondrial. A forma aniônica do ácido graxo é então transportada de volta ao espaço intermembranas onde pode ser protonado e um novo ciclo recomeça. (C) Perda de especificidade do carreador leva ao desacoplamento. No caso mais extremo, a modificação da estrutura da proteína permite a passagem de solutos de 1000 Da através de um poro constituído pelo carreador modificado..... 31

PÁG.

Esquema 1: A cadeia respiratória mitocondrial e principais sítios de vazamento de elétrons..... 24

Esquema 2: A cadeia respiratória mitocondrial da *Cândida parapsilosis*..... 27



RESUMO

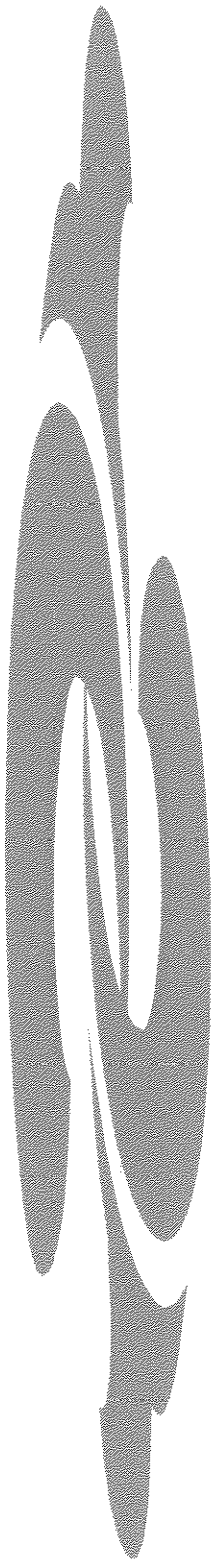
Nesta Tese, estudamos o transporte de Ca^{2+} em esferoplastos permeabilizados de *Candida parapsilosis* preparados por uma nova técnica utilizando-se a enzima liticase. Caracterizou-se um mecanismo de captação de Ca^{2+} intracelular não mitocondrial, insensível ao ortovanadato porém sensível ao inibidor de V-H^+-ATPase bafilomicina A_1, nigericina e FCCP. A acidificação deste compartimento, no qual o Ca^{2+} é acumulado, foi acompanhada utilizando-se o corante fluorescente alaranjado de acridina. A acidificação interna foi estimulada pelo quelante de Ca^{2+} EGTA e inibida por Ca^{2+}. Esses resultados adicionados ao fato de que o Ca^{2+} induz a alcalinização deste compartimento sugerem fortemente a presença de um antiporter Ca^{2+}/nH^+ na membrana deste compartimento ácido. Uma outra observação interessante foi o fato de que, assim como nos tripanossomatídeos, o composto antioxidante BHT inibe a V-H^+-ATPase. Além disso, o agente antifúngico cetoconazol promoveu uma rápida alcalinização desse compartimento ácido de maneira dose-dependente e na mesma faixa de concentração encontrada no plasma sangüíneo de pacientes tratados com essa droga.

Uma proteína desacopladora (UCP) foi identificada em mitocôndrias de *C. parapsilosis* (CpUCP), uma levedura não-fermentativa. A atividade da CpUCP, investigada em mitocôndrias livre de ácidos graxos, foi estimulada por ácido linoléico (LA) e inibida por GTP. A atividade da CpUCP, após a adição de LA, foi verificada pelo aumento da respiração em estado 4, devido ao decréscimo no $\Delta\Psi$ e a diminuição da razão ADP/O dissipando assim, a energia da oxidação fosforilativa. Essa dependência de voltagem do fluxo de elétrons indica que o LA teve um efeito puramente protonofórico. A descoberta da CpUCP demonstra que as proteínas tipo UCP ocorrem em pelo menos quatro reinos eucarióticos: animal, vegetal, fungos e protista.

As mitocôndrias de *C. parapsilosis* possuem uma oxidase alternativa (AOX) constitutiva, além da cadeia respiratória clássica (CRC) e da cadeia respiratória paralela (PAR), ambas terminado em citocromo c oxidases diferentes.

A AOX da *C. parapsilosis* foi identificada imunologicamente e verificou-se que a mesma apresenta uma regulação tipo-fúngica na qual o GMP age como estimulador e o LA como inibidor.

A análise da inibição da respiração, através da determinação da razão ADP/O e da respiração em estado 3, demonstraram que (i) o oxigênio pode ser reduzido pelas três oxidases terminais através das quatro vias respiratórias, implicando em uma interligação entre CRC e PAR, (ii) a somatória das capacidades da CRC, AOX e PAR é maior do que a respiração total (sem aditividade) e seu aumento pode ser progressivo dependendo do estado redox da ubiquinona, primeiro via dos citocromos, depois AOX e finalmente PAR. Através dessas evidências, foi proposto nesta tese um novo modelo para a cadeia respiratória de *C. parapsilosis*.



1. INTRODUÇÃO GERAL

A incidência de infecções severas causadas por espécies de *Candida* tem aumentado em importância em diversos hospitais (COLOMBO *et al*, 1996, STILL *et al*, 1995), especialmente entre pacientes oncológicos ou submetidos a transplantes de medula óssea (CANCELAZ *et al*, 1994, CHAO *et al*, 1994). Foi recentemente publicado que em pacientes oncológicos, *Candida* são responsáveis por 46 % das infecções sistêmicas, das quais 7 % estão relacionadas a *C. parapsilosis*. Além disso infecções fúngicas tem se tornado a maior causa de mortalidade entre pacientes imunocomprometidos (especialmente aqueles com SIDA) (GEORGOPAPADAKOU & TKACZ, 1995, REX, *et al*, 1993, BOSSCHE, MARICHAL & ODDS, 1994). Ainda mais perturbador são os freqüentes relatos de que os fungos vem desenvolvendo resistência aos agentes antifúngicos disponíveis (REX, RINALDI & PFALLER, 1995; BOSSCHE, MARICHAL & ODDS, 1994). Isso vem promovendo um crescente interesse nos estudos tanto de patógenos fúngicos como no desenvolvimento de novos agentes antifúngicos.

Nos últimos 30 anos, anfotericina B, conhecida por sua nefrotoxidade, era a única droga disponível para o controle de infecções fúngicas. A aprovação dos imidazólicos e os triazólicos nos anos 80 e 90, representaram um grande avanço em se tratando de eficácia e segurança para o tratamento de infecções fúngicas sistêmicas. Por outro lado, o uso extensivo, em especial de fluconazol, tem propiciado um aumento de relatos de resistência (GHANNOUM, M. A. & RICE, L.B., 1999).

A *Candida parapsilosis* ATCC22019 é uma cepa padrão utilizada principalmente para controle de qualidade e validação de testes de suscetibilidade a antifúngicos, uma vez que seu espectro de sensibilidade a anfotericina B e fluconazol é bem determinado. Sua responsabilidade por casos de endocardite, endoftalmite e afinidade por tecidos traumatizados tem sido constantemente relatados na literatura (DARWAZAH, BERG & FARIS, 1999) , entretanto seus meios de infecção e propagação ainda não estão bem definidos e podem envolver tanto o ambiente hospitalar quanto a veiculação através das mãos dos profissionais da área de saúde.

1.1. HOMEOSTASE INTRACELULAR DE CÁLCIO

O íon Ca^{2+} exerce papel essencial no controle de uma série de funções celulares devido a sua propriedade de transmitir sinais para o interior da célula (CARAFOLI, 1987; GUNTER *et al*, 1994).

O controle da contração muscular ou a liberação de neurotransmissores são exemplos óbvios mas o Ca^{2+} parece estar presente em muitos outros processos, incluindo plasticidade sináptica, proliferação e morte celular. Para se acomodar ao controle de tantas funções, os mecanismos responsáveis pela geração dos sinais de Ca^{2+} são muito diversos. Alguns produzem grandes e localizadas flutuações de Ca^{2+} enquanto outros produzem longas e baixas elevações do Ca^{2+} que aparecem como ondas repetitivas (BERRIDGE & DUPONT, 1994).

Para que o Ca^{2+} atue como segundo mensageiro é necessário que sua distribuição entre os compartimentos extra e intracelulares seja regulada por mecanismos de alta precisão e que possam ocorrer rápidas flutuações na sua concentração. Assim são necessárias três condições fundamentais:

- alto gradiente eletroquímico de Ca^{2+} através da membrana plasmática;

- proteínas de membrana capazes de se complexar com o Ca^{2+}, transporta- lo através da membrana e liberá-lo do outro lado;

- proteínas solúveis com capacidade de ligar o Ca^{2+} reversivelmente e processar a informação trazida pelo íon.

A concentração do Ca^{2+} no citosol é da ordem de 10^{-7} M e é cerca de 10.000 vezes menor do que a concentração extracelular, da ordem de 10^{-3} M. Assim pequenos aumentos na permeabilidade da membrana ao Ca^{2+} geram um rápido fluxo do íon para o interior das células (CARAFOLI, 1987).

A distribuição do Ca^{2+} intracelular é controlada por processos de transporte do íon através da membrana plasmática e das membranas das organelas subcelulares como retículo endoplasmático, núcleo e mitocôndrias, (Figura 1) (CARAFOLI, 1987; GUNTER

& PFEIFFER, 1990; GUNTER *et al*, 1994). As membranas de células eucarióticas possuem proteínas que participam de quatro tipos de transporte de Ca^{2+}: ATPases, que possuem alta afinidade pelo Ca^{2+} e são responsáveis pela sua regulação fina; além dos trocadores iônicos, canais e transportadores eletroforéticos que possuem baixa afinidade pelo Ca^{2+}, porém alta velocidade de transporte e são responsáveis pelas variações rápidas nas concentrações do íon (CARAFOLI, 1987; BASSANI, BASSANI & BERS, 1992).

O influxo de Ca^{2+} para dentro da célula é mediado por canais específicos modulados por voltagem (VOCs), canais modulados por receptores (ROCs) ou canais operados pelo armazenamento (SOCs). A saída pode ocorrer por processo ativo mediado pela Ca^{2+}-ATPase ou por trocador Na^{+}/Ca^{2+} particularmente em células de tecidos excitáveis (BERRIDGE, 1997). Em células não excitáveis a entrada de cálcio se dá por um mecanismo chamado de capacitativo no qual a prolongada depleção dos estoques intracelulares de Ca^{2+}, por exemplo por uma estimulação constante, é necessária para ativar a entrada de Ca^{2+} (BERRIDGE, 1997).

No retículo endo(sarco)plásmatico a entrada de Ca^{2+} é catalisada pela Ca^{2+}-ATPase, que transloca 2 Ca^{2+} para cada ATP hidrolisado (DE MEIS & VIANNA, 1979). O efluxo de Ca^{2+} se dá por um canal estimulado por inositol trifosfato (IP_3) (BERRIDGE, 1993) e cafeína (receptor de rianodine) (SORRENTINO & VOLPE, 1993). A alta afinidade da Ca^{2+}-ATPase ao cátion confere ao retículo um importante papel na regulação da concentração de Ca^{2+} livre no citosol. Em 1991, DE MEIS mostrou que a própria Ca^{2+}-ATPase do retículo sarcoplasmático pode ser desacoplada por fenotiazínicos, propanolol e alprenolol, servindo como um "canal" para efluxo rápido de Ca^{2+}, independente da presença de IP_3 e receptores de rianodine.

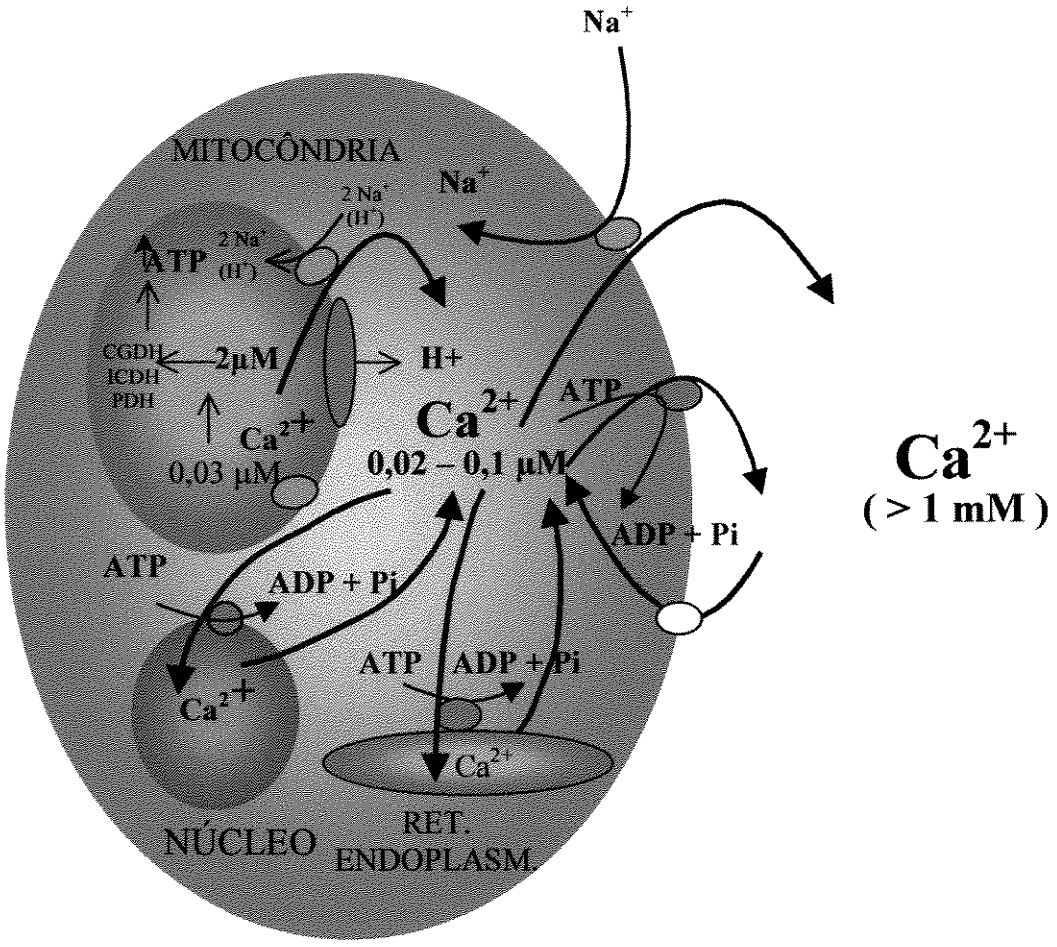


Figura 1: Esquema sumarizando os mecanismos de transporte de Ca^{2+} localizados nas membranas plasmática, nuclear, mitocondrial e do retículo endoplasmático em uma célula eucariótica típica.

A presença de uma outra organela que também está envolvida na homeostase de Ca^{2+} intracelular foi identificada em diferentes microorganismos. PHILOSOPH & ZILBERSTEIN, 1989 foram os primeiros a descrever a mobilização de Ca^{2+} de um compartimento intracelular pela nigericina em *Leishmania donovani.* Em 1994, VERCESI, MORENO & DOCAMPO identificaram pela primeira vez a presença de um compartimento ácido, sensível a nigericina em *Trypanosoma brucei* e o denominaram de acidocalcisoma. Posteriormente os acidocalcisomas foram encontrados em *T. cruzi* (DOCAMPO *et al*, 1995), *L. mexicana amazonensis* (LU *et al*, 1997), *Toxoplasma gondii* (MORENO & ZHONG, 1996) e recentemente em *Plasmodium chabaudi* e *P. falciparum* (PASSOS & GARCIA, 1998).

As características fisiológicas dos acidocalcisomas foram primeiramente descritas utilizando-se células permeabilizadas (VERCESI, MORENO & DOCAMPO, 1994, DOCAMPO *et al*, 1995). A captação de Ca^{2+} ocorre em uma reação catalisada por uma Ca^{2+}-ATPase vacuolar em troca por H^+, a qual é inibida por vanadato. O gradiente de H^+ é formado por uma H^+-ATPase vacuolar sensível a bafilomicina A_1 ou por uma H^+-pirofosfatase (Ppase) sensível a aminometilinodifosfato (AMDP). Um transporte de Cl^-, através de um canal de Cl^-, está associado a função da H^+-ATPase vacuolar. A liberação de Ca^{2+} ocorre em troca de H^+ e é favorecida pela troca de Na^+ -H^+. Os acidocalcisomas são ricos em pirofosfato, Mg^{2+}, Ca^{2+}, Na^+ e Zn^{2+}. Nem todas essas bombas e trocadores estão necessariamente presentes em todos os acidocalcisomas descritos e sua composição interna pode variar (Figura 2).

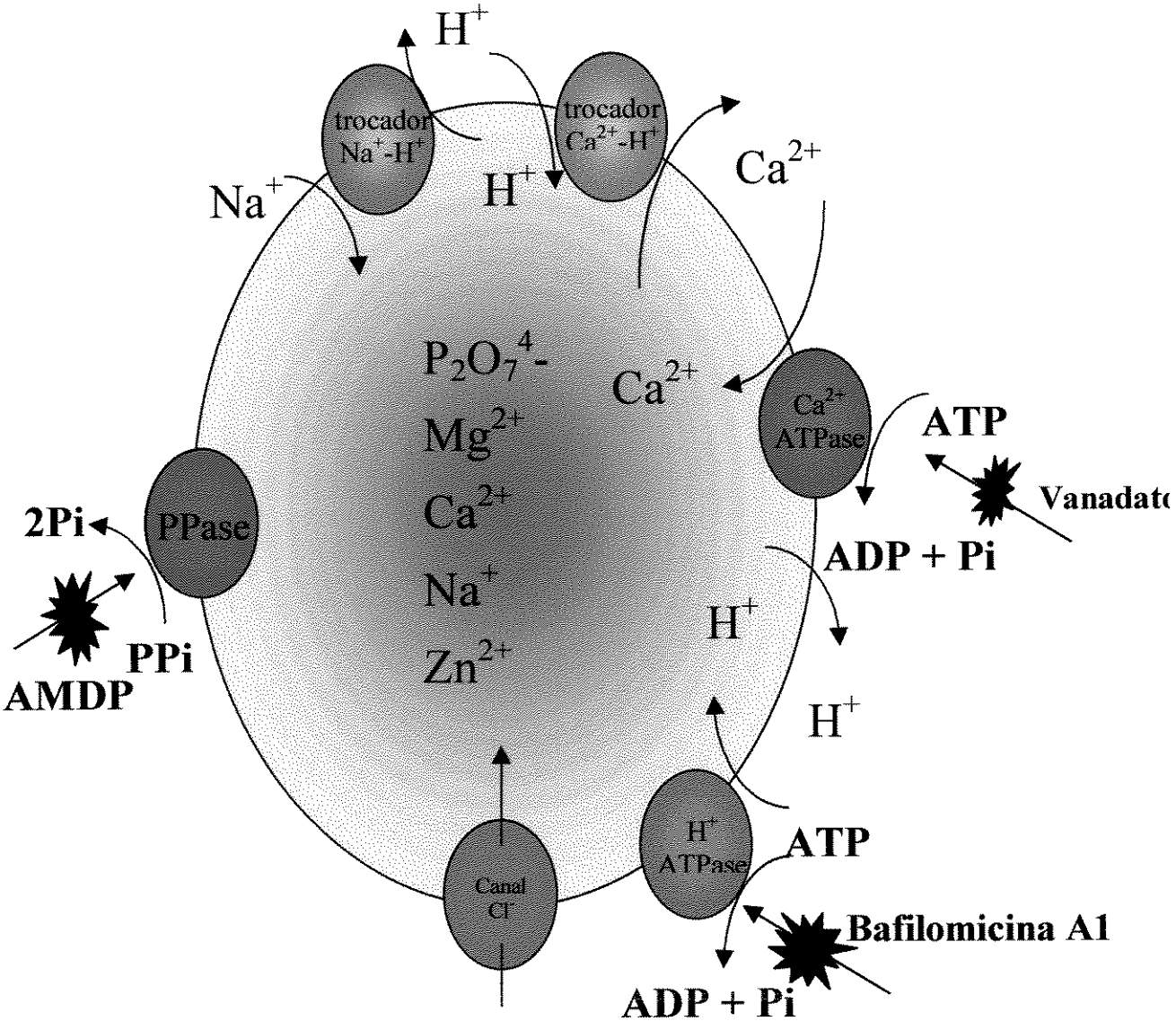


Figura 2: Modelo de um acidocalcisoma. (adaptado de DOCAMPO & MORENO, 1999).

1.1.1. Transporte de cálcio pela mitocôndria

Em contraste com a membrana celular e o retículo endoplasmático, a mitocôndria não possui ATPase transportadora de Ca^{2+}. Este íon entra na mitocôndria por um processo eletroforético em resposta ao potencial elétrico da membrana gerado durante a respiração ou hidrólise do ATP, por um uniporter sensível a vermelho de rutênio (REED & BYGRAVE, 1974). O efluxo de Ca^{2+} mitocondrial ocorre por um processo distinto da via de entrada , onde um íon Ca^{2+} interno é trocado por dois íons Na^{+} (em tecidos excitáveis) ou $2H^{+}$ (em tecidos não excitáveis) (GUNTER & PFEIFFER, 1990).

Em mitocôndrias isoladas, quando estes dois transportadores operam simultaneamente, estabelece-se uma condição de equilíbrio na qual a concentração de Ca^{2+} extra mitocondrial situa-se na faixa entre 0,5 e 1,0 μM (NICHOLLS, 1978). Assim, nas condições *in situ* onde a concentração de Ca^{2+} citossólica é geralmente inferior a 0,1 μM este sistema de transporte parece ser inoperante (HANSFORD, 1985; McCORMACK & DENTON, 1986). Entretanto, quando a concentração de Ca^{2+} citossólica aumenta em resposta a estímulos externos, como no caso de hormônios (vasopressina, angiotensina e catecolaminas) (COBBOLD & RINK, 1987) que ativam processos que demandam energia o sistema de captação de Ca^{2+} se torna operante e a concentração de Ca^{2+} intramitocondrial aumenta para a faixa de 2-10 μM (HANSFORD, 1985 e McCORMACK & DENTON, 1986). Nestas concentrações, o íon Ca^{2+} estimula três desidrogenases mitocondriais (piruvato, isocitrato e α-cetoglutarato desidrogenase) que catalisam reações regulatórias do Ciclo de Krebs, estimulando o metabolismo oxidativo com conseqüente aumento da produção de energia (HANSFORD, 1991).

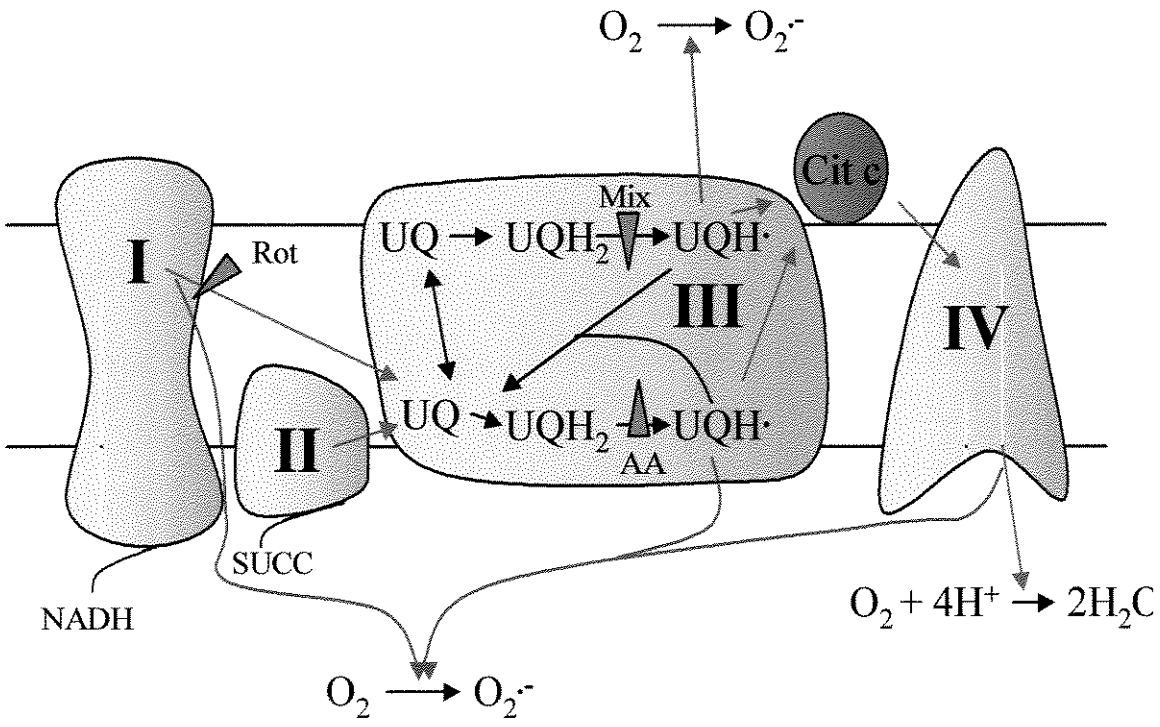
Sabe-se que o Ca^{2+} tem papel determinante no desenvolvimento de uma variedade de processos patológicos e toxicológicos (NICOTERA *et al*, 1990; TRUMP & BEREZESKY, 1995). Depósitos de cálcio têm sido encontrados em tecidos necróticos (SHANNE *et al*, 1979). A manutenção de elevadas concentrações de Ca^{2+} citosólico, causada por isquemia, estresse oxidativo ou algumas toxinas, resulta em acúmulo mitocondrial de Ca^{2+} e ativação de várias enzimas catabólicas Ca^{2+}- dependentes, tais como fosfolipases, proteases e endonucleases, que conduzem a morte celular (TRUMP &

BEREZESKY, 1995). O acúmulo de grandes quantidades de Ca^{2+} na matriz mitocondrial resulta em disfunção mitocondrial e danos celulares devido a permeabilização da membrana mitocondrial interna e inativação da fosforilação oxidativa, por um processo conhecido como transição de permeabilidade da membrana mitocondrial (TPM) (FAGIAN *et al*, 1990).

1.2. A CADEIA RESPIRATÓRIA MITOCONDRIAL

A cadeia respiratória mitocondrial contém as bombas redox que geram o potencial protônico transmembrana e promove a fosforilação oxidativa. Elétrons provenientes de diferentes substratos são colecionados na forma de NADH e transferidos para o átomo de ferro central da NADH desidrogenase (complexo I – esquema I, página 23). O complexo I então transfere elétrons para a forma oxidada da coenzima Q (UQ), levando a sua redução (UQH_2). Elétrons originários do succinato são transferidos para a UQ pelo complexo II, resultando também em sua redução. Em alguns tecidos, a UQ pode ser reduzida também pela glicerol-3-fosfato desidrogenase, na presença de glicerol-3-fosfato, ou pela ubiquinona oxidoredutase, como conseqüência da β-oxidação de ácidos graxos.

A UQH_2 é então desprotonada, resultando na formação do ânion semiquinona ($UQH^{\cdot}$), responsável pela doação de elétrons ao citocromo *c* (cit c). Existem dois *pools* distintos de $UQH^{\cdot}$, um localizado na face citoplasmática da membrana mitocôndria interna, e outro na face matricial da membrana mitocondrial interna. As duas formas de $UQH^{\cdot}$ são combinadas quando oxidadas, regenerando UQ e doando seus elétrons para o citocromo *c* (cit c). O citocromo *c* então transfere elétrons para a citocromo *c* oxidase (complexo IV), responsável pela passagem de elétrons para o oxigênio, que resulta na formação de água. Esta passagem de elétrons para o oxigênio se dá em quatro passos consecutivos envolvendo um elétron (NICHOLLS & FERGUSON, 1982; TURRENS, 1997).



Esquema 1: A Cadeia Respiratória Mitocondrial e Principais Sítios de Vazamento de Elétrons. Os complexos respiratórios mitocondriais (marcados em algarismos romanos) se encontram inseridos na membrana mitocondrial interna, enquanto o citocromo *c* (cit c) está intimamente associado a essa membrana. Estes componentes mitocondriais realizam a transferência de elétrons de substratos como NADH ou succinato (SUCC) para o O_2. As setas azuis representam transferências eletrônicas. As setas vermelhas representam os sítios onde é possível ocorrer vazamento de elétrons com redução monoeletrônica do O_2. Os triângulos verdes representam os sítios respiratórios inibidos por rotenona (rot), antimicina A (AA) e mixotiazol (mix). (KOWALTOWSKI & VERCESI, 1999)

Foi demonstrado anteriormente que diferentes componentes da cadeia respiratória podem converter O_2 a $O_2^{\cdot-}$ quando reduzidos. Na cadeia respiratória plenamente funcional, isso ocorre com aproximadamente 1-2% do oxigênio consumido (BOVERIS & CHANCE, 1973). O $O_2^{\cdot-}$ originado pela cadeia respiratória pode ser gerado pela NADH desidrogenase (BOVERIS & CHANCE, 1973; TURRENS & BOVERIS, 1980) ou pela coenzima Q (CADENAS *et al.*, 1977; TURRENS, ALEXANDRE & LEHNINGER, 1985; BOVERIS & CHANCE, 1973) (veja esquema I). Apesar da citocromo *c* oxidase ser o local onde é normalmente realizada a transferência de elétrons para o oxigênio, e desse processo ser realizado em quatro passos de um elétron cada, a liberação de $O_2^{\cdot-}$ pela citocromo *c* oxidase é surpreendente baixa (TURRENS, 1997). Isso se deve a capacidade da citocromo *c* oxidase de se ligar fortemente aos intermediários de oxigênio parcialmente reduzidos, impedindo seu desligamento antes da redução completa. A geração de $O_2^{\cdot-}$ ao nível do átomo central de ferro da NADH desidrogenase é maior, e pode ser estimulada pela presença de substratos respiratórios convertidos a NADH, como o malato, glutamato e piruvato. A presença de rotenona, um inibidor da transferência de elétrons entre o complexo I e a UQ, também estimula sensivelmente a geração de $O_2^{\cdot-}$ pela NADH desidrogenase (TURRENS & BOVERIS, 1980; TURRENS 1997) (ver esquema I).

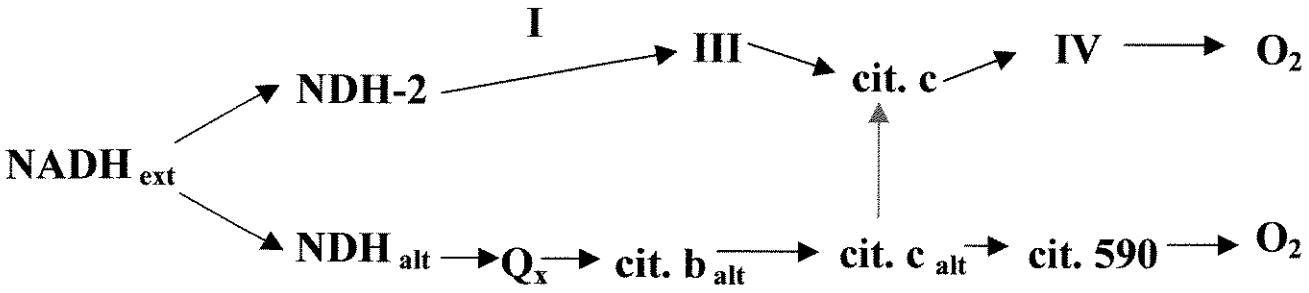
A coenzima Q é, provavelmente, o sítio mais importante de vazamento de elétrons na cadeia respiratória mitocondrial. Isso se deve a doação de elétrons da $UQH^{\cdot}$ (que é um radical livre) para o oxigênio molecular. O vazamento de elétrons ao nível da coenzima Q é estimulado por succinato, cianeto e antimicina A (BOVERIS, CADENAS, STOPPANI, 1976; CADENAS *et al.*, 1977; TURRENS, 1997; TURRENS, ALEXANDRE, & LEHNINGER, 1985; KOWALTOWSKI, COSTA & VERCESI., 1998a). A antimicina A tem um efeito estimulatório notavelmente grande, porque bloqueia a formação de $UQH^{\cdot}$ na face matricial da membrana mitocondrial interna, promovendo o acúmulo de $UQH^{\cdot}$ formado anteriormente na face citossólica da membrana (veja esquema I). O mixotiazol, um inibidor da formação de $UQH^{\cdot}$ na face citossólica da membrana mitocondrial interna, previne a formação de $O_2^{\cdot-}$ pela coenzima Q (TURRENS; ALEXANDRE. & LEHNINGER, 1985; DAWSON, 1993; HANSFORD, HOGUE & MILDAZIENE, 1997; TURRENS, 1997; KOWALTOWSKI, COSTA & VERCESI, 1998a).

1.3. A CADEIA RESPIRATÓRIA MITOCONDRIAL DA *Candida parapsilosis*

A levedura *Candida parapsilosis* apresenta características muito peculiares a nível bioenergético. Sua mitocôndria possui duas cadeias respiratórias, a cadeia respiratória clássica (vias dos citocromos - CRC) e uma segunda via oxidativa (PAR) (GUÉRIN *et al,* 1989) que é muito diferente das oxidases alternativas encontradas em diversas plantas e microorganismos (DEGN, LLYOD & HILL, 1977; MOORE & SIEDOW, 1991).

Em 1994, foi descrito por GUÉRIN & CAMOUGRAND um modelo da cadeia respiratória dessa levedura no qual a segunda via alternativa (PAR), que é insensível a antimicina A mas inibida por amital (CAMOUGRAND *et al,* 1988), SHAM e altas concentrações de mixotiazol (CAMOUGRAND, ZNIBER & GUÉRIN, 1991) ou cianeto (GUÉRIN *et al,* 1989), seria paralela a cadeia dos citocromos com a possibilidade dos elétrons serem desviados para a cadeia dos citocromos a nível do complexo bc*1* (GUÉRIN & CAMOUGRAND, 1986; CAMOUGRAND *et al,* 1993). Anteriormente, GUÉRIN *et al,* 1989 e CAMOUGRAND, VELOURS & GUÉRIN, 1991 haviam descrito que a oxidação de substratos exógenos como NADH, NADPH e glicerol trifosfato se dava preferencialmente através dessa segunda via alternativa e propuseram que esta seria uma reposição funcional da via fermentativa, que não está presente nesta levedura, permitindo assim que a mesma cresça em ambientes não fermentativos e na presença de drogas que inibem o fluxo de elétrons mitocondriais (CAMOUGRAND, GUÉRIN & VELOURS, 1986). (ver esquema II)

No terceiro trabalho desta tese, apresentamos um novo modelo para a cadeia respiratória da levedura *Candida parapsilosis,* baseado em dados tanto da razão ADP/O dessa mitocôndria quanto das capacidades respiratórias do sistema na presença de diferentes substratos respiratórios.



Esquema 2: A Cadeia Respiratória Mitocondrial da *Candida parapsilosis* (Modelo proposto por GUERIN & CAMOUGRAND, 1994). Os complexos respiratórios estão identificados por algarismos romanos. cit c, citocromo c e cit c alt., citocromo c da cadeia alternativa. As setas indicam a direção preferencial dos elétrons e a seta vermelha a possível interligação entre a cadeia paralela (ou alternativa) e a cadeia dos citocromos.

1.4. AS PROTEÍNAS DESACOPLADORAS - UCPS

A primeira UCP foi descoberta em tecido adiposo marrom de ratos por RICQUIER & KADER em 1976 e posteriormente denominada UCP1. A UCP1 está relacionada com a termogênese de animais recém-nascidos e durante a hibernação, sendo que a sua expressão é específica de tecido adiposo marrom e altamente regulada (BOSS, MUZZIN & GIACOBINO, 1998).

Recentemente foram isolados genes que codificam para novos membros da família das proteínas desacopladoras em mamíferos. A UCP2 é expressa em vários tecidos humanos e sua expressão em tecido adiposo branco está relacionada ao estado nutricional. Sugere-se que esta proteína esteja relacionada com hiperinsulinemia e obesidade desempenhando papel importante no diabetes melitus (FLEURY *et al*, 1997). Já a UCP3

estaria envolvida na termogênese de músculos esqueléticos com atividade glicolítica (BOSS *et al*, 1997). A UCP4 e BMCP1 (brain mitochondrial carrier protein-1) são também homólogas a UCP1 e ambas expressas no sistema nervoso central de humanos (SANCHIS *et al*, 1998, MAO *et al*, 1999) o que indica que estas proteínas estariam envolvidas na produção de calor e no metabolismo basal do cérebro. Proteínas homólogas a UCPs de mamíferos também foram identificadas em plantas (LALOI et al., 1997), protistas (JARMUSZKIEWICZ *et al*., 1999) e fungos (JARMUSZKIEWICZ *et al*., 2000 - 2° Trabalho desta Tese).

1.4.1. Características estruturais da UCP

O transporte de metabólitos através da membrana mitocondrial interna é realizado por carreadores específicos que encontram-se mergulhados na bicamada lipídica. A maioria destes carreadores transportam ânions (ADP, ATP, fosfato inorgânico, malato, aspartato, etc) que são necessários não apenas a fosforilação oxidativa, mas também na integração de todos as vias metabólicas da célula. Todos estes carreadores pertencem a família de carreadores mitocondriais e possuem uma estrutura similar, peso molecular por volta de 30 kDa e ponto isoelétrico alto. Estas proteínas de membrana são caracterizadas por uma estrutura tripartida composta por três domínios de aproximadamente 100 aminoácidos sendo que cada domínio consiste de duas α-hélices transmembrana separadas por loops hidrofílicos extra-membrana (PALMIERI *et al*, 1996).

As UCPs fazem parte da família de carreadores mitocondriais devido a alta homologia com os outros carreadores (AQUILA *et al*, 1985). A topologia da UCP 1 foi elegantemente determinada através de estudos imunológicos (MIROUX *et al*., 1993). Esta proteína possui 6 α-hélices transmembrana, sendo que ambas as extremidades, N- e C-terminais, se localizam do lado citosólico. O modelo também prediz dois *loops* hidrofílicos no espaço mitocondrial intermembrana e três do lado matricial (figura 3). Sugere-se que a UCP1 seja ativa na forma de homodímeros, como é o caso para os outros membros da família de carreadores mitocondriais (KLINGERBERG & APPEL, 1989).

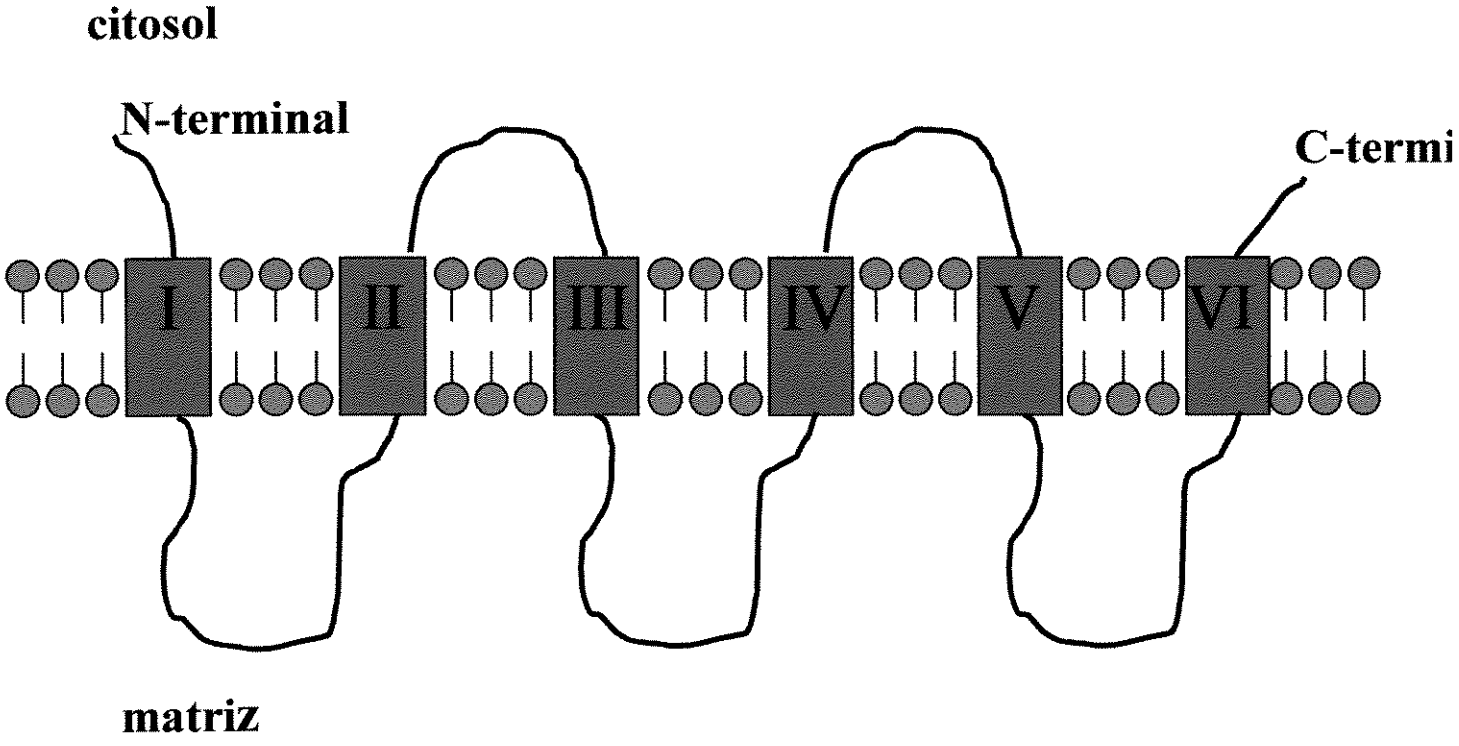


Figura 3: Modelo topológico da UCP. As α-hélices estão representadas por retângulos vermelhos e numeradas de I a VI.

1.4.2. Mecanismos de desacoplamento

As UCPs permitem a passagem de prótons presentes no espaço intermembrana de volta a matriz mitocondrial, contornando a ATP sintetase e conseqüentemente dissipando o potencial eletroquímico de prótons ($\Delta\mu H^+$). A atividade da UCP é dependente da presença de ácidos graxos livres (JABUREK, 1999, KLINGENBERG & HUANG, 1999) e inibida alostericamente por nucleotídeos de purina di- ou trifosfatados (HEATON *et al*, 1978, RIAL, POUSTIE & NICHOLLS, 1983).

Atualmente três principais modelos para o mecanismo de desacoplamento das UCPs são discutidos na literatura. O primeiro (figura 4A) propôs que ácidos graxos livres

(FFA para Free Fatty Acids) ligam-se a UCP e que os seus grupos carboxílicos funcionam como doadores de H^+ a resíduos protonáveis/deprotonáveis localizados no sítio da enzima (KLINGENBERG, 1990, WINKLER & KLINGENBERG, 1994). Neste modelo FFA não são translocados através da membrana. Evidências da existência de grupamentos doadores/receptores de H^+ foram obtidas através de mutações sítio-específicas. Dois resíduos de histidina altamente conservados (His^{145} e His^{147} de UCP1 de hamster) seriam os receptores finais de H^+, "empurrando" os prótons em direção a matriz mitocondrial (BIENENGRAEBER, ECHTAY & KLINGENBERG, 1998). Recentemente, ECHTAY, WINKLER & KLINGENBERG, 2000 e ECHTAY *et al,* 2001(a) demonstraram que o transporte de prótons pelas UCPs 1, 2 e 3, necessitam da presença de um cofator lipofílico, a ubiquinona (Q) na sua forma oxidada. O FFA ancorado na membrana mitocondrial, captaria um H^+ matricial, transferindo-o a ubiquinona através de uma ponte de hidrogênio. Este complexo FFAH-Q catalisaria a passagem do H^+ a um resíduo aceptor da UCP que em seguida atravessaria a membrana por um mecanismo de transferência entre os resíduos protonáveis da enzima.

O segundo modelo propõe que a UCP não seria um transportador de H^+, mas sim um carreador de ânions de ácidos graxos para o exterior da mitocôndria, os quais atravessam a membrana mitocondrial na forma protonada, por um mecanismo de "flip-flop" (SKULACHEV, 1991). O pH básico da matriz mitocondrial permite a dissociação do H^+, dissipando assim o $\Delta\mu H^+$. Este ciclo fútil resulta no transporte indireto de um H^+ para cada ciclo com conseqüente desacoplamento mitocondrial (GARLID *et al.*, 1996).

O terceiro modelo sugere que a perda de especificidade do carreador através de uma mutação/deleção levaria ao desacoplamento (GONZÁLEZ-BARROSOS *et al*, 1997). Em um caso extremo, a modificação da estrutura do carreador permitiria a passagem de solutos de até 1000 Da.

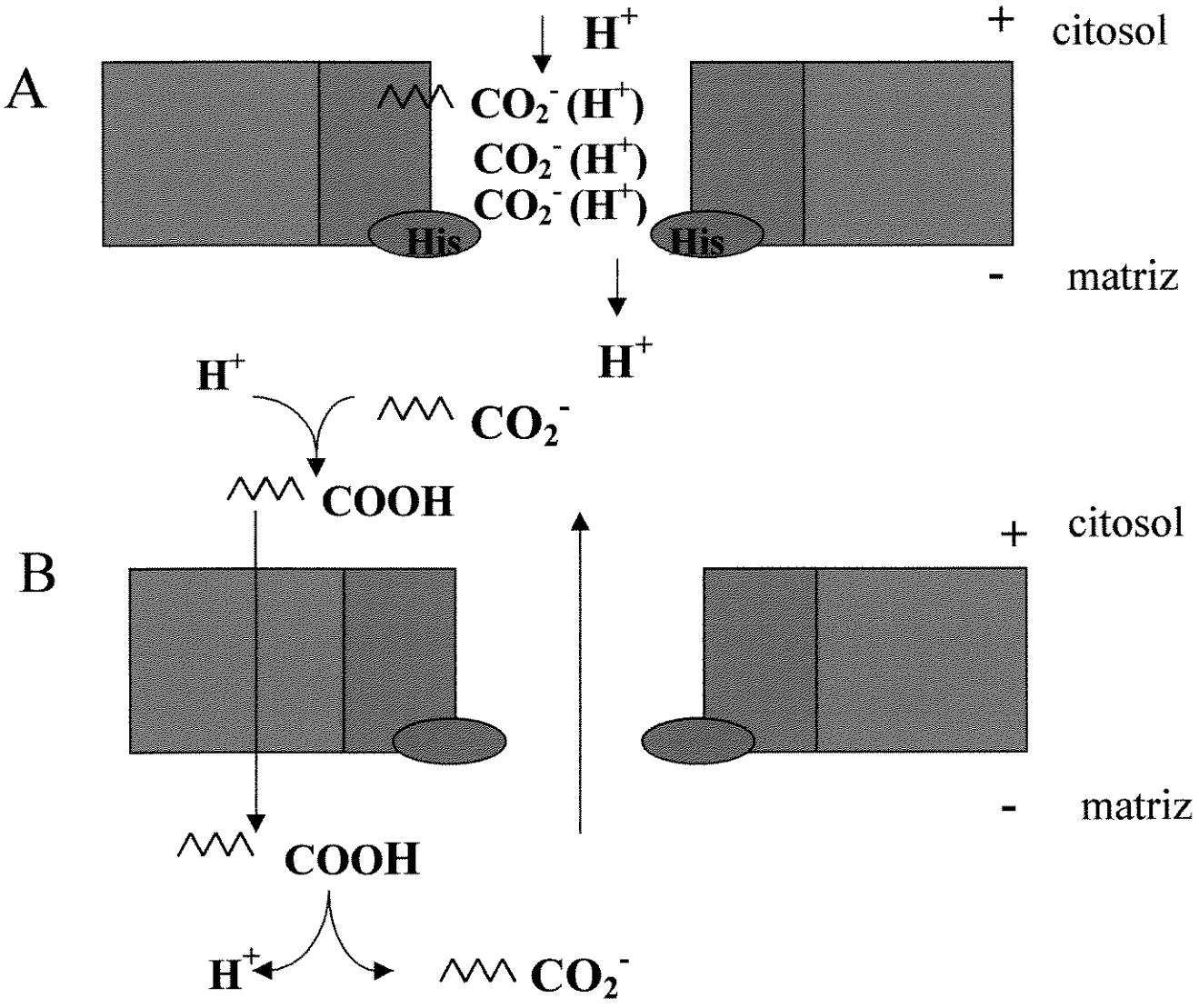


Figura 4: Modelos propostos de mecanismo de ação da UCP1. A UCP está representada como um retângulo laranja inserida na membrana mitocondrial interna (retângulo cinza). A orientação do potencial de membrana está indicada. (A) UCP funciona como um transportador de H^+. O grupo carboxil do ácido graxo funciona como cofator no transporte de H^+. (B) Hipótese do ciclo de FFA. O ácido graxo protonado se movimenta livremente na fase lipídica da membrana em direção a matriz mitocondrial. A forma aniônica do ácido graxo é então transportada de volta ao espaço intermembranas onde pode ser protonado e um novo ciclo recomeça. (C) Perda de especificidade do carreador leva ao desacoplamento. No caso mais extremo, a modificação da estrutura da proteína permite a passagem de solutos de 1000 Da através de um poro constituído pelo carreador modificado.

1.4.3. A proteína desacopladora de plantas

A UCP de plantas foi descoberta em nosso laboratório em 1995. Esta proteína com cerca de 32 kDa, foi isolada de mitocôndrias de batata (*Solanum tuberosum*), e da mesma forma que a UCP1, aumenta a condutividade da membrana a prótons quando incorporada em lipossomas de lecitina (VERCESI *et al.*, 1995). Também foi demonstrado que a UCP de plantas reconstituída em proteolipossomos é responsável pelo transporte de H^+ mediado por FFA e sensível a nucleotídeos de purina de uma maneira similar a UCP1 (JEZEK *et al.*, 1997). Além disso, em mitocôndrias isoladas de batata foi observado que FFA induzem o inchamento mitocondrial dependente de H^+ na presença de valinomicina (JEZEK *et al.*, 1996). Ácido linoléico (LA), um ácido graxo naturalmente abundante em plantas, estimula fortemente a respiração no estado 4 e simultaneamente induz a queda do potencial de membrana em mitocôndrias de batata (VERCESI *et al*, 1995, JEZEK *et al*, 1996) e tomate verde (JARMUSZKIEWCIZ, 1998).

A existência de uma proteína similar a UCP em mitocôndrias de plantas foi confirmada através da clonagem de *StUCP* (*Solanum tuberosum* UCP) em uma biblioteca de cDNA batata (LALOI *et al.*, 1997). Pouco tempo depois dois genes homólogos em *Arabdopsis thaliana*, *AtUCP1* (MAIA *et al*, 1998) e *AtUCP2* (WATANABE *et al*, 1999), e em repolho (*Symplocarpus foetidus*), *SfUCP1* (ITO, 1999), que codificam peptídeos com alta similaridade as UCPs de mamíferos foram também clonados. A seqüência de aminoácidos deduzida destes genes são fortemente similares sendo que AtUCP1 e StUCP possuem 81% de identidade entre elas enquanto que AtUCP2 possui apenas 66 e 67% de identidade com AtUCP1 e StUCP, respectivamente. A % de identidade com as UCPs de mamíferos varia entre 37-45%. Através do uso de anticorpos policlonais, também detectou-se a presença da UCP em vários frutos como tomate, banana, manga, pêra, maçã, morango, mamão, melão e abacaxi (JEZEK *et al*, 1998).

As UCPs de plantas possuem a estrutura típica dos carreadores mitocondriais, contendo três subunidades com 100 resíduos de aminoácidos, cada uma com dois domínios presentes em todos os transportadores mitocondriais (LALOI *et al.*, 1997). A seqüência de aminoácidos das UCPs de plantas contém os três resíduos de arginina (Arg^{83}, Arg^{182},

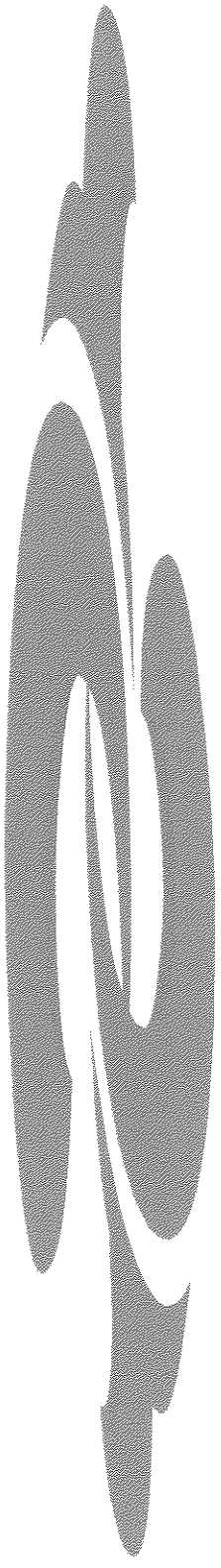
Arg^{276}) descritos em UCP1 como essenciais para a ligação e inibição por nucleotídeos (MURDZA-INGLIS *et al.*, 1994, MODRIANSKY *et al.*, 1997, ECHTAY *et al.*, 2001b).

Ao contrário das UCPs 1, 3 e 4 de mamíferos, que são preferencialmente expressas em tecido adiposo marrom, músculo esquelético e cérebro, respectivamente, as UCPs de plantas são expressas constitutivamente, como UCP2, e fortemente induzidas após exposição ao frio (LALOI *et al.*, 1997, NANTES *et al.*,1997, MAIA *et al.*, 1998).

1.5. A OXIDASE ALTERNATIVA – AOX

A respiração insensível ao cianeto foi observada pela primeira vez em 1929 por GENEVOIS em brotos de ervilha. Mas apenas no final dos anos 70, uma quinona oxidase insensível ao cianeto foi isolada a partir de mitocôndrias de *Arum maculatum* e a respiração resistente ao cianeto foi atribuída a esta enzima batizada oxidase alternativa (AOX) (HUQ & PALMER, 1978, RICH, 1978). Com a criação de anticorpos monoclonais contra AOX de *Sauromatum guttatum*, a identificação de AOX foi possível não só em uma grande variedade de plantas (DAY *et al.*, 1995, SIEDOW & UMBACH, 1995) mas também em fungos (LAMBOWITZ *et al.*, 1989, SAKAJO *et al*, 1991), trypanosomatidea (CLARKSON *et al.*, 1989) e ameba (JARMUSKZIEWICZ *et al.*, 1997) e em *C. parapsilosis* (MILANI *et al,* no prelo - 3° Trabalho desta Tese).

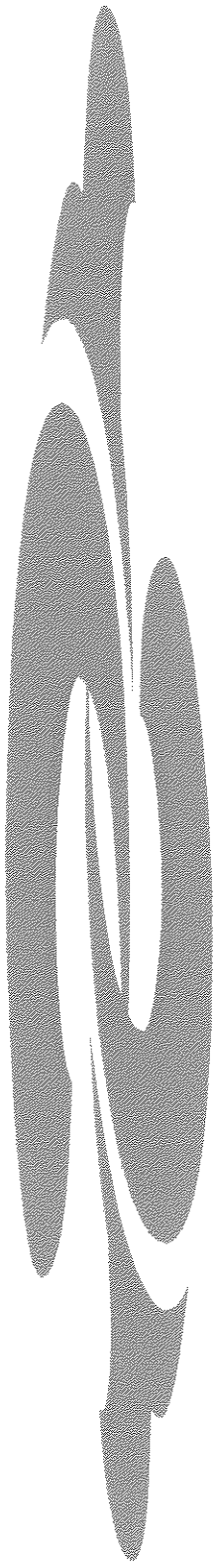
A AOX retém elétrons da cadeia principal de transporte de elétrons ao nível da ubiquinona (Q). Quando AOX é ativa, o fluxo de elétrons é desviado dos complexos III e IV, ejetores de prótons (H^+), e o gradiente eletroquímico final de prótons é menor. A passagem de elétrons através da AOX pode ser especificamente inibida por vários compostos como ácidos hidroxâmicos (ácido benzohidroxâmico – BHAM, ácido salicilhidroxâmico – SHAM) (SCHONBAUM *et al*, 1971), n-propilgalato (SIEDOW & GRIVIN, 1980) e disulfirano (GROVER & LATIES, 1978).



2. OBJETIVOS

O conjunto de trabalhos apresentados teve como objetivos a caracterização funcional da UCP e AOX e também de organelas intracelulares ácidas (vacúolos ácidos) em mitocôndrias e esferoplastos de *Candida parapsilosis* respectivamente. A escolha dessa levedura como modelo se deu ao fato de as infecções fúngicas terem se tornado uma das maiores causas de morte entre pacientes imunocomprometidos e também aos freqüentes relatos de que os fungos vem desenvolvendo resistência aos agentes antifúngicos disponíveis no mercado. Além disso, essa cepa possui relativa facilidade de cultura e crescimento necessárias à padronização das técnicas.

Espera-se que através da caracterização de funções mitocondriais e dos mecanismos de transporte de Ca^{2+} em *Candida parapsilosis* possam ser identificados possíveis novos alvos para a ação eficaz de drogas assim como o desenvolvimento de metodologias para uma avaliação rápida e precisa da suscetibilidade das mesmas frente aos agentes antifúngicos disponíveis ou possíveis alternativas terapêuticas.



3. RESULTADOS

3.1. 1° trabalho

Milani, G., Schreiber, A.Z and Vercesi, A.E. (2001) Characterization of Ca^{2+} uptake by an intracellular acidic compartment in *Candida parapsilosis* FEBS Letters, 500: 80-84.

FEBS Letters 500 (2001) 80–84 FEBS 25000

Ca^{2+} transport into an intracellular acidic compartment of *Candida parapsilosis*

Graziela Milani, Angelica Z. Schereiber, Anibal E. Vercesi*

Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas 13083 970, SP, Brazil

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Abstract In this report, we study Ca^{2+} transport in permeabilized *Candida parapsilosis* spheroplasts prepared by a new technique using lyticase. An intracellular non-mitochondrial Ca^{2+} uptake pathway, insensitive to orthovanadate and sensitive to the V-H^+-ATPase inhibitor bafilomycin A_1, nigericin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was characterized. Acidification of the compartment in which Ca^{2+} accumulated was followed using the fluorescent dye acridine orange. Acidification was stimulated by the Ca^{2+} chelator EGTA and inhibited by Ca^{2+}. These results, when added to the observation that Ca^{2+} induces alkalization of a cellular compartment, provide evidence for the presence of a Ca^{2+}/nH^+ antiporter in the acid compartment membrane. Interestingly, like in acidocalcisomes of trypanosomatids, the antioxidant 3,5-dibutyl-4-hydroxytoluene inhibits the V-H^+-ATPase. In addition, the antifungal agent ketoconazole promoted a fast alkalization of the acidic compartment. Ketoconazole effects were dose-dependent and occurred in a concentration range close to that attained in the plasma of patients treated with this drug. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: *Candida parapsilosis*; Ca^{2+} transport; Ketoconazole; Lyticase; Intracellular Ca^{2+}

1. Introduction

The incidence of severe fungal infections has increased during the last decades [1,2] and fungal infections have become a major cause of mortality among imunocompromised patients, including those with AIDS [2–4]. Disturbingly, frequent reports that fungi are developing resistance to antifungal agents have surfaced [4,5]. In oncological patients, *Candida albicans* accounted for 54% and *Candida parapsilosis* for 7% of all systemic *Candida* infections [4]. Current chemotherapy against these parasites has many flaws, including low specificity, toxicity and drug resistance [1]. It is, therefore, very important to search for biological targets in these parasites that could be exploited for the rational development of improved therapies. In this search, understanding of the mechanisms involved in fungal control of Ca^{2+} homeostasis is very important, since disruption of Ca^{2+} homeostasis leads to cell death [8]. Cell death is also intimately linked to energetic metabolism, and we have recently characterized aspects of *C. parapsilosis* energy conservation systems [7].

Intracellular Ca^{2+} ions play a crucial role in controlling many biological processes [9] which are dependent on Ca^{2+} compartmentalization, governed by several transport systems operating in a highly regulated fashion [9]. Cells have developed a variety of molecular devices, such as channels, pumps and transporters, to regulate Ca^{2+} influx and efflux across the plasma membrane and between intracellular stores, adjusting cytoplasmic Ca^{2+} concentrations [9]. Mammalian cells contain very active Ca^{2+}-ATPases and Ca^{2+}/Na^+ antiporters in their plasma membrane, able to promote the fast exchange of intracellular Ca^{2+} with the extracellular pool. In contrast, most Ca^{2+} transport into and out of the cytoplasm of fungal cells is mediated by their vacuolar membranes [13]. Vacuoles are the largest compartments in yeast cells and are postulated to function as lysosomes and storage compartments [10]. In most plant and fungal cells, the vacuoles occupy over 50% of the cell volume and their membrane contains numerous carriers, transporters, channels and enzymes. Little is known about the mechanisms involved in Ca^{2+} homeostasis in *C. parapsilosis*, which is assumed to transport Ca^{2+} in a manner similar to *Saccharomyces cerevisiae* [11].

In this report we demonstrate that *C. parapsilosis* possesses a non-mitochondrial, nigericin-sensitive Ca^{2+} compartment that functions in a manner dependent on the pH gradient formed by a bafilomycin A_1-sensitive H^+-ATPase. A Ca^{2+}/nH^+ antiporter apparently mediates Ca^{2+} uptake and release by this acidic compartment. Furthermore, ketoconazole, an antimycotic drug, promotes a fast and extensive alkalization of the acidic compartment, followed by Ca^{2+} release.

2. Materials and methods

2.1. *C. parapsilosis* culture

C. parapsilosis CCT 3834 (ATCC 22019) cells were cultured at 37°C under vigorous aeration in complete liquid medium (2% glycerol, 2% Bacto-peptone (Difco), 1% Bacto-yeast extract (Difco) until the mid-exponential phase.

2.2. *Spheroplast preparation*

Cultured *C. parapsilosis* cells were harvested by centrifugation from a liter of culture media, and cells were washed with cold water, followed by a wash using buffer A (1 M sorbitol, 10 mM $MgCl_2$ and 50 mM Tris–HCl, pH 7.8). Cells were resuspended in buffer A (3 ml/g of cells) containing 30 mM dithiothreitol (DTT). After 15 min incubation at room temperature with shaking, cells were harvested by centrifugation, resuspended in buffer A containing lyticase (1 mg/g of

*Corresponding author. Fax: (55)-19-3788 9434.
E-mail: anibal@obelix.unicamp.br

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; AO, acridine orange; BHT, 3,5-dibutyl-4-hydroxytoluene

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cells) and 1 mM DTT and incubated at 30°C until approximately 90% of the cells converted to spheroplasts (60–80 min). The digestion was stopped by the addition of an equal volume of ice-cold buffer A, and spheroplasts were washed twice with the same buffer. Protein concentration of the final suspension was determined using the biuret assay [14] in the presence of 0.2% deoxycolate.

2.3. Measurement of Ca^{2+} movements

Variations in free Ca^{2+} concentrations were followed by measuring the changes in the absorbance spectrum of arsenazo III [15], using a SLM Aminco DW 2000 spectrophotometer at the wavelength pair 675–685 nm [16,17]. The concentrations of the ionic species and complexes at equilibrium were calculated by employing an interactive computer program as described before [18].

2.4. Proton pump activity

Acidification of permeabilized cells was followed by measuring the changes in the absorbance or fluorescence spectrum of acridine orange (AO) [19], using a SLM Aminco DW2000 spectrophotometer at the wavelength pair 493–530 nm or a Hitachi F4500 fluorescence spectrophotometer at the wavelength pair 510–550 nm.

Each experiment was repeated at least three times with different cell preparations, and the figures show representative experiments.

2.5. Chemicals

ATP, calcium ionophore A_{23187}, sodium orthovanadate, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), succinate, arsenazo III, EGTA, nigericin, antimycin A, myxothiazol, oligomycin, ammonium chloride and lyticase were purchased from Sigma. AO was from Molecular Probes (Eugene, OR, USA). All other reagents were of analytical grade.

3. Results

3.1. Ca^{2+} uptake by C. parapsilosis spheroplasts: inhibition by nigericin and bafilomycin A_1

Fig. 1 shows that, when *C. parapsilosis* spheroplasts were added to a reaction medium containing MgATP, succinate and 8 μM free Ca^{2+}, most of the Ca^{2+} present was taken up and retained by the spheroplasts. The subsequent addition of the Ca^{2+} ionophore A_{23187} promoted a release of the accumulated cation [trace (a)]. Nigericin, a K^+/H^+ exchanger, was also able to release part of the accumulated Ca^{2+} [trace (c)]. The simultaneous presence of antimycin A, oligomycin and myxothiazol (see [7]), which prevent mitochondrial energization and Ca^{2+} uptake [21] or sodium orthovanadate, an inhibitor of the P-type Ca^{2+}-ATPase, caused no difference either on the rate or extent of Ca^{2+} uptake (the trace is superimposable with trace (a)). The presence of a V-H^+-PPase [22] in these vacuoles was also ruled out in our experiments.

Acidic compartments may possess an ATP-driven H^+ pump that maintains their interior at a pH lower than that of the cytoplasm [20]. These H^+ pumps are bafilomycin A_1-sensitive [21,23]. Indeed, trace (b) shows that when *C. parapsilosis* spheroplasts were added to reaction medium containing nigericin or bafilomycin A_1, Ca^{2+}-transport activity was completely inhibited (the traces with bafilomycin A_1 and nigericin are superimposable). The sensitivity of this Ca^{2+}-transporting activity to bafilomycin A_1 confirms that Ca^{2+} is being taken up by a compartment acidified by a V-type proton ATPase [24].

3.2. ATP-dependent vacuolar acidification in C. parapsilosis spheroplasts: effects of nigericin, NH_4Cl and bafilomycin A_1

In order to confirm the presence of a V-H^+-ATPase in these cells we used AO, a tertiary amine that becomes concentrated in acidic compartments and changes both its absorbance and fluorescence properties as a consequence of its stacking [19] as a probe. Trace (a) shows that the addition of ATP to a reaction medium containing antimycin A, myxothiazol, oligomycin, EGTA and *C. parapsilosis* spheroplasts caused a significant time-dependent decrease in the absorbance of AO compatible with its accumulation in acidic compartments. This uptake was inhibited by bafilomycin A_1 [trace (c)]. The addition of bafilomycin A_1 also reversed the decrease in AO absorbance [trace (b)]. EGTA, present in trace a, decreased the concentration of Ca^{2+} from 8 μM to less than 1 nM and stimulated both the rate and extent of AO absorbance decrease, when compared to trace (b). Under all experimental conditions tested, nigericin, FCCP or NH_4Cl reversed the decrease in AO absorbance induced by ATP, confirming that the decrease in AO absorbance was caused by its accumulation into acidic compartments.

3.3. Calcium-induced AO release from C. parapsilosis acidic vacuoles

We also investigated the effect of Ca^{2+} on the *C. parapsilosis* ATP-dependent compartment acidification, in a reaction medium containing antimycin A, myxothiazol and oligomycin. Fig. 3 shows that 40 μM $CaCl_2$ [trace (b)] reversed the decrease in AO absorbance promoted by the spheroplasts.

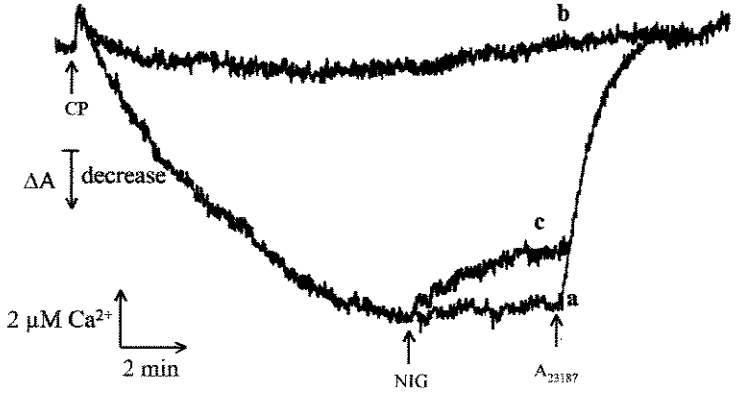

Fig. 1. Ca^{2+} uptake by *C. parapsilosis* spheroplasts. The *C. parapsilosis* spheroplasts (1 mg of protein/ml) were added to standard reaction media (125 mM sucrose, 65 mM KCl, 10 mM HEPES, pH 7.2, 2.5 mM potassium phosphate, 1 mM Mg Cl_2) containing 5 mM succinate, 40 μM arsenazo III and 0.5 mM ATP. Trace (a), control; trace (b), 1.3 μM nigericin and/or 2.5 μM bafilomycin A_1 present from the beginning of the experiment and trace (c), 1.3 μM nigericin added where indicated. Calcium ionophore A_{23187} (1 μM) was added where indicated by the arrow.

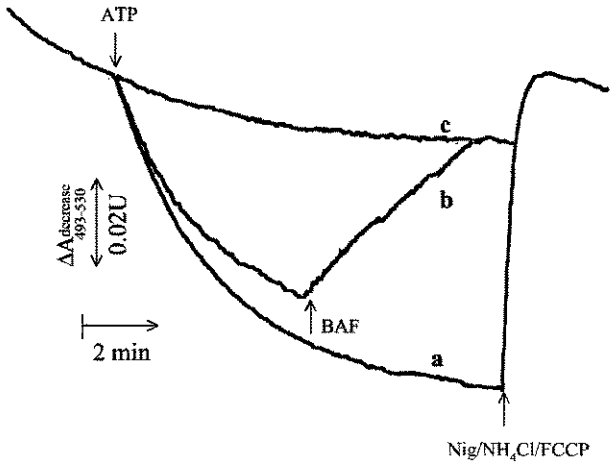

Fig. 2. ATP-driven AO accumulation by *C. parapsilosis* spheroplasts. The spheroplasts (1 mg of protein/ml) were added to the standard reaction media containing 2 μM antimycin A, 2 μg/ml oligomycin, and 2 μg/ml myxothiazol. AO (3.3 μM) was added 3 min after the cells. Trace (a), EGTA (1 mM) present in the reaction media; trace (b), 4 μM bafilomycin A_1 added where indicated by the arrow and trace (c), bafilomycin A_1 (4 μM, from the beginning of the experiment) or absence of ATP (traces were superimposable). ATP (0.5 mM), 20 mM NH_4Cl, 1 μM FCCP and 1.3 μM nigericin were added where indicated.

Addition of 20 mM NH_4Cl completed the release of AO. This AO release induced by Ca^{2+} also occurs when vanadate was present in the incubation medium, indicating that this process is totally independent of cation transport by a P-type Ca^{2+}-ATPase [trace (a)]. Furthermore, the presence of 20 μM EGTA, which removes most of the contaminant Ca^{2+} present in the reaction medium (about 8 μM), increased the extent of ATP-dependent vacuolar acidification in the permeabilized cells [trace (a)], indicating a close relationship between Ca^{2+} uptake and vacuolar acidification. Both the insensitivity of Ca^{2+} uptake to vanadate (Fig. 1) and the vacuolar alkalization promoted by this cation (Fig. 2 traces (a) and (b)) suggest the presence of a Ca^{2+}/nH^+ antiporter in the vacuoles of these cells [11,12]. The stimulation of AO uptake by EGTA (Fig. 2) and its release by Ca^{2+} (Fig. 3) indicate that this dye and Ca^{2+} are, in fact, being accumulated in the same compartment.

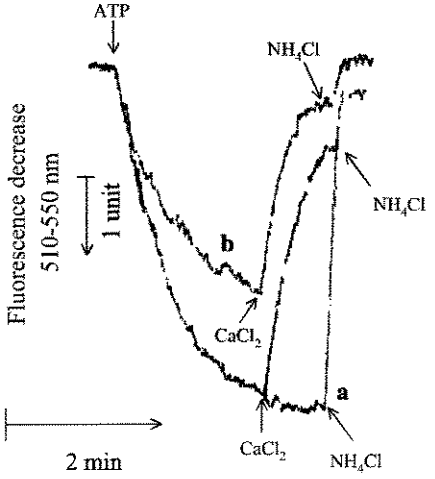

Fig. 3. Ca^{2+} effect on ATP-driven AO accumulation in *C. parapsilosis* spheroplasts. The spheroplasts (1 mg of protein/ml) were added to standard reaction media containing 2 μM antimycin A, 2 μg/ml oligomycin, 2 μg/ml myxothiazol and 20 μM EGTA. AO (3.3 μM) was added 3 min after the cells. ATP (0.5 mM), 40 μM $CaCl_2$ and 20 mM NH_4Cl were added where indicated. Trace (a), 400 μM vanadate present from the beginning of the experiment and trace (b), control.

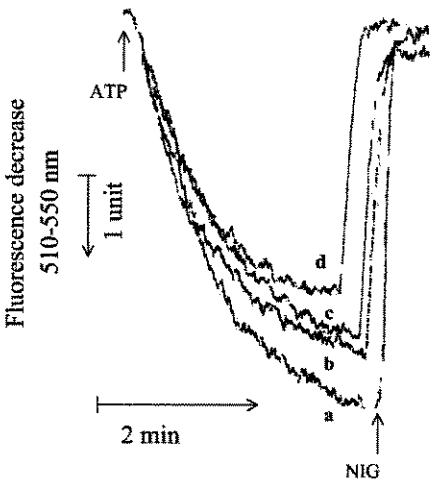

Fig. 4. Inhibition by BHT of ATP-driven AO accumulation in *C. parapsilosis* spheroplasts. The spheroplasts (1 mg of protein/ml) were added to the standard reaction medium containing 2 μM antimycin A, 2 μg/ml oligomycin, 2 μg/ml myxothiazol and 1 mM EGTA. AO (3.3 μM) was added 3 min after the cells. ATP (0.5 mM) and 1.3 μM nigericin were added where indicated. BHT was added 30s before ATP. Trace (a), control; trace (b), 5 μM BHT; trace (c), 10 μM BHT; trace (d), 20 μM BHT.

3.4. *Effect of 3,5-dibutyl-4-hydroxytoluene (BHT) on ATP-driven AO accumulation by C. parapsilosis spheroplasts*

We have previously demonstrated [29] that BHT, at commonly used antioxidant concentrations [25], has an inhibitory effect on both $V\text{-}H^+$-ATPases and Na^+/H^+ antiporters of the acidocalcisomes present in *Trypanosoma brucei* procyclic trypomastigotes. At the same concentration, and depending on the preincubation time, BHT presented a stimulatory or inhibitory effect on the vacuolar H^+-ATPase present in those trypanosomes [29].

In Fig. 4 we studied the effects of different concentrations of BHT on AO uptake in *C. parapsilosis* spheroplasts. When ATP was added 30 s after BHT was included in the medium, a progressive inhibition of AO accumulation was detected with increasing BHT concentrations (5–20 μM) [traces (b–d)].

3.5. *Vacuole alkalization by the antimycotic drug ketoconazole*

Ketoconazole (R41.400; Janssen Pharmaceutica) is an imidazole derivative with broad-spectrum activity against fungi and pathogenic yeast [26]. It is proposed that ketoconazole and other azole-based antimycotic agents (fluconazole, itraconazole) act by inhibiting the synthesis of ergosterol, the predominant component of the fungal cell membrane [27].

Since Ca^{2+}-containing vacuoles are not present in most mammalian cells, they can be exploited as important targets for drugs against diseases caused by fungi. In this regard, we studied the effect of the antimycotic drug ketoconazole [28] on the retention of AO by the acidic vacuoles in *C. parapsilosis* (Fig. 5). Ketoconazole (16–128 μg/ml, 75–600 nM) induced a progressive alkalization of the acidocalcisome [traces (b–e)]. As expected, this was followed by Ca^{2+} release (not shown).

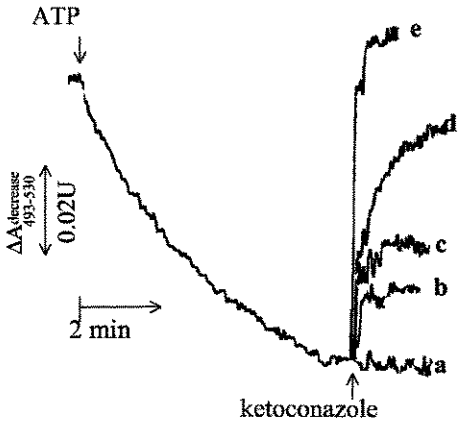

Fig. 5. Effect of ketoconazole on ATP-driven AO accumulation in *C. parapsilosis* spheroplasts. The spheroplasts (1 mg of protein/ml) were added to the standard reaction medium containing 2 μM antimycin A, 2 μg/ml oligomycin, 2 μg/ml myxothiazol and 0.5 mM EGTA. AO (3.3 μM) was added 3 min after the cells. Ketoconazole was added at the following concentrations: Trace (a), control; trace (b), 16 μg/ml; trace (c), 32 μg/ml; trace (d), 64 μg/ml and trace (e), 128 μg/ml.

The concentration range studied was the same used in drug susceptibility tests, with the difference that the cell concentration used in these experiments was at least three orders of magnitude higher than that used in these tests [6]. Other azole-based antimycotic agents, such as fluconazole and itraconazole, had no effect on the retention of AO by the acidic vacuoles.

4. Discussion

This study demonstrates that lyticase can be used to prepare *C. parapsilosis* spheroplasts, permeable to ions, nucleotides and respiratory substrates, without affecting the functional integrity of their internal vacuoles. Previous results [7] demonstrated that the functional properties of their mitochondria are also preserved after this treatment.

Our experiments provide strong evidence for the presence of acidic Ca^{2+} storage vacuoles in *C. parapsilosis*. First, we demonstrate inhibition and release of Ca^{2+} by the K^+/H^+ ionophore nigericin and the vacuolar H^+-ATPase inhibitor bafilomycin A_1 (Fig. 1). Also using permeabilized spheroplasts, we show that Ca^{2+} induces alkalization of the intracellular vesicles, as measured by shifts in AO fluorescence (Fig. 2). The mitochondrial inhibitors used (antimycin A, myxothiazol and oligomycin) had no effect on Ca^{2+} uptake by these permeabilized cells, suggesting that mitochondria, although able to carry out oxidative phosphorylation and other energy-linked functions [7], are not actively participating in Ca^{2+} homeostasis. The inhibition of Ca^{2+} uptake by the vacuolar H^+-ATPase inhibitor bafilomycin A_1 (Fig. 1) indicates that the internal acidic pH generated by the vacuolar proton pump promotes Ca^{2+} uptake and retention. Furthermore, EGTA, which decreases medium Ca^{2+} concentration (Fig. 2), increased the rate of ATP-dependent vacuolar acidification in *C. parapsilosis* spheroplasts, again indicating a close relationship between Ca^{2+} uptake and vacuolar acidification. The insensitivity of Ca^{2+} uptake to orthovanadate indicates that this transport is not mediated by a P-type Ca^{2+}-ATPase and that this enzyme is not present in these vacuoles where Ca^{2+} movements seem to be mediated by a Ca^{2+}/nH^+ antiporter, dependent on Ca^{2+} and pH gradients.

The possible physiological roles of this integrated system for Ca^{2+} and H^+ transport (H^+-ATPase and Ca^{2+}/nH^+) could be many: regulation of cytosolic Ca^{2+} concentration, a large-scale Ca^{2+} storage system to provide a homeostatic reserve, or a detoxification system to prevent the effects of excess free Ca^{2+} in the cytosol when the cells are submitted to an unfavorable environment. Alternatively, the regulation of cytosolic pH could be very important in many physiological situations. In many microorganisms, relatively small increases in cytosolic pH halt cell division and activate the expression of different genes [37]. On the other hand, increases in cytosolic Ca^{2+}, mediated by Ca^{2+} influx through the plasma membrane, could induce H^+ release from the acidic vacuoles, protecting the cells against alkaline pH.

We have previously reported [29] that the antioxidant BHT has effects on both H^+-ATPases and Na^+/H^+ antiporters from *T. brucei*. These effects are not related to alterations in the fluidity or viscosity of the bilayer, as expected with a hydrophobic molecule [35]. Using *C. parapsilosis* spheroplasts, we also demonstrated an inhibitory effect of BHT (Fig. 4), which is probably due to its effect on the H^+-ATPase, since we could not detect a Na^+/H^+ antiporter in these cells.

Acidic vacuoles containing Ca^{2+} have been previously described in *Dictyostelium discoideum* [30], trypanosomatids [31,34,38] and some mammalian cells [32,33], although this finding is controversial [36]. The acidic vacuole in *C. parapsilosis* could represent a potential target for the chemotherapy against this fungus. In this regard, the antimycotic drug ketoconazole promoted a fast alkalization of these vacuoles (Fig. 5) that, in addition to its effect on the synthesis of ergosterol [39], could be involved in the mechanism of its antimycotic action. This is a new and singular effect described for ketoconazole, since the other two azole-based antimycotic agents tested (fluconazole and itraconazole) had no effect on the *C. parapsilosis* acidic vacuole. Although all these compounds inhibit ergosterol synthesis [39], there is a heterogeneity of action among them and ketoconazole is the only drug known to have effects on several membrane-bound enzymes [39], a property which seems to be in agreement with its unique effect on the acidic vacuole of *C. parapsilosis*. In this regard, μM concentrations of ketoconazole can be detected in the plasma of patients using therapeutic doses of this drug [40]. The Ca^{2+}-containing *C. parapsilosis* acid vacuoles seem to be similar to those present in plant cells [24] and differ from those present in most trypanosomatids [22] because they lack Ca^{2+}-ATPase, V-H^+-PPase and Na^+/H^+ exchanger.

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3.2. 2° trabalho

Jarmuszkiewicz, W.; Milani,G.; Fortes, F.; Schreiber, A.Z.; Sluse, F.E. and Vercesi, A.E. (2000) First evidence and characterization of an uncoupling protein in fungi kingdom: CpUCP of *Candida parapsilosis.* FEBS Letters, 467: 145-149

First evidence and characterization of an uncoupling protein in fungi kingdom: CpUCP of *Candida parapsilosis*

Wiesława Jarmuszkiewicz[c], Graziela Milani[a], Fabiane Fortes[a], Angelica Z. Schreiber[d], Francis E. Sluse[b], Anibal E. Vercesi[a,*]

[a] *Departamento de Patologia Clinica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, CP 6111, 13083-970 Campinas, SP, Brazil*
[b] *Laboratory of Bioenergetics, Centre of Oxygen, Research and Development, Institute of Chemistry B6, University of Liège, Sart Tilman, B-4000 Liège, Belgium*
[c] *Department of Bioenergetics, Adam Mickiewicz University, Fredry 10, 61-701 Poznan, Poland*
[d] *Laboratorio de Microbiologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-970 Campinas, SP, Brazil*

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Abstract An uncoupling protein (UCP) was identified in mitochondria from *Candida parapsilosis* (CpUCP), a non-fermentative parasitic yeast. CpUCP was immunodetected using polyclonal antibodies raised against plant UCP. Activity of CpUCP, investigated in mitochondria depleted of free fatty acids, was stimulated by linoleic acid (LA) and inhibited by GTP. Activity of CpUCP enhanced state 4 respiration by decreasing $\Delta\Psi$ and lowered the ADP/O ratio. Thus, it was able to divert energy from oxidative phosphorylation. The voltage dependence of electron flux indicated that LA had a pure protonophoretic effect. The discovery of CpUCP proves that UCP-like proteins occur in the four eukaryotic kingdoms: animals, plants, fungi and protists.

Key words: Yeast mitochondrion; Uncoupling protein; *Candida parapsilosis*

1. Introduction

Mitochondria generate most of the aerobic cell's energy from the reduction of oxygen by their respiratory chain. However, these internal power plants release damaging reactive oxygen species arising from the non-catalyzed partial reduction of molecular oxygen at the level of complexes utilizing or producing ubiquinol [1,2]. Mitochondrial function [3] and mitochondrial proteins [4] are the first targets of such free radical side production. Every condition, which increases the reduced state of electron carriers of the respiratory chain, leads to an increase in reactive oxygen production. Conversely, processes that decrease the reduced state of electron carriers will decrease this damaging production [5].

The energy-dissipating systems such as alternative oxidase (AOX) and uncoupling protein (UCP) have been shown to act in this way in vitro [6,7] and in vivo [8]. AOX consumes ubiquinol independently to phosphate potential and UCP dissipates the proton electrochemical gradient ($\Delta\mu H^+$), allowing an increase of electron flux in the cytochrome pathway at the expense of ubiquinol. Therefore, these two energy-dissipating enzymes can be seen as endogenous protectors of mitochondria, able to prevent deterioration and energy deprivation of the cell. The cost of that protection is a decrease in oxidative phosphorylation yield [9,10].

After the emergence of eukaryotic cells, such intramitochondrial protectors should have appeared quite soon, and if so, they should presently be very widespread. AOX has been early recognized to exist not only in specialized thermogenic tissues of plants but also in plant non-thermogenic tissues and in a large variety of organisms like trypanosomes, fungi, amoeba and other microorganisms [10,11]. In contrast, evidence of the existence of UCP was restricted to mammal brown adipose tissue until 1995 when it was proved also to exist in plants [12]. More recently, several UCP-like proteins have been described in mammal non-thermogenic tissues [13–15], in fishes [16] and in a primitive amoeba, *Acanthamoeba castellanii* [17]. The presence of UCP in *A. castellanii* has allowed hypothesizing the presence of this protein in the whole eukaryotic world. However, no UCP had been shown to occur in the fungi kingdom until now.

The present study demonstrates the existence and characterizes function of an UCP in the parasitic non-fermentative yeast *Candida parapsilosis.*

2. Materials and methods

2.1. Strain and culture

C. parapsilosis CCT 3834 (ATCC 22019) was grown at 37°C under vigorous aeration in complete liquid medium (2% glycerol, 2% Bactopeptone (Difco), 1% Bacto-yeast extract (Difco)) until middle stationary phase.

2.2. Isolation of mitochondria

For standard preparation, 1 l of culture was harvested by centrifugation, and cells were washed once with cold water and once with A buffer (1 M sorbitol, 10 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.8). Cells were resuspended in A buffer (3 ml per g of cells) additionally containing 30 mM dithiothreitol (DTT). After a 15 min incubation at room temperature with shaking, cells were harvested by centrifugation, resuspended in A buffer containing lyticase (1 mg per g of cells) and 1 mM DTT and incubated at 30°C until about 90% of cells converted to spheroplasts (60–80 min). The digestion was stopped by the addition of an equal volume of ice-cold A buffer, and spheroplasts were washed with A buffer. The pellet was resuspended in B1

*Corresponding author. Fax: (55)-19-7881118.
E-mail: anibal@obelix.unicamp.br

Abbreviations: AOX, alternative oxidase; BHAM, benzohydroxamate; BSA, bovine serum albumin; CAT, carboxyatractyloside; CpUCP, uncoupling protein of *C. parapsilosis*; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FFA, free fatty acid; LA, linoleic acid; UCP, uncoupling protein; $\Delta\Psi$, mitochondrial transmembrane electrical potential

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buffer (0.6 M mannitol, 1 mM EDTA, 0.5% bovine serum albumin (BSA), 1 mM PMSF, 10 mM Tris–HCl, pH 7.4). Spheroplasts were mechanically broken using a Dounce homogenizer for a maximum of 10 up-and-down strokes. Cell debris was pelleted by centrifugation for 10 min at $1000 \times g$. Mitochondria were pelleted from the supernatant by 10 min centrifugation at $10\,500 \times g$ and washed with B2 buffer (0.6 M mannitol, 1 mM EDTA, 1% BSA, 10 mM Tris–HCl, pH 7.0). The presence of BSA in the medium allowed chelation of free fatty acid (FFA) from the mitochondrial suspension. The last washing was made in B2 medium without BSA and EDTA. The mitochondrial protein concentration was determined by the biuret method [18].

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting of UCP

A different amount of mitochondrial protein was solubilized in the sample buffer containing 1% (w/v) SDS, 0.1 M Tris–HCl, pH 6.8, 10% glycerol, 0.005% (v/v) bromophenol blue and 0.5% β-mercaptoethanol, and boiled for 4–5 min. Electrophoresis (SDS–PAGE) was carried out using a 5% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel containing 4.5 M urea, followed by Western blotting as described before [17,19]. Antibodies against UCP protein of potato (*Solanum tuberosum*) (generously supplied by Dr. P. Arruda) were used at a dilution of 1:1000. The bands were detected by chemiluminescence (Amersham ECL system).

2.4. Oxygen uptake and membrane potential

Oxygen uptake was measured polarographically using a Clark-type electrode (Yellow Springs Instruments) in 1.3 ml of standard incubation medium (28°C) containing: 125 mM sucrose, 65 mM KCl, 10 mM HEPES pH 7.2, 2.5 mM KH_2PO_4 and 1 mM $MgCl_2$, with 0.4–0.5 mg of mitochondrial protein. The membrane potential of mitochondria was measured under the same conditions as oxygen uptake (additionally with 3 μM tetraphenylphosphonium, TPP^+) using a TPP^+-specific electrode according to Kamo et al. [20]. For calculation of the mitochondrial transmembrane electrical potential ($\Delta\Psi$) value, the matrix volume of yeast mitochondria was assumed as 2.0 μl/mg protein. All measurements were made in the presence of 1.5 mM benzohydroxamate (BHAM). Details of measurements are included in the legends of figures.

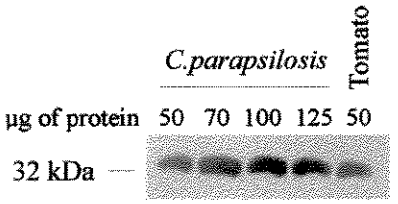

Fig. 1. Immunological identification of CpUCP protein in yeast *C. parapsilosis* mitochondria. Detection of UCP in mitochondria isolated from green tomato fruit (control) is also shown. Primary antibodies were raised against potato UCP. The amount of protein loaded on lane is indicated.

3. Results

3.1. Immunological detection of C. parapsilosis UCP (CpUCP)

Polyclonal antibodies developed against potato UCP cross-react widely not only with various plant tissues or plant species [21] but also with the UCP of a primitive soil ameboid protozoan, *A. castellanii* [17]. These antibodies were used in total mitochondrial proteins to evidence the presence of UCP in *C. parapsilosis* (CpUCP). A single protein band with an appropriate similar molecular mass (32 000 Da) was revealed in green tomato fruit (control) and *C. parapsilosis*, indicating cross-reactivity of plant antibodies with the fungi protein (Fig. 1). A similar band was observed with monoclonal antibodies developed against *Arabidopsis thaliana* protein (not shown).

3.2. Determination of coupling parameters in C. parapsilosis mitochondria

In addition to the classical respiratory chain, *C. parapsilosis* possesses an alternative pathway, which differs from the AOX

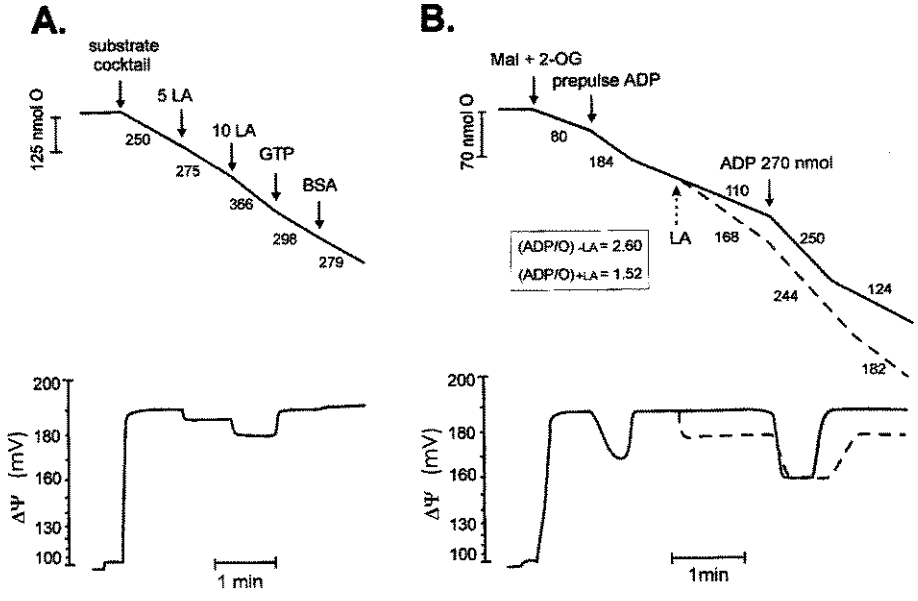

Fig. 2. Influence of LA on respiratory rates, coupling parameters and $\Delta\Psi$ of *C. parapsilosis* mitochondria depleted of FFAs. A: Mitochondria (0.5 mg of protein) were incubated in the presence of 1.5 mM BHAM, 1 μM CAT. Substrate cocktail (5 mM malate, 5 mM pyruvate, 5 mM succinate and 1 mM NADH); 5 or 10 μM LA; 2 mM GTP; and 0.5% BSA were added where indicated. B: Mitochondria (0.5 mg of protein) were incubated in the presence of 1.5 mM BHAM and in the presence (solid lines) or absence (dotted lines) of 15 μM LA. Additions: malate (5 mM)+2-oxoglutarate (5 mM), 120 μM (prepulse) and 207 μM ADP (pulse). The total amount of oxygen consumed during state 3 respiration was used for calculation of the ADP/O ratio. Numbers on the traces refer to O_2 consumption rates in nmol O/min/mg protein. Membrane potential changes are shown in mV.

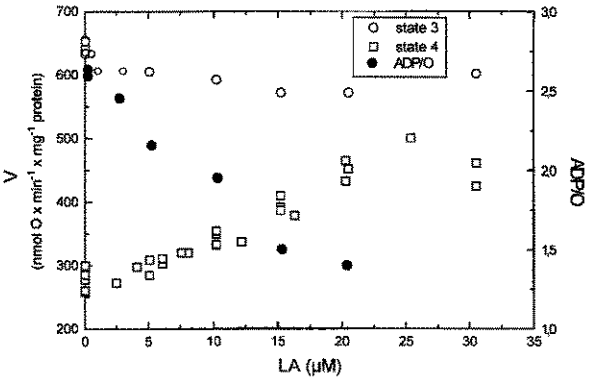

Fig. 3. Concentration dependence of LA effect on phosphorylating respiration (state 3), resting respiration (state 4) and ADP/O ratio. Effect of LA on respiratory rates (left abscissa axis, open symbols) were measured with mitochondria incubated with substrate cocktail (5 mM malate, 5 mM pyruvate, 5 mM succinate and 1 mM NADH), 1.5 mM BHAM, and 1 μM CAT. State 3 respiration was measured in the presence of 207 μM ADP or 2 mM ADP (saturating conditions). Increasing concentrations of LA (1–30 μM) were obtained by successive additions when the steady-state respiration rate was achieved. Several oxygen traces were needed to cover the full investigated range of LA concentrations. Effect of LA on ADP/O ratio (right abscissa, solid symbols) was measured with 5 mM malate+5 mM 2-oxoglutarate as oxidizable substrates as described in Fig. 2B.

well described in plants and some microorganisms. This second respiratory chain, fully energy-dissipating, parallels the classical one and has specific components: external NADH dehydrogenase, quinone (Qx), cytochrome *b*, cytochrome c_{alt} and alternative terminal oxidase sensitive to salicylhydroxamate. Two possible sites of electron partitioning with the main pathway exist, one upstream of complex III and the second at the level of the two cytochromes *c* [22]. Such network complexity leads to a wide range of ADP/O values and respiratory rates according to the reducing substrate and the electron route even in the presence of salicylhydroxamate. In a representative experiment, the ADP/O ratios were 1.08, 2.55, 0.79 and 0.49 and the state 3 rates were 276, 258, 594 and 428 nmol O/min/mg protein for malate+pyruvate, malate+2-oxoglutarate, substrate cocktail (malate+pyruvate+succinate+NADH) and NADH alone, respectively.

Substrate conditions were chosen to lead to the highest ADP/O value (malate+2-oxoglutarate) and to the highest respiratory rates (substrate cocktail) in order to describe the specific effects of linoleic acid (LA) in *C. parapsilosis* mitochondria (Fig. 2A,B). Simultaneous measurements of oxygen consumption and $\Delta\Psi$ in the presence of BHAM were performed with mitochondria depleted of endogenous FFA by isolation in the presence of BSA (see Section 2). State 4 respiration (plus carboxyatractyloside (CAT), plus BHAM) was stimulated by the successive addition of LA (+47% at 10 μM LA) with a parallel decrease in $\Delta\Psi$ from 190 to 180 mV (−5%) (Fig. 2A). The addition of 2 mM GTP partly reversed stimulation of respiration and restored $\Delta\Psi$. The subsequent addition of BSA slightly decreased respiration. Presence of both, GTP and BSA, totally cancelled the LA-induced stimulation of respiration. This stimulation in state 4 suggests the existence of a FFA-linked H^+ re-uptake in *C. parapsilosis* mitochondria. The equality between starting state 4 respiratory rate and the GTP+BSA-inhibited rate confirmed the absence of endogenous FFA in mitochondria. The ADP/O ratios and state 3 respiration were measured during ADP pulses (Fig. 2B) in the absence or presence of LA. Pulse duration was defined with the help of $\Delta\Psi$ measurements. LA (15 μM) increased the state 4 respiratory rate and decreased $\Delta\Psi$ in state 4 while not modifying respiration and $\Delta\Psi$ in state 3. Respiratory control and ADP/O were clearly lowered in the presence of LA, suggesting the LA-induced dissipation of the H^+ electrochemical gradient possibly through CpUCP.

3.3. Concentration dependence of LA effect

State 4 (with 1 μM CAT) and state 3 respiration were measured in the presence of BHAM, substrate cocktail and increasing concentration of LA (Fig. 3). State 4+CAT respiratory rates increased with LA concentration up to 85% of state 3 rates that remained almost constant. The same behavior of respiratory rates (increase in state 4+CAT, no increase in state 3) was observed in the other substrate conditions (not shown).

The effect of LA concentration on ADP/O ratios was determined with malate+2-oxoglutarate as oxidizable substrates. A decrease in the ADP/O ratio was observed with an increasing LA concentration up to 20 μM (Fig. 3). The effect of LA was also checked on the ADP/O ratios with the other substrates. LA decreased the ADP/O ratios in every condition in the following manner in a representative experiment: from 2.59 to 1.4 at 20 μM LA (with malate+2-oxoglutarate), from 0.89 to a value lower than 0.5 at 5 μM LA (with substrate cocktail), from 1.09 to 0.68 at 20 μM LA (with malate+pyruvate) and from 0.5 to a non-measurable value with NADH. These results mean that the LA-induced H^+ recycling can efficiently divert energy from oxidative phosphorylation in state 3 respiration even if the state 3 respiratory rates are not modified.

3.4. Voltage dependence of electron flux

Fig. 4 reports the relation between $\Delta\Psi$ and respiratory rate measurements with substrate cocktail and in the presence of

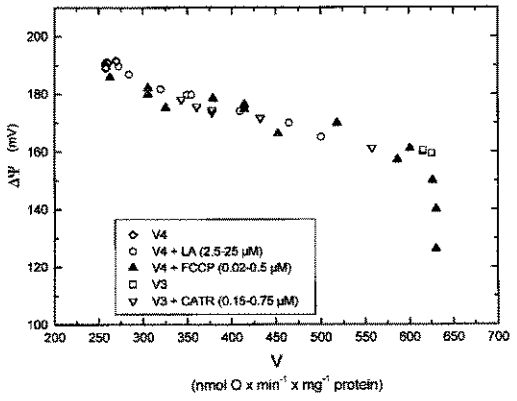

Fig. 4. Relation between $\Delta\Psi$ and mitochondrial respiration in the presence of BHAM. All measurements were made in the presence of substrate cocktail (5 mM malate, 5 mM pyruvate, 5 mM succinate and 1 mM NADH), 1.5 mM BHAM. State 4 (V4) was measured in the presence of 1 μM CAT. State 3 (V3) was reached by the addition of 2 mM ADP. (◇) State 4, (○) state 4 with increasing concentrations of LA (2.5–25 μM), (▲) state 4 with increasing concentration of FCCP (0.02–0.5 μM), (□) state 3, and (▽) state 3 with increasing concentration of CAT (0.15–0.75 μM).

BHAM in five conditions: (1) in state 4+CAT (◇), (2) in state 4+CAT with increasing LA concentration (from 2.5 to 25 μM) (○), (3) in state 4+CAT with increasing carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) concentration (0.02–0.5 μM) (▲), (4) in state 3 (□) and (5) in state 3 with increasing CAT concentration (0.15–0.75 μM) (▽). The maximum uncoupled respiration (at ≥0.2 μM FCCP) was 630 nmol O/min/mg protein, i.e. not higher than the state 3 respiratory rate (615–625 nmol O/min/mg protein), suggesting that ATP synthase activity is not a limiting factor in the oxidative phosphorylation rate with these oxidizable substrates (substrate cocktail). On the contrary, maximum respiratory rate measured with 25 μM LA (30 μM led to a decrease in rate) was lower (500 nmol O/min/mg protein) compared to FCCP- (fully uncoupled) and ADP- (fully coupled) induced respiratory rates, indicating that maximum electron flux was not reached with the LA-induced H^+ recycling system. However, a set of conditions (state 3 with increasing CAT concentration, state 4 with both FCCP and LA increasing concentrations) constituted a single force-flow relationship, indicating that LA had a pure protonophoretic effect like the artificial protonophore, FCCP.

4. Discussion

The results reported in this paper support the existence of the UCP-like protein in mitochondria from the pathogenic yeast *C. parapsilosis*. The immunodetection of CpUCP with antibodies raised against the UCP from potato mitochondria indicates a close molecular relationship not only with plant UCP but also with amoeba protein [17].

C. parapsilosis mitochondria fully depleted of endogenous FFA exhibit a stimulation of state 4 respiration linked to the addition of LA as well as a decrease in $\Delta\Psi$ in state 4, suggesting the existence of a FFA-induced H^+ re-uptake.

A single force-flow relationship is observed for state 4 respiration enhanced by LA or FCCP as well as for state 3 respiration progressively inhibited by CAT. Therefore, modulation of $\Delta\Psi$ either by CAT (increase in $\Delta\Psi$), by H^+ permeabilization with FCCP or by LA addition exerts the same control of the flow (oxygen consumption rate). These results prove the exclusive protonophoric effect of LA: namely that LA does not act directly on the respiratory chain activity (no intrinsic uncoupling or slips).

Stimulation of state 4 by LA is observed in the presence of CAT, an inhibitor of adenylic carrier. In such conditions, this carrier cannot participate in the FFA-induced H^+ re-uptake through FFA anion translocation. In state 4, respiration stimulated by 10 μM LA is inhibited by 2 mM GTP (60% of the LA-induced stimulation) and $\Delta\Psi$ lowered by LA is restored by GTP. As purine nucleotides are known to inhibit UCP-like proteins, it is likely that CpUCP is involved in the protonophoric uncoupling effect of LA either by LA^- translocation [23] or by LA-stimulated H^+ transport [24]. It seems that the sensitivity of CpUCP is similar to that of UCP in potato [25] and of UCP2 in mammals [24] ($K_i \approx 1$ mM).

Although as with tomato and amoeba *A. castellanii* mitochondria, in *C. parapsilosis* mitochondria, LA does not stimulate respiration in state 3 (which is probably limited by the rate of electron supply to the respiratory chain), its uncoupling effect is evidenced by a decrease in ADP/O ratio. Thus, the apparent insensitivity of state 3 respiration to LA is accompanied by an important decline in energy conservation by oxidative phosphorylation. It can be concluded that *C. parapsilosis* mitochondria possess a UCP-like protein (CpUCP) with uncoupling properties similar to those of the other members of the UCP family.

The discovery of UCP in *A. castellanii* [17], an ameboid protozoan positioned in the molecular phylogenetic tree of eukaryotes on a branch basal to the divergence points of plants, animals and fungi [26,27], has allowed us to hypothesize its existence in the whole eukaryotic world and to propose its emergence, as specialized proteins for H^+ recycling very early during phylogenesis [17]. The evidence presented in this paper of the existence of an UCP-like protein in the yeast *C. parapsilosis* demonstrates that the UCP family occurs also in the fungi kingdom even if absent from *Saccharomyces cerevisiae*. Moreover, *C. parapsilosis* also possesses a redox energy-dissipating respiratory chain quite complex when compared to plant and *A. castellanii* AOX. Thus, the presence of two energy-dissipating systems, that may alter the coupling between respiration and ATP synthesis, can be used to maintain a balance between supply of reducing substrates and energy and carbon demand for biosynthesis in the *Candida* cells; this could also hold true for every non-thermogenic tissue and monocellular [28]. Moreover, these two energy-dissipating systems can be postulated to take part in the defense system against reactive oxygen species in the living cell by decreasing the reducing power and the local oxygen concentration [29] and also to play a role in cell or tissue aging as shown in fruit ripening [19].

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3.3. 3° trabalho

Milani, G., Jarmuszkiewicz, W., Schreiber, A.Z., Vercesi, A.E. and Sluse, F.E. (2001) Respiratory chain network of *Candida parapsilosis*: ADP/O ratio appraisal of the multiple electron pathways. FEBS Letters **508: 231-235.**

Respiratory chain network in mitochondria of *Candida parapsilosis*: ADP/O appraisal of the multiple electron pathways

Graziela Milani[a,1], Wiesława Jarmuszkiewicz[b,1], Claudine M. Sluse-Goffart[c], Angelica Z. Schreiber[d], Anibal E. Vercesi[a], Francis E. Sluse[c,*]

[a] *Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-970 Campinas, SP, Brazil*
[b] *Department of Bioenergetics, Adam Mickiewicz University, Fredry 10, 61-701 Poznan, Poland*
[c] *Laboratory of Bioenergetics, Centre of Oxygen, Research and Development, Institute of Chemistry B6, University of Liège, Sart Tilman, B-4000 Liege, Belgium*
[d] *Laboratorio de Microbiologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-970 Campinas, SP, Brazil*

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Abstract In this study we demonstrated that mitochondria of *Candida parapsilosis* contain a constitutive ubiquinol alternative oxidase (AOX) in addition to a classical respiratory chain (CRC) and a parallel respiratory chain (PAR) both terminating by two different cytochrome *c* oxidases. The *C. parapsilosis* AOX is characterized by a fungi-type regulation by GMP (as a stimulator) and linoleic acid (as an inhibitor). Inhibitor screening of the respiratory network by the ADP/O ratio and state 3 respiration determinations showed that (i) oxygen can be reduced by the three terminal oxidases through four paths implying one bypass between CRC and PAR and (ii) the sum of CRC, AOX and PAR capacities is higher than the overall respiration (no additivity) and that their engagement could be progressive according to the redox state of ubiquinone, i.e. first cytochrome pathway, then AOX and finally PAR. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mitochondrion; Electron transport route; Alternative oxidase; *Candida parapsilosis*

1. Introduction

It has been recently shown that a cyanide-resistant respiration occurs frequently in yeasts, essentially in those incapable of aerobic fermentation [1]. In order to rationalize this finding, it has been proposed that the aerobic fermentation and the cyanide-resistant respiration are two strategies developed by yeasts as alternatives to the cytochrome pathway respiration, when the activity of this pathway is restricted, in order to allow catabolism to proceed. The cyanide-resistant ubiquinol alternative oxidase (AOX) is widespread in eukaryotes as higher plants, many fungi and some protozoa. It branches at the ubiquinone (Q) level bypassing two sites of energy conservation in the cytochrome pathway. The regulatory features of AOX differ among organisms [2–4]. In plant mitochondria, the activity of AOX is stimulated by α-keto acids and regulated by the redox state of the enzyme (oxidation/reduction of dimeric form). The fungal and protozoan AOX generally exists as monomer and is independent of organic acid stimulation but can be stimulated by purine nucleotides. Several functions have been assigned to AOX as the free redox energy-dissipating enzyme: participation in thermogenesis in spadices of Araceae, regulatory function when excess of reducing power occurs, response to various stress conditions in plants, and decreasing mitochondrial reactive oxygen species generation [2,5–8].

Candida parapsilosis is a parasitic non-fermentative yeast presenting a huge natural resistance to a large spectrum of antibiotics [9]. This resistance to drugs, acting on different mitochondrial functions, has been assigned to the appearance of two types of alternative electron flux pathways: (i) an inducible cyanide-resistant AOX branched at the level of Q, similar to the plant AOX, and (ii) a secondary parallel respiratory chain (PAR) involving alternative quinone (Qx), cytochrome *b* ($cyt c_{PAR}$), cytochrome *c* ($cyt c_{PAR}$), and terminal oxidase ($ox c_{PAR}$), insensitive to antimycin A (AA) and inhibited by amytal and a high concentration of salicylohydroxamate and cyanide [10–12]. It has been proposed that AOX and PAR drive electrons to oxygen from Krebs' cycle intermediates and from cytosolic NADH, respectively. It has been also hypothesized that partitioning of electron flux between the classical respiratory chain (CRC) and PAR could occur at the level of Q–Qx and cyt*c*–$cyt c_{PAR}$ (electron transfer would be from $cyt c_{PAR}$ to cyt*c* according to their redox potential) and that CRC and PAR activities could be additive, i.e. the sum of their capacities would be equal to the total measured respiration.

The present study demonstrates that in mitochondria of *C. parapsilosis*, AOX is constitutive and regulated in a fungi-type manner. We show that the AOX, PAR and CRC activities are not additive and that one bypass exists between PAR and CRC in the Q–oxygen span. We also determine the electron flux capacities and phosphorylation yield of various electron pathways.

*Corresponding author. Fax: (32)-4-366 2878.
E-mail address: f.sluse@ulg.ac.be (F.E. Sluse).

[1] G.M. and W.J. contributed equally to this work.

Abbreviations: AA, antimycin A; AOX, alternative oxidase; BHAM, benzohydroxamate; BSA, bovine serum albumin; CRC, classical respiratory chain; DTT, dithiothreitol; PAR, parallel respiratory chain; $cyt b_{PAR}$, cytochrome *b* of PAR; cyt*c*, $cyt c_{PAR}$, cytochromes *c* of CRC and PAR, respectively; $ox c_{PAR}$, terminal cyt*c* oxidase of PAR; Q, ubiquinone; Qx, alternative quinone

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2. Materials and methods

2.1. Cell culture and mitochondrial isolation

C. parapsilosis CCT 3834 (ATCC 22019) was grown at 37°C under vigorous aeration in complete liquid medium (2% glycerol, 2% Bactopeptone (Difco), 1% Bacto-yeast extract (Difco)) until middle stationary phase. Mitochondria were isolated and purified as described before [13]. Mitochondrial protein concentration was determined by the biuret method [14].

2.2. SDS–PAGE and immunoblotting of AOX

Mitochondrial protein was solubilized in the sample buffer (1% (w/v) SDS, 60 mM Tris–HCl, pH 6.8, 10% glycerol, 0.004% bromophenol blue, with or without reducing agent, 1 mM dithiothreitol (DTT)) and boiled for 5 min. Electrophoresis was carried out in a manner similar to that of Laemmli [15] using 5% polyacrylamide stacking gel and 12% polyacrylamide resolving gel, followed by Western blotting. Bio-Rad prestained low molecular mass markers were used. Antibodies against AOX proteins of *Sauromatum guttatum* (generously supplied by Dr. T.E. Elthon) were used at a dilution of 1:1000. AOX bands were visualized using the Amersham ECL system.

2.3. Oxygen uptake

Oxygen uptake was measured polarographically using a Clark-type electrode in 1.3 ml of standard incubation medium (28°C) containing: 125 mM sucrose, 65 mM KCl, 10 mM HEPES pH 7.2, 2.5 mM KH_2PO_4, 1 mM $MgCl_2$, plus or minus 0.2% bovine serum albumin (BSA) with 0.4–0.5 mg of mitochondrial protein. Concentrations of respiratory chain inhibitors: 4 μM AA (to block complex III of CRC), 1 or 10 mM KCN (to block cytc oxidases, complex IV or complex IV and oxc_{PAR}, respectively), 2 or 10 mM benzohydroxamate (BHAM) (to block AOX or AOX and $cytb_{PAR}$, respectively). Details of measurements are included in the legends of the figures. The ADP/O ratio was determined by the ADP pulse (0.17 mM) method. The total amount of oxygen consumed during state 3 respiration was used to calculate the ADP/O ratio.

3. Results and discussion

3.1. Regulation and constitutive character of AOX in C. parapsilosis mitochondria

The huge resistance to drugs of *C. parapsilosis* was attributed to activity of an inducible AOX and development of PAR in mitochondria. As in our study of *C. parapsilopsis* cells grown in the absence of drugs, any evidenced mitochondrial electron transport pathway is constitutive. As shown in Fig. 1A, respiration with external NADH was inhibited partly by 4 μM AA that blocks complex III of CRC. Oxygen consumption was further inhibited by 10 mM KCN that blocks the two terminal cytc oxidases of CRC and PAR. The remaining cyanide-resistant respiration was significantly stimulated by 0.6 mM GMP and fully inhibited by 2 mM BHAM, an inhibitor of AOX. These results indicate that the GMP-stimulated AOX activity is present constitutively in *C. parapsilosis* mitochondria. Moreover, the AOX protein of *C. parapsilosis* was immunodetected with antibodies raised against AOX protein of *S. guttatum* as shown in Fig. 1B. A single protein band, with a molecular mass around 38 kDa, was revealed both in the absence and presence of reducing agent (1 mM DTT) suggesting that AOX of *C. parapsilosis* exists as monomer.

In isolated mitochondria of *C. parapsilosis*, the AOX activity, measured as cyanide (10 mM)-resistant respiration, was not sensitive to DTT and pyruvate (not shown), while it was stimulated by GMP in concentration-dependent manner (Fig. 2A). The results presented in Figs. 1 and 2 clearly indicate that *C. parapsilosis* AOX does not reveal regulatory features of the plant-type oxidase that is stimulated by DTT and pyruvate inducing, respectively, a change in the redox state and a chemical modification (thiohemiacetal formation) of the enzyme at the level of a single cysteine [3–4]. Similarly to other tested fungal (*Neurospora crassa* and *Pichia stipitis*) and protozoan (*Acanthamoeba castellanii*) AOXs [16,17], monomeric AOX of *C. parapsilosis* is insensitive to organic acid stimulation but is stimulated by GMP. Stimulation of *C. parapsilosis* AOX by GMP reveals lower affinity ($S_{0.5}$ = 70 μM) (Fig. 2A) compared to *A. castellanii* AOX ($S_{0.5}$ = 30 μM) (Hryniewiecka, unpublished data).

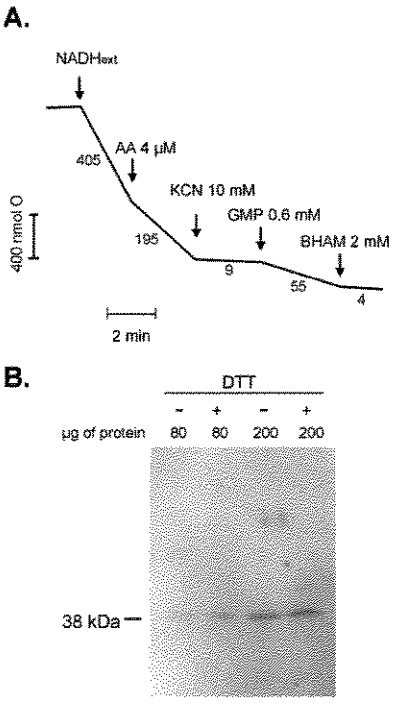

Fig. 1. Presence of constitutive AOX in *C. parapsilosis* mitochondria. A: Oxygen uptake was measured with external NADH (1 mM) as reducing substrate. Inhibitors: 4 μM AA, 10 mM KCN, and 2 mM BHAM were used to block complex III, the two terminal cytc oxidases (of CRC and PAR), and AOX, respectively. GMP (0.6 mM) was used to activate the AOX activity. Numbers on the trace refer to O_2 consumption rates in nmol O min^{-1} mg^{-1} protein. B: Immunodetection. Where indicated, 1 mM DTT was added to the sample buffer. Amount of protein loaded on lane is indicated.

On the other hand, as shown in Fig. 2B, the *C. parapsilosis* AOX activity was negatively regulated by linoleic acid, an abundant fatty acid. It has been shown recently that as in mitochondria of mammal brown adipose and some non-thermogenic tissues, many plants, and some protozoa [18,19], mitochondria of *C. parapsilosis* possess an uncoupling protein that enables free fatty acid-activated H^+ re-entry into matrix, dissipating the proton motive force in mitochondria [13]. The inhibitory effect of linoleic acid on cyanide-resistant respiration was found in some plants and *Hansenula anomala* [20–22]. However, the plant-type AOX is inhibited by a low concentration of free fatty acids [22]. Thus the sensitivity to linoleic acid of *C. parapsilosis* AOX ($I_{0.5}$ = 33 μM) (Fig. 2B) is lower than that of plant AOX (50% inhibition by 10 μM linoleic acid) [22], while protozoan *A. castellanii* AOX is insensitive to linoleic acid [23]. Different sensitivities of AOX activity to fatty acids among these organisms could reflect

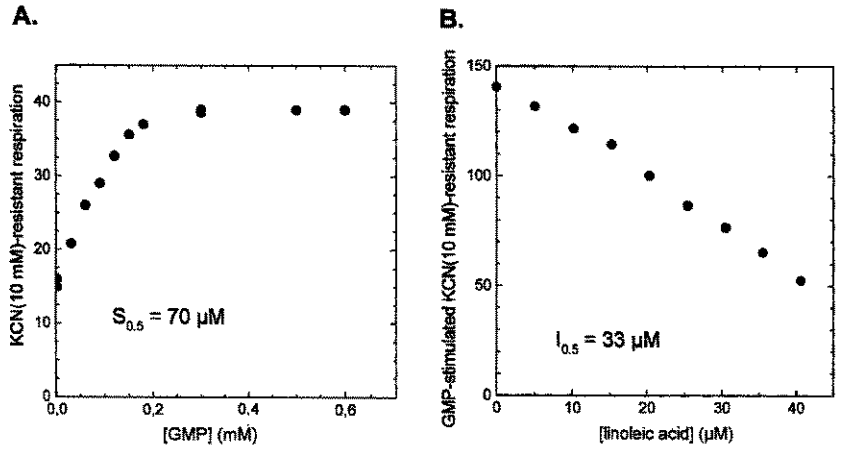

Fig. 2. Concentration dependence of GMP stimulation (A) and linoleic acid inhibition (B) of cyanide (10 mM)-resistant respiration. Oxygen uptake in the presence of 10 mM KCN (to exclude respiration through CRC and PAR) was measured (A) with 1 mM external NADH or (B) with cocktail of internal NADH substrates (5 mM 2-oxoglutarate+5 mM malate+5 mM pyruvate). Increasing concentrations of GMP and linoleic acid were obtained by successive additions when steady state respiration was achieved. When the sensitivity of the cyanide-resistant respiration to linoleic acid was measured (B), BSA (that chelates free fatty acids) was omitted in incubation medium. Respiratory rates are in nmol O min^{-1} mg^{-1} protein. The half maximum stimulation ($S_{0.5}$) of 10 mM cyanide-resistant respiration by GMP was 70 µM GMP and the half maximum inhibition ($I_{0.5}$) of 10 mM cyanide-resistant respiration by linoleic acid was 33 µM.

different functional connection between the two energy-dissipating systems, AOX and uncoupling protein.

3.2. Respiratory chain network in mitochondria of C. parapsilosis

3.2.1. The three terminal oxidases and the four electron transport pathways. As shown above, in mitochondria of *C. parapsilosis* grown in the absence of any drugs, AOX is constitutive. Thus, three terminal oxidases are present constitutively in these mitochondria, namely, AOX which is inhibited by 2 mM BHAM, the cytc oxidase (complex IV) which is inhibited by 1 mM KCN, and the parallel cytc_{PAR} oxidase which was claimed to be inhibited by high KCN and hydroxamate (BHAM) concentrations (10 mM) [11,12]. According to the electron partitioning between CRC and PAR previously proposed by Guérin et al. [14], four different electron pathways were possible from ubiquinol to oxygen: CRC, AOX, PAR, and the fourth pathway: cytb_{PAR} → cytc_{PAR} → cytc → complex IV. In this study, we verified activity of electron transport pathways in the mitochondrial respiratory network of *C. parapsilosis* using different inhibitors (and their concentrations). We evidenced that the fourth pathway is the following: complex III → cytc → cytc_{PAR} oxidase (oxc$_{PAR}$) (Fig. 3). Indeed, as shown in Table 1, the difference between the respiration in the presence of 2 mM BHAM+4 µM AA with and without 1 mM KCN was negligible (9 nmol O min^{-1} mg^{-1} protein), while the respiration in the presence of 10 mM BHAM and 1 mM KCN was significant (84 ± 10, S.E.M., nmol O min^{-1} mg^{-1} protein). This last result also indicates that 10 mM BHAM acts at the level of cyt*b* of PAR and not

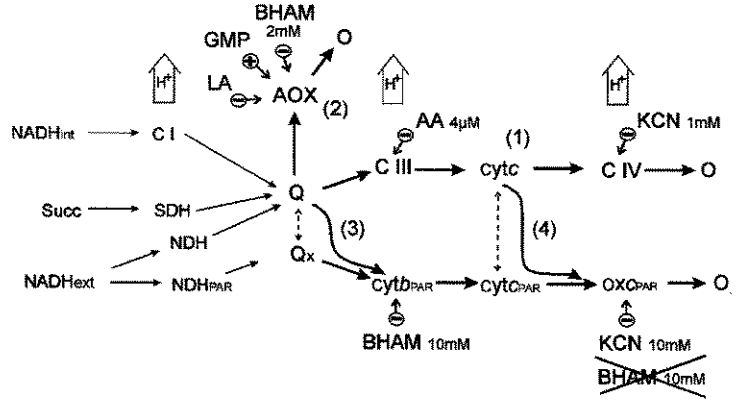

Fig. 3. Respiratory network of *C. parapsilosis* mitochondria: three terminal oxidases and four electron transport paths. Complex I (C I), complex III (C III) and complex IV (C IV, cytc oxidase) are the three complexes of CRC. Respiratory substrates: succinate, Succ; $NADH_{int}$, internal NADH; $NADH_{ext}$, external NADH. SDH, NDH, NDH_{PAR} are succinate and external NADH dehydrogenases. Components of the PAR: NDH_{PAR}, Qx, cytb_{PAR}, cytc_{PAR} (cytochrome *c*) and oxc$_{PAR}$ (second terminal cytc oxidase). Numbers between brackets are four electron transport paths, namely: (1) cytochrome pathway of CRC (C III → cytc → C IV), (2) AOX, (3) PAR (cytb_{PAR} → cytc_{PAR} → oxc$_{PAR}$) working when Q is very reduced, and (4) fourth pathway (C III → cytc → cytc_{PAR} → oxc$_{PAR}$) working when complex IV is blocked. Targets of inhibitors and activator are indicated by arrows. Dotted lines indicate putative interactions. Sites of H^+ pumping are shown.

Table 1
Quantitative analysis of electron routes to oxygen in *C. parapsilosis* mitochondria: ADP/O and respiratory capacity determinations

Conditions	ADP/O (±S.E.M.)	Electron routes to O_2 (according to numbering in Fig. 3)		Respiratory rates (±S.E.M.) (nmol O min^{-1} mg^{-1} protein)
		possible	effective	
No inhibitors+GMP 0.6 mM	2.06±0.08	1, 2, 3, 4	1, 2 (CRC+AOX)	466±47
BHAM 10 mM	2.49±0.05	1, 4	1 (CRC)	347±30
BHAM 10 mM+AA 4 μM (or +KCN 10 mM)	0	no	no	15±2
BHAM 2 mM+KCN 1 mM	1.49±0.06	3, 4	4 (CRC→oxc_{PAR})	84±8
BHAM 10 mM+KCN 1 mM	1.69±0.08	4	4 (CRC→oxc_{PAR})	84±10
KCN 10 mM+GMP 0.6 mM	0.68±0.03	2	2 (AOX)	166±15
KCN 10 mM+AA 4 μM+GMP 0.6 mM	0.74±0.05	2	2 (AOX)	155±21
BHAM 2 mM+AA 4 μM	0.90±0.07	3	3 (PAR)	52±2
BHAM 2 mM+AA 4 μM+KCN 1 mM	0.77±0.08	3	3 (PAR)	43±1
KCN 1 mM+AA 4 μM+GMP 0.6 mM	0.92±0.02	2, 3	2 (AOX)	147±8
BHAM 2 mM	2.33±0.10	1, 3, 4	1 (CRC)	365±42

Oxygen uptake was measured as described in Section 2 in the presence of three substrates feeding the respiratory chain at the level of complex I (5 mM 2-oxoglutarate+5 mM malate+5 mM pyruvate). Conditions of respiration measurements (i.e. presence of inhibitors and activator) are given in the first column. ADP/O was measured by ADP pulse (170 mM) method. Electron transport routes are numbered according to the scheme described in Fig. 3. Possible and effective electron routes to O_2 are given in the third and fourth columns. Data are average of 3–4 measurements (±S.E.M.).

at the level of the terminal oxidase of PAR as suggested by Guérin et al. [10–12]. Fig. 3 shows the current scheme of respiratory network of *C. parapsilosis* mitochondria.

3.2.2. Quantitative description of the four electron transport pathways. On the basis of electron transport pathway network and sensitivity to inhibitors (Fig. 3) we performed a quantitative description of electron flux capacities and phosphorylation yield of the different pathways in *C. parapsilosis* mitochondria. Using 11 conditions of incubation (Table 1), with substrates of complex I (2-oxoglutarate+malate+pyruvate), we determined ADP/O ratio values, respiratory rates, and effective main electron routes to oxygen. Table 1 presents results of experiments where the total respiratory rate (no inhibitors, +GMP) was 466±47 (S.E.M.) nmol O min^{-1} mg^{-1} protein and ADP/O was 2.06±0.08 (S.E.M.). In the presence of 10 mM BHAM, the ADP/O was 2.49±0.05 (S.E.M.) indicating that in these conditions the three coupling sites of CRC were used but not the fourth pathway. Thus, in the presence of 10 mM BHAM, the main pathway was CRC with a capacity of 347±30 (S.E.M.) nmol O×min^{-1}×mg^{-1} protein. When complex III was blocked by AA in the presence of 10 mM BHAM, the respiratory rate was very low and ADP/O not measurable. Similarly, the residual respiration was measured in the presence of 10 mM BHAM and 10 mM KCN. When complex IV was inhibited by 1 mM KCN and AOX by 2 mM BHAM (or when AOX and $cytb_{PAR}$ were blocked by 10 mM BHAM), the fourth pathway (CRC→cyt*c*→oxc_{PAR}) was forced to work (because of a high level of cyt*c* reduction) as can be concluded from the ADP/O (1.49±0.06 (S.E.M.) or 1.69±0.08 (S.E.M.)) implicating two coupling sites. The capacity of this fourth pathway was 84±10 (S.E.M.) nmol O min^{-1} mg^{-1} protein. When AOX was the main effective pathway (in the presence of 0.6 mM GMP and 10 mM KCN with or without 4 μM AA), ADP/O was 0.68±0.03 (S.E.M.) or 0.74±0.05 (S.E.M.) corresponding to the engagement of the first phosphorylation site and the capacity was 166±15 (S.E.M.) or 155±21 (S.E.M.) nmol O min^{-1} mg^{-1} protein. Under conditions when AOX was blocked by 2 mM BHAM, complex III by 4 μM AA, the respiratory capacities and ADP/O in the absence or presence of additionally added 1 mM KCN (that blocks complex IV) were similar (52±2 (S.E.M.) and 43±1 (S.E.M.) nmol O min^{-1} mg^{-1} protein, and 0.9±0.07 (S.E.M.) and 0.77±0.08 (S.E.M.), respectively) indicating that electron flux from PAR to complex IV (i.e. the fourth pathway of Guérin et al. [10–12]) was not efficient. Thus under these conditions, electron flux took place entirely through PAR reaching oxc_{PAR}. When complexes III and IV were blocked (by 4 μM AA and 1 mM KCN), ADP/O and capacity were characteristic of AOX acting with complex I (0.92±0.02 (S.E.M.) and 147±8 (S.E.M.) nmol O min^{-1} mg^{-1} protein) thereby indicating that PAR was not engaged yet, likely because of too low Q reduction level when AOX is engaged. Finally, in the absence of any inhibitor, the ADP/O ratio was 2.06±0.08 (S.E.M.) and in the presence of 2 mM BHAM the ADP/O ratio was 2.33±0.10 (S.E.M.). When compared to the control ADP/O (i.e. ADP/O measured when the main non-proton pumping pathways, AOX and PAR, were excluded), 2.49±0.05 (S.E.M.), these results mean that PAR is poorly engaged when complex III is active, even when AOX is blocked, and suggest that in the absence of inhibitors electrons are mainly shared out between AOX and the cytochrome pathway.

3.2.3. Additivity of the various electron transport pathways. Our results on the respiratory pathway capacities measured with isolated mitochondria are restricted to situations in which one of the activities is functioning while the other are blocked. Even if these measurements do not reflect the true contributions of respective electron pathways into overall respiration (because any change in one inevitably effects the others), they do allow relative comparisons. Thus, according to the results described in Table 1, the capacities of the three electron pathways are not additive. Indeed, the sum of the calculated general average capacities of CRC (359±19 (S.E.M.) nmol O min^{-1} mg^{-1} protein), AOX (156±8 (S.E.M.) nmol O min^{-1} mg^{-1} protein) and PAR (47±3 (S.E.M.) nmol O min^{-1} mg^{-1} protein) is 562±30 (S.E.M.) nmol O min^{-1} mg^{-1} protein and is higher than the overall measured respiration (466±47 (S.E.M.) nmol O min^{-1} mg^{-1} protein). Thus, at least with the tested respiratory substrates (i.e. substrates of complex I) and in our conditions of cell growing, the additivity of electron pathways is not observed.

Our results on respiratory network demonstrate that in mi-

tochondria of *C. parapsilosis*, grown without respiratory chain inhibitors, three terminal oxidases and four electron pathways leading to oxygen exist. Screening of the respiratory network by ADP/O determination with substrates of complex I indicates that the cytochrome pathway and AOX share the electrons of the Q pool. The capacity of the cytochrome pathway was more than twice the AOX capacity. On the other hand, engagement of PAR was only effective when both the cytochrome pathway and AOX were blocked, thus when the reduced state of quinone was very high. Moreover, the PAR capacity was only one tenth of the total respiration. We have also shown that the bypass between the cytochrome pathway and PAR occurred from cyt*c* to the terminal oxidase of PAR when complex IV is blocked, thus when cyt*c* is very reduced. Finally, it can be concluded that the capacities of three pathways (AOX, PAR and cytochrome pathway) are not additive, as their sum is higher than the total measured respiration, and that their engagement in the overall respiration could be progressive according to the redox state of Q: first cytochrome pathway, then AOX, and then PAR.

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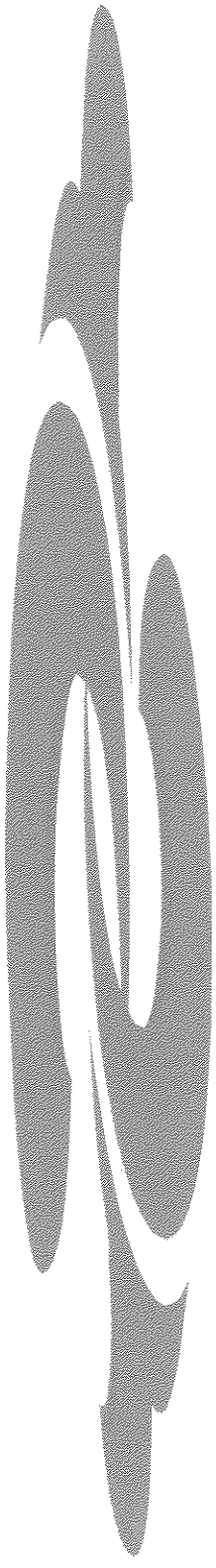


4. CONCLUSÕES

4.1. CONCLUSÕES DO 1° TRABALHO

Neste trabalho, a importância da preparação do material, especialmente a eliminação da parede celular das leveduras estudadas foi o fator limitante para a obtenção de resultados confiáveis e reprodutíveis. A obtenção de esferoplastos de *C. parapsilosis,* utilizando-se da enzima liticase, permeáveis a íons, nucleotídeos e substratos respiratórios sem afetar a integridade funcional das organelas intracelulares, em especial ass mitocôndrias, demonstrou que a liticase pode ser seguramente utilizada na obtenção de esferoplastos permeabilizados.

Foi demonstrada a presença de vacúolos ácidos que captam Ca^{2+} nessas leveduras. A nigericina, um ionóforo de K^+/H^+, e a bafilomicina A_1, inibidor da H^+ ATPase vacuolar, inibiram a captação de Ca^{2+} assim como liberaram o Ca^{2+} captado pelos esferoplastos de *C. parapsilosis*. Uma outra evidência verificada foi a alcalinização das vesículas intracelulares por Ca^{2+}. Os inibidores mitocondriais clássicos (antimicina A, mixotiazol e oligomicina) não tiveram efeito na captação de Ca^{2+} sugerindo que as mitocôndrias, embora capazes de produzir ATP e exercer outras funções (JARMUSZKIEWICZ *et al*, 2000 - 2° Trabalho) não apresentam participação ativa na homeostase de Ca^{2+} dessas células. A inibição da captação de Ca^{2+} pela bafilomicina A_1 assim como o aumento da acidificação vacuolar na presença de EGTA, que diminui a concentração de Ca^{2+} do meio, indicam uma forte relação entre a captação de Ca^{2+} e a acidificação vacuolar. A captação de Ca^{2+} foi insensível ao ortovanadato indicando que esse transporte não é mediado por uma P-type Ca^{2+} -ATPase e que provavelmente esta enzima não está presente nestes vacúolos aonde o transporte de Ca^{2+} parece ser mediado por um antiporter Ca^{2+}/nH^+ dependente do gradiente de Ca^{2+} e pH.

Nosso laboratório já havia anteriormente demonstrado, utilizando-se células de *T. brucei*, os efeitos do antioxidante BHT tanto nas H^+-ATPases quanto no antiporter Na^+/H^+ vacuolar desses tripanossomatídeos (VERCESI *et al*, 1997). Os efeitos observados não estão relacionados a alterações na fluidez ou viscosidade da camada bilipídica (MICHELANGELLI *et al*, 1990). O uso do BHT teve efeito inibitório na acidificação dos vacúolos presentes em *C. parapsilosis*, provavelmente devido ao seu efeito na H^+-ATPase, visto que a presença de um antiporter Na^+/H^+ não foi detectada nestas células.

Inúmeras hipóteses foram formuladas na tentativa de se explicar um possível papel fisiológico desse sistema integrado de transporte de Ca^{2+} e H^+ (H^+-ATPase e Ca^{2+} /nH^+), entre elas estão: regulação da concentração citosólica de Ca^{2+}, pH ou com a função de um sistema de estoque de Ca^{2+}, funcionando como uma reserva homeostática ou ainda como um sistema de desintoxicação para prevenir os efeitos deletérios que o excesso de Ca^{2+} livre no citossol pode causar quando as células são submetidas a um ambiente desfavorável.

Vacúolos ácidos contendo Ca^{2+} foram previamente descritos em *D. discoideum* (RONNEY & GROSS, 1992), muitos tripanossomatídeos (SHIGEMATSU *et al*, 1982; DOCAMPO et al, 1995) e em algumas células de mamíferos (THÉVENOD & SCHULTZ, 1988; THÉVENOD et al, 1989). A identificação da presença de vacúolos ácidos em *C. parapsilosis* pode servir como um alvo em potencial para o tratamento quimioterápico das infecções fúngicas visto que, a droga antimicótica cetoconazol induziu uma rápida alcalinização destes vacúolos (Fig. 5). Este foi um novo e particular efeito descrito para o cetoconazol, cuja ação antifúngica descrita até o momento se baseava apenas em seu mecanismo inibitório na síntese do ergosterol presente na parede celular (GHANNOUM & RICE, 1999).

Os vacúolos ácidos contendo Ca^{2+} identificados pela primeira vez em *C. parapsilosis* se assemelham aos vacúolos presentes em células de plantas (NELSON & HARVEY, 1999) e diferem daqueles presentes na maioria dos tripanossomatídeos (DOCAMPO & MORENO, 1999) devido a ausência da Ca^{2+}-ATPase, V-H^+-Ppase e do trocador Na^+/H^+.

4.2. CONCLUSÕES 2° TRABALHO

Os resultados apresentados neste trabalho dão suporte à existência de uma proteína desacopladora, tipo UCP, na mitocôndria da levedura patogênica *C. parapsilosis*. A imunodetecção da CpUCP utilizando-se anticorpos desenvolvidos contra UCP de mitocôndria de batata indica uma relação molecular muito próxima dessa UCP não apenas com a UCP de plantas, mas também com a proteína da ameba (JARMUSZKIEWICZ *et al*, 1999).

A mitocôndria de *C. parapsilosis* completamente livre de ácidos graxos endógenos (FFA) teve sua respiração em estado 4 estimulada, pela adição de ácido linoléico (LA) assim como uma diminuição no potencial de membrana mitocondrial ($\Delta\psi$) em estado 4 sugerindo a existência de uma recaptação de prótons (H^+) induzida por FFA.

Uma relação de fluxo de força única é observada na respiração em estado 4, complementada por LA ou FCCP, assim como para a respiração em estado 3 inibida por CAT. Entretanto, a modulação do $\Delta\psi$, tanto por CAT (aumento no $\Delta\psi$) quanto pela permeabilização à prótons induzida pela adição de LA, levam a uma mesma taxa de consumo de oxigênio. Esses resultados provam um exclusivo efeito protonofórico do LA, visto que o LA não possui ação direta na atividade da cadeia respiratória.

O LA produz uma estimulação do estado 4, na presença de CAT, um inibidor do transportador de nucleotídeos de adenina. Nessas condições esse transportador não pode participar da recaptação de H^+, induzida por FFA, através da translocação do ânion de FFA. Em estado 4, a respiração estimulada por 10 μM de LA é inibida por 2 mM GTP (60% da estimulação induzida por LA) e o $\Delta\psi$, diminuído pelo LA, é restabelecido pelo GTP. Os nucleotídeos de purina são conhecidos por inibirem as proteínas tipo UCP, sendo portanto muito provável que a CpUCP esteja envolvida no efeito desacoplador protonofórico mediado pelo LA, tanto por translocação do LA^- (JABUREK *et al,* 1999) quanto por transporte de H^+ estimulado por LA (KLINGENBERG & HUANG, 1999). A sensibilidade da CpUCP é muito semelhante a da UCP de batata (JEZEK, COSTA & VERCESI, 1996) e da UCP2 em mamíferos (KLINGENBERG & HUANG, 1999) ($K_i \approx 1$ mM).

Assim como nas mitocôndria de tomate e ameba *A. castelanii*, nas mitocôndrias de *C. parapsilosis* a respiração em estado 3 não é estimulada por LA (a qual está provavelmente limitada a velocidade de fornecimento de elétrons para a cadeia respiratória), o seu efeito desacoplador é apenas evidenciado devido a diminuição da razão ADP/O. A aparente insensibilidade da respiração em estado 3 ao LA é acompanhada por um importante declínio na conservação de energia pela fosforização oxidativa. Podemos concluir que a mitocôndria de *C. parapsilosis* possui uma proteína tipo UCP (CpUCP) com propriedades desacopladoras similares aos membros da família de UCP.

A descoberta de UCP em *A. castellanii* (JARMUSZKIEWICZ *et al,* 1999), um protozoário amebóide posicionado na árvore filogenética molecular dos eucariontes numa ramificação basal que diverge para os diferentes reinos das plantas, animais e fungos (WAINRIGTH *et al,* 1993; GRAY, BURGER & LANG, 1999) nos permitiu hipotetizar a existência de UCP em todo o universo eucariótico e a propor a emergência dessa proteína, especializada em reciclar H^+, muito cedo durante a filogênese (JARMUSZKIEWICZ *et al,* 1999). As evidências apresentadas neste trabalho em relação a existência de um proteína tipo UCP na levedura *C. parapsilosis* demonstram que a família das UCPs aparece também no reino dos fungos, mesmo que ausentes em *Saccharomyces cerevisiae*. Além disso, *C. parapsilosis* possuem uma cadeia respiratória com um sistema de dissipação de energia muito complexo quando comparado as oxidases alternativas de plantas e *A. Castellanii*. Assim, a existência de dois sistemas de dissipação de energia, que podem alterar o acoplamento entre a respiração e a síntese de ATP, podem ser usados com o objetivo de manter o balanço entre o suprimento de substratos redutores e energia e a demanda de carbono para biosíntese nas células de *Candida*; isso também pode ser extrapolado para todos os tecidos não termogênicos e unicelulares (SLUSE & JARMUSZKIEWICZ, 2000). Além disso, esses dois sistemas de dissipação de energia podem ser postulados como parte do sistema de defesa contra espécies reativas de oxigênio na célula viva reduzindo o seu poder redutor e a concentração local de oxigênio (SKULACHEV, V.P., 1998) e também desempenhar um papel no envelhecimento da célula ou tecido, como demonstrado no amadurecimento de frutos (ALMEIDA *et al*, 1999).

4.3. CONCLUSÕES 3° TRABALHO

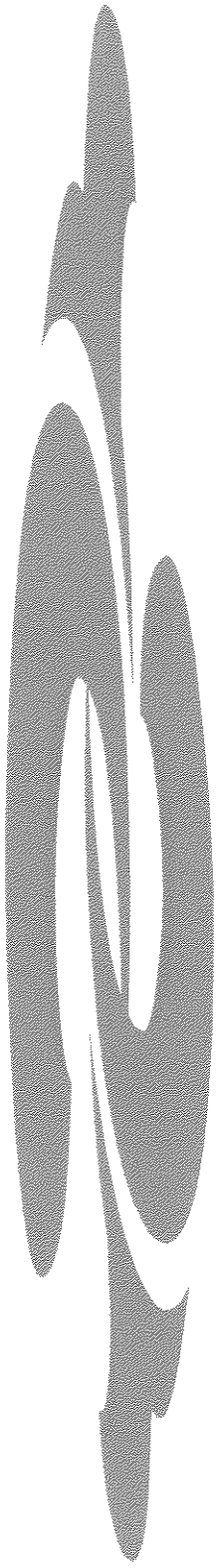
A larga resistência da *C. parapsilosis* as drogas é atribuída a atividade de uma oxidase alternativa (AOX) induzida e ao desenvolvimento de uma cadeia respiratória paralela (PAR) nas suas mitocôndrias. Em nossos estudos, as células de *C. parapsilosis* foram cultivadas na ausência de drogas, portanto qualquer evidência, tanto de AOX quanto de PAR, no transporte de elétrons pela mitocôndria pode ser considerada como constitutiva. De fato, os resultados apresentados neste trabalho, indicam a presença de uma AOX

constitutiva nessas mitocôndrias (detectada imunologicamente Fig. 1B) e também evidenciada através do estímulo da respiração por GMP (sustentada por NADH e acrescida de antimicina A, a qual bloqueia complexo III da cadeia respiratória dos citocromos – CRC) e pela completa inibição dessa respiração após a adição de 2 mM BHAM, conhecido inibidor da AOX (Fig. 1A).

Em mitocôndrias de *C. parapsilosis*, a atividade da AOX, medida através da respiração resistente ao cianeto (10 mM), se mostrou insensível ao DTT e piruvato, mas estimulada por GMP de uma maneira dose-dependente (Fig. 2A). Os resultados aqui apresentados indicam que a AOX de *C. parapsilosis* não apresenta uma regulação semelhante a AOX de plantas, que é estimulada por DTT e induzida por piruvato, respectivamente por uma mudança no seu estado redox ou por uma modificação química da enzima (formação de tiohemeacetal) a partir de uma única cisteína (SIEDOW & UMBACH, 2000; JOSEPH-HORNE, HOLLOMON & WOOD, 2001). Características semelhantes foram demonstradas em oxidases alternativas de outros fungos (*N. crassa* e *P. stipitis*) e protozoários (*A. castellanii*) (UMBACH & SIEDOW, 2000; JARMUSZKIEWICZ *et al*, 1997). Além da estimulação da AOX por GMP, a Fig. 2B mostra que a AOX, identificada em *C. parapsilosis,* é inibida por ácido linoléico (LA) um ácido graxo livre muito abundante.

As mitocôndrias de *C. parapsilosis*, cultivadas na ausência de inibidores da cadeia respiratória, possuem três oxidases terminais e quatro rotas de elétrons que levam ao oxigênio. Através da determinação das razões ADP/O, com substratos do complexo I, verificamos que a via dos citocromos e a AOX compartilham elétrons a nível do pool de ubiquinona. A capacidade da via dos citocromos é duas vezes maior do que a capacidade da AOX. Por outro lado, um aumento na capacidade da via paralela (PAR) é apenas eficaz quando tanto CRC e AOX estão bloqueadas, ou seja, quando o estado de redução da quinona é muito alto. A capacidade da PAR é apenas um décimo da respiração total. A interligação entre CRC e PAR ocorre entre o cyt*c* e a oxidase terminal da PAR, quando complexo IV está bloqueado, portanto quando o cyt*c* está muito reduzido. Para finalizar, concluímos que as capacidades das três vias (AOX, PAR e via dos citocromos) não são aditivas, diferente do que havia sido anteriormente postulado por GUÉRIN &

CAMOUGRAND, 1994, pois a sua soma é maior do que a respiração total medida e o seu engajamento na respiração total pode ser progressivo de acordo com o estado redox da ubiquinona: primeiro via dos citocromos, depois AOX e então PAR. A partir dos resultados descritos neste trabalho, foi proposto um novo modelo para a cadeia respiratória da *C. parapsilosis* e como CRC, PAR e AOX estão interligadas (Fig.3).

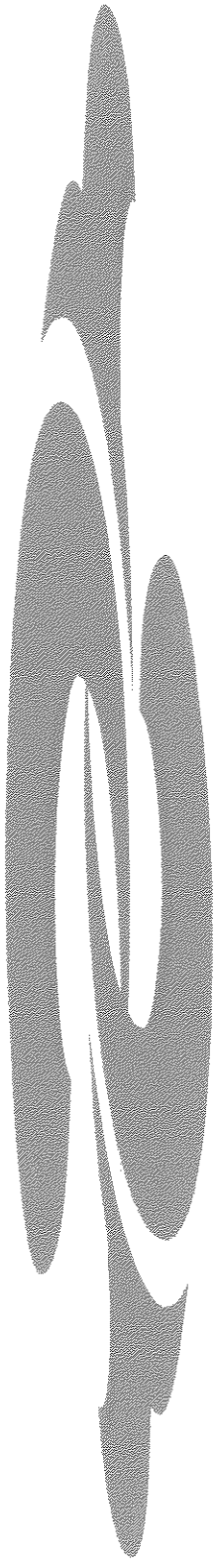


5. SUMMARY

In this thesis, we studied Ca^{2+} transport in permeabilized *Candida parapsilosis* spheroblasts prepared by a new technique using lyticase. An intracellular non-mitochondrial Ca^{2+}-uptake pathway, insensitive to orthovanadate and sensitive to the V-H^+-ATPase inhibitor bafilomycin A_1, nigericin and FCCP was characterized. Acidification of this Ca^{2+} compartment was followed using the fluorescent dye acridine orange. Acidification was stimtlated by the Ca^{2+} chelator EGTA and inhibited by Ca^{2+}. These results, when added to the observation that Ca^{2+} induces alkalization of a cellular compartment, provided evidence for the presence of a Ca^{2+}/nH^+ antiporter in the acidic compartment membrane. Interestingly, like in the acidocalcisomes of trypanosomatids, the antioxidant BHT inhibits the V-H^+-ATPase. In addition, the antifungal agent ketoconazole promoted a fast alkalization of this acidic compartment. Ketoconazole effects were dose-dependent and occurred in a concentration range close to that attained in the blood plasma of patients treated with this drug.

An uncoupling protein (UCP) was identified in mitochondria from *C. parapsilosis* (CpUCP), a non- fermentative parasitic yeast. Activity of CpUCP, investigated in mitochondria depleted of free fatty acids, was stimulated by linoleic acid (LA) and inhibited by GTP. Activity of CpUCP enhanced state 4 respiration by decreasing $\Psi\Delta$and lowered the ADP/O ratio. Thus, it was able to divert energy from oxidative phosphorylation. The voltage dependence of electron flux indicated that LA had a pure protonophoretic effect. The discovery of CpUCP proves that UCP-like proteins occur in four eukaryotic kingdoms: animals, plants, fungi and protists.

We have also demonstrated that mitochondria of *C. parapsilosis* contain a constitutive ubiquinol alternative oxidase (AOX) in addition to a classical respiratory chain (CRC) and a parallel respiratory chain (PAR) both terminating by two different cytochrome c oxidases. The *C. parapsilosis* AOX is characterized by a fungi-type regulation by GMP (as a stimulator) and linoleic acid (as an inhibitor). Inhibitor screening of the respiratory network by the ADP/O ratio and state 3 respiration determinations showed that (i) oxygen can be reduced by the three terminal oxidases through four paths implying one bypass between CRC and PAR, (ii) the sum of CRC, AOX and PAR capacities is higher than the overall respiration (no additivity) and that their engagement could be progressive according to the redox state of ubiquinone, i.e. first cytochrome pathway, then AOX and finally PAR.



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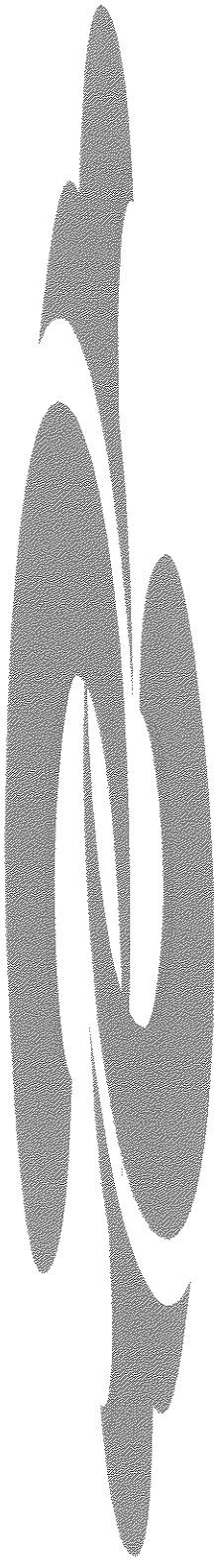

7. ANEXOS

CURRICULUM VITAE

1. INFORMAÇÕES PESSOAIS

1.1. Nome: Graziela Milani Narezzi

1.2. Filiação: Marcos Antonio Milani

Edna Casagrande Milani

1.3. Naturalidade: São Paulo, SP, Brasil

1.4. Data de Nascimento: 07/09/1972

1.5. Residência e Domicílio: Vila Residencial Park Avenida, rua dois, 32 13338-000 Indaiatuba – SP Tel: 19 3894-6006

1.6. e-mail grazielamilani@terra.com.br

2. ESCOLARIDADE

2.1. 1979-1986

1º Grau

Colégio Sagrado Coração de Jesus, São Paulo, SP

2.2. 1987-1989

2º Grau

Colégio São Luís, São Paulo, SP

2.3. 1990-1994

Bacharelado em Ciências Biológicas

Modalidade Médica-Escola Paulista de Medicina (UNIFESP-EPM)

2.4. 1997-1998

Mestrado em Biologia Molecular

Departamento de Bioquímica

Universidade Federal de São Paulo (UNIFESP / EPM)

Orientador: Prof. Anibal Eugênio Vercesi

3. AUXÍLIOS RECEBIDOS

3.1. Bolsa de Iniciação Científica CNPq/PIBIC/EPM (agosto/1992 - julho/1993)

3.2. Bolsa de Iniciação Científica FAPESP (janeiro/1994 - dezembro/1994)

3.3. Bolsa de Mestrado em Biologia Molecular CNPq (fevereiro/1995 - agosto/1995)

3.4. Auxílio-Ponte FAEP/UNICAMP (novembro/1997)

3.5. Bolsa de Doutorado CAPES (março/1998 em andamento)

3.6. Auxílio Viagem para participação em Congresso no Exterior FAEP/UNICAMP (Fevereiro 2000) US$ 1.356,00

3.7.Auxílio Viagem para participação em Congresso no Exterior CPG/FCM/UNICAMP (Setembro 2000) US$ 600,00

4. ESTÁGIOS

4.1. Estagiário no Laboratório de Anatomia

Departamento de Morfologia

Escola Paulista de Medicina

Janeiro/1991 à junho/1993

Professores Responsáveis Dr. Ricardo L. Smith

Dr. Marco A. de Angeli

4.2. Estagiário no Laboratório de Biologia Molecular

Departamento de Bioquímica

Escola Paulista de Medicina

Julho/1993 à Outubro/1993

Professor Responsável: Dra. Lucia O. Sampaio

4.3. Estagiário no Laboratório de Bioenergética

Departamento de Bioquímica

Universidade Estadual de Campinas

Janeiro/1994 à Janeiro/1995

Professor Responsável: Dr. Aníbal E. Vercesi

5. TRABALHOS APRESENTADOS EM CONGRESSOS

5.1. **Milani, G.**; de Angelis, M.A. e Smith, R.L. (1993). Morfologia da retina do Ituí-cavalo (*Apteronotus albifrons*). XVI Congresso Brasileiro de Anatomia. VII Congresso luso Brasileiro de Anatomia, Escola paulista de Medicina. (comunicação oral) São Paulo, SP, *Livro de Resumos número 233*

5.2. **Milani, G.**; de Angelis, M.A. e Smith, R.L. (1993). Morfologia da retina do Ituí-cavalo (*Apteronotus albifrons*). I Congresso Acadêmico de Iniciação Científica, São Paulo, SP, 25 à 29 de Outubro. *Livro de Resumos, pg 95*

5.3. **Milani, G.;** Gadelha, F.R.; Thonson, L.; Radi, R. and Vercesi, A.E (1995). Mitochondrial Membrane potential and Calcium Transport in Permeabilized Walker 256 Tumor Cells; Effect of prooxidants. XXIV Reunião anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Maio 6-9. *Livro de Resumos, pg 23* (A5)

5.4. Thonson, L.; **Milani, G.**; Radi, R.; Vercesi, A. and Gadelha, F.R. (1995) Effect of Peroxynitrite on Mitochondrial Functions and Calcium Transport by *Trypanossoma Cruzi* Epimastigotes. XXIV Reunião anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Maio 6-9. *Livro de Resumos, pg* 23 (A6)

5.5. **Milani, G.**, Catisti, R. and Vercesi, A.E. (1997) Mitochondrial Membrane Potential and Calcium Transport in Permeabilized Walker 256 Tumor Cells: effect of NAD(P^+) redox state. VI Simpósio Internacional de Medicina Ortomolecular. São Paulo, 13 a 15 de agosto.(comunicação oral 19). *Livro de Resumos, pg* 141.

5.6.- **Milani, G.,** S.T.O. Saad and Vercesi, A.E. (1998) Bcl-2 may protect Walker 256 tumor cells against the citotoxic effects of Ca^{2+} and oxidative stress. Reunião anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Maio 23-26. *Livro de Resumos, pg* 194.

5.7. **Milani, G.,** S.T.O. Saad and Vercesi, A.E. (1998) Bcl-2 may protect Walker 256 tumor cells against the citotoxic effects of Ca^{2+} and oxidative stress. 10^{th} European Bioenergetics Conference, 27 Junho - 2 Julho, Göteborg, Sweden. *Livro de Resumos, pg 167, Abstract published on Biochimica et Biophysica Acta, vol 10.*

5.8. **Milani, G.,** Bernardes, C.F., Schreiber, A.Z., Barros-Mazon, S. and Vercesi, A.E. (1999) Mitochondrial respiration and oxidative phosphorilation in *Candida parapsilosis* spheroblasts. Reunião anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Maio 1999. *Livro de Resumos, pg 0*4.

5.9. **Milani, G.,** Kowaltowski, A.J., Saad, S.T.O., Metze, K. and Vercesi, A.E. Characteristics of mitochondrial Ca^{2+} uptake in two tumor cell lines with distinct Bcl-2 expressions. Biophysical Society, New Orleans, U.S.A. (12-16 de Fevereiro de 2000). *Abstract published on Biophysical Journal* (Plataform 824, pg 140 A)

5.10. Jarmuszkiewicz, W.; **Milani,G.;** Fortes, F.; Schreiber, A.Z.; Sluse, F.E. and Vercesi, A.E. (2000) First evidence and characterization of an uncoupling protein in fungi kingdom: CpUCP of *Candida parapsilosis.* XXIX Reunião anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Maio 2000. *Livro de Resumos, pg* 01 (A 2)

5.11. Sluse, F.E.; Jarmuszkiewicz, W.; **Milani, G.**; Fortes, F.; Schreiber, A.Z. and Vercesi, A.E. (2000) First evidence and characterization of an uncoupling protein in fungi kingdom: CpUCP of *Candida parapsilosis*. The 175th Meeting of The Belgian Society of Biochemistry and Molecular Biology (BsBMB), 26 de Maio de 2000, Leuven, Belgica.

5.12. **Milani, G.** Schreiber, A.Z. and Vercesi, A.E. (2000) Ca^{+2} transport in acidic vacuoles of the pathogenic yeast *Candida parapsilosis.* 11th European Bioenergetics Conference, 09 Setembro - 14 Setembro, Sussex, England. *Livro de Resumos, pg* 205 (I-27). *Abstract published on Biochimica et Biophysica Acta, vol 11.*

5.13. **Milani, G.,** Jarmuszkiewicz, W., Schreiber, A.Z., Vercesi, A.E. and Sluse, F.E. (2001) Respiratory chain network of *Candida parapsilosis*: ADP/O ratio appraisal of the multiple electron pathways. XXX Reunião anual da Sociedade Brasileira de Bioquímica e Biologia Molecular, Maio 2001. *Livro de Resumos, pg.*

6. PUBLICAÇÕES EM REVISTAS ESPECIALIZADAS

6.1. Jarmuszkiewicz, W.; **Milani,G.;** Fortes, F.; Schreiber, A.Z.; Sluse, F.E. and Vercesi, A.E. (2000) First evidence and characterization of an uncoupling protein in fungi kingdom: CpUCP of *Candida parapsilosis.* FEBS Letters, **467**: 145-149

6.2. **Milani, G.,** Kowaltowski, A.J., Saad, S.T.O., Metze, K. and Vercesi, A.E. (2001) Walker tumor cells express larger amounts of the antiapoptotic protein Bcl-2 and presents higher resistance to toxic concentrations of Ca^{2+} than the tumor cells K 562 Drug and Development and Research, **52**: 508-514.

6.3. **Milani, G.,** Schreiber, A.Z and Vercesi, A.E. (2001) Characterization of Ca^{2+} uptake by an intracellular acidic compartment in *Candida parapsilosis* FEBS Letters, **500**: 80-84.

6.4. **Milani, G.,** Jarmuszkiewicz, W., Schreiber, A.Z., Vercesi, A.E. and Sluse, F.E. (2001) Respiratory chain network of *Candida parapsilosis*: ADP/O ratio appraisal of the multiple electron pathways FEBS Letters **508: 231-235**.

7. INFORMAÇÕES COMPLEMENTARES

7.1. Prova de Qualificação para Defesa de Tese de Doutorado

Apresentada e aprovada em: 11 de Maio de 2000

Titulo: Acidocalcisomas em *Candida parapsilosis*: um possível alvo quimioterápico.

7.2. Fluência de fala, leitura e escrita em Inglês e Italiano.