UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

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ESTUDO DA MODULAÇÃO E DO PAPEL FUNCIONAL DA ENZIMA LISINA-CETOGLUTARATO REDUTASE EM MILHO

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Genética e Biologia Molecular na área de Genética Vegetal e Melhoramento.

Orientador: Prof. Dr. Paulo Arruda

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pelo apoio, pelo carinho...

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AASA	Ácido α-aminoadípico-δ-semialdeído
Ask1	Asparto quinase insensível à retroinibição por lisina
b-ZIP	Motivo básico-zíper de leucinas.
cDNA	DNA complementar ao RNA
gcn4	Mutação "*general control of nitrogen 4*"
cyPPDK-1	Gene que codifica a piruvato ortofosfato diquinase 1 citossólica
DAP	Dias após a polinização
De-B30	Mutação "*defective endosperm B30*"
dek	Mutação "*defective kernel*"
DHDPS	Dihidrodipicolinato sintase
fl2	Mutação "*floury2*"
GAD	Glutamato decarboxilase
GCN4	Proteína ou fator GCN4
HSD	Homoserina desidrogenase
lg	Mutação "*indeterminate gamatophyte*"
kDa	Unidade de peso molecular aparente de proteínas
LKR	Lisina-cetoglutarato redutase
LYS1	Sacaropina desidrogenase, formadora de lisina
Lys1	Gene que codifica a SDH de levedura, formadora de lisina
LYS9	Sacaropina desidrogenase, formadora de glutamato
Lys9	Gene que codifica a SDH de levedura, formadora de glutamato
mc	Mutação "*mucronate*"
O2	Loco Opaco2
o2	Mutação "*opaco2*"
O2	Proteína ou fator Opaco2
o6	Mutação "*opaco6*"
o7	Mutação "*opaco7*"
Rnase	Ribonuclease
SDH	Sacaropina desidrogenase
ZLYSSDH	Gene que codifica a LKR/SDH de milho

RESUMO

As sementes de cereais podem ser consideradas fontes de alimento com baixa qualidade nutricional, devido à carência no aminoácido essencial lisina. O baixo nível de lisina é o resultado de uma complexa interação entre o metabolismo deste aminoácido e a biossíntese das proteínas de reserva da semente. Existem fortes evidências de que o nível de lisina livre nas células do endosperma é limitante para a síntese de proteínas ricas neste aminoácido. Por muito tempo se acreditou que a retro-inibição exercida pela lisina sobre enzimas-chave envolvidas em sua síntese fosse o fator crítico no controle da sua disponibilidade na semente. Entretanto, estudos recentes indicam que o controle do catabolismo de lisina é um fator determinante no controle do nível desse aminoácido na semente.

A lisina é catabolisada nos eucariotos superiores através da "via da sacaropina", cujos dois primeiros passos são catalisados pelas atividades enzimáticas da lisina cetoglutarato redutase (LKR) e sacaropina desidrogenase (SDH). Em milho, à semelhança do que ocorre em mamíferos, estas duas atividades residem em um único polipeptídeo bifuncional, cuja forma nativa é um dímero. Para caracterizar a função e modulação desta enzima, foram realizados diferentes ensaios durante o transcurso desta tese. Foi demonstrado que o domínio LKR é seletivamente ativado por Ca^{2+}, alta concentração de sal, solventes orgânicos e Mg^{2+}. O aumento da atividade de redutase dependente de Ca^{2+} pode ser inibida por antagonistas de calmodulina, indicando que o Ca^{2+} modula a atividade da enzima via calmodulina ou diretamente via algum domínio com semelhança estrutural à calmodulina. Experimentos de proteólise limitada foram conduzidos para determinar a relação estrutura-função da LKR/SDH. A digestão com elastase separou a enzima bifuncional de 125 kDa em dois polipeptídeos de 65 e 57 kDa, contendo os domínios funcionais de LKR e SDH, respectivamente. A proteólise não afetou a atividade de SDH, enquanto que a de LKR foi inativada em função do tempo de proteólise e da concentração de elastase. Após o aumento do tempo de incubação ou aumento na concentração de elastase, a atividade de LKR foi recuperada parcialmente, indicando a presença de um domínio inibitório da atividade de LKR na enzima inteira. Polipeptídios isolados contendo atividade de

SDH, obtidos por proteólise limitada e cromatografia de troca iônica, inibiram a atividade de polipeptídeos contendo atividade de LKR. Os peptídeos contendo atividade de LKR mantiveram a propriedade de serem ativados por Ca^{2+}, mas ficaram insensíveis a alta concentração de sal.

Um clone de cDNA, designado de *ZLKRSDH*, codificador da enzima LKR/SDH foi isolado de sementes em desenvolvimento de milho. O polipeptídeo predito a partir da seqüência nucleotídica indica que a anzima possui o domínio LKR na porção N-terminal e o SDH no C-terminal similares às proteínas monofuncionais LYS1 e LYS9 de leveduras, respectivamente. A proteína LKR/SDH está localizada no citoplasma das camadas celulares de subaleurona do endosperma. Transcritos do gene *ZLKRSDH* e polipeptídeos de LKR/SDH apresentam acúmulo durante estágios intermediários do desenvolvimento do endosperma, que é acompanhado pelas atividades de LKR e SDH. Esta análise foi conduzida em sementes de milho normal e *opaco2*. No endosperma mutante, os níveis de mRNA foram reduzidos em mais de 90%, com concomitante redução do acúmulo de polipeptídeos e das atividades de LKR e SDH. Isto sugere que o nível de LKR/SDH no endosperma é controlado em parte pelo ativador transcricional Opaco2. Além disto, o mutante *opaco2*, conhecido por conter um endosperma rico em lisina, mostrou uma redução severa da atividade de redutase (~18 vezes menor que no endosperma selvagem), o que não ocorreu com a atividade de desidrogenase (~2 vezes menor). Estas diferenças no comportamento das duas atividades provavelmente decorre de diferenças no padrão de dimerização e fosforilação na proteína nativa. De fato, o tratamento da LKR/SDH com fosfatase alcalina leva a perda da atividade de LKR devido a defosforilação. Esta atividade pode ser recuperada após tratamento com caseína quinase I e II, mas apenas na presença de lisina. Por outro lado, a atividade de redutase é encontrada apenas em dímeros, enquanto que a atividade de desidrogenase é encontrada em dímeros e monômeros. Verificou-se que o Ca^{2+} desempenha papel importante na dimerização da enzima. Um modelo de modulação da enzima é descrito e o papel de um putativo zíper de leucinas e um "EF-hand" de baixa afinidade no domínio LKR é discutido.

SUMMARY

Cereal seed are considered a poor source of protein because of the relative low content of lysine in its endosperm. The low lysine content of the endosperm is generally associated with the high accumulation of zein, which is known to contain small amounts of lysine. There are several evidences that free lysine is limiting for the synthesis of high-lysine proteins. Recent studies indicated that lysine catabolism is the rate limiting step that controls the free lysine levels in the cereal seed. A better understanding of lysine catabolism is important for future genetic manipulation which would lead to an overproduction and accumulation of lysine in plants.

The saccharopine pathway is utilized to degrade excess lysine in higher eukaryotes. Lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH) are enzymatic activities that catalyze the first two steps of this pathway. In maize, these enzymatic activities reside on a single bifunctional polypeptide, the native form of which is a dimeric protein. The LKR domain of the bifunctional enzyme was shown to be activated by Ca^{2+}, high salt concentration, organic solvents and Mg^{2+}. The Ca^{2+}-dependent enhancement of LKR activity was inhibited by the calmodulin antagonists W7 and calmidazolium, indicating that the enzyme activity is modulated by a calmodulin or that Ca^{2+} binds the enzyme directly by a calmodulin-like domain. Limited proteolysis was used to assess the structure-function relationship of the enzyme. Digestion with elastase separated the bifunctional 125 kDa polypeptide into two polypeptides of 65 and 57 kDa, containing the functional domains of LKR and SDH, respectively. Proteolysis did not affect SDH activity, while LKR showed a time- and protease- concentration-dependent inactivation followed by reactivation. The SDH-containing polypeptides inhibited the enzymatic activity of LKR-containing polypeptides. When separated from the SDH domain by limited proteolysis and ion exchange chromatography, the LKR domain retained its Ca^{2+} activation property, but was no longer activated by high salt concentrations. These results suggest that the LKR activity of the native enzyme is normally inhibited such that after modulation, the enzyme

undergoes a conformational alteration to expose the catalytic domain for substrate binding. Next, we have isolated a cDNA clone, designated *ZLKRSDH*, encoding the LKR/SDH protein from maize. The predicted polypeptide has an N-terminal LKR domain and a C-terminal SDH domain that are similar to the yeast LYS1 and LYS9 monofunctional proteins, respectively. The maize LKR/SDH protein is located in the cytoplasm of subaleurone endosperm cell layers. Transcripts, polypeptides, as well as enzyme activities showed a parallel upregulation and downregulation during endosperm development. The developmental expression of *ZLKRSDH* was examined in normal and *opaque2* maize seeds. In the mutant endosperm, mRNA levels were reduced by more than 90%, with a concomitant reduction on polypeptide levels and LKR/SDH activity. These results suggest that lysine levels in the endosperm are likely to be controlled, in part, at the transcriptional level by the Opaque2 transcription factor. The maize mutant *o2*, known to have a lysine-rich endosperm, shows a severe impairment in reductase activity (~18 fold lower compared to the wild type levels) which is not paralleled by a similar effect on dehydrogenase activity (~2 fold reduction). The differences in the behavior of these two activities are probably the result of distinct phosphorylation and dimerization patterns exhibited by the native enzyme. The dephosphorylated enzyme, which lacks reductase activity, recovered its activity upon treatment with casein kinases I and II, but only in the presence of lysine. The reductase activity is present only in dimers, whereas the dehydrogenase activity is present in dimers and monomers. Ca^{2+} plays an important role in the dimerization of this bifunctional enzyme. A model of the dimeric LKR/SDH is described and the possible roles of a leucine-zipper and a low-affinity EF-hand in the reductase domain are discussed.

INTRODUÇÃO

Dentre as plantas cultivadas, os cereais são as mais importantes considerando a área plantada, a produção e a contribuição para a alimentação humana e animal. Em 1998, mais de 691 milhões de hectares foram cultivados com cereais, gerando uma produção final de 2094 milhões de toneladas de grãos. Três espécies contribuíram com 85% deste total: Trigo (588 milhões de toneladas produzidas), arroz (563 milhões de toneladas produzidas) e milho (604 milhões de toneladas produzidas)(FAO, 1998). O grande sucesso no cultivo de cereais deve-se a vários fatores, entre eles a sua alta produtividade, facilidade de colheita e a capacidade de espécies e cultivares de se adaptarem a diferentes condições climáticas (Lazzeri e Shewry, 1993).

O principal produto resultante do cultivo de cereais é, sem dúvida, o grão, apesar de caules e folhas serem utilizados para silagem. Em termos botânicos, o grão é uma cariopse, tipo de fruto em que a parede da semente (testa) encontra-se fundida com a parede do fruto (pericarpo). A semente é constituída de dois órgãos, o endosperma triplóide e o embrião diplóide. O endosperma, o principal órgão de reserva, é diferenciado em dois tecidos na semente madura – o endosperma amiláceo e a camada de aleurona. O endosperma amiláceo é um tecido morto, envolvido pela aleurona, uma camada celular que continua viva após a maturação da semente, atuando como fonte de enzimas hidrolíticas durante a germinação (Lazzeri e Shewry, 1993).

As sementes contêm três grupos principais de substâncias de reserva. Nos cereais, o principal é o amido, que, usualmente, compreende 60 a 70 % do peso seco da semente. Já proteínas e lipídeos correspondem de 8 a 12% e 2 a 8%, respectivamente (Bewley e Black, 1978). Embora o conteúdo de proteínas seja relativamente pequeno, este componente tem importância vital para a alimentação humana, uma vez que o homem consome em média mais proteína proveniente de cereais do que de origem animal ou mesmo de outras espécies vegetais. Entretanto, as proteínas dos cereais são consideradas de

reduzido valor biológico, pois são deficientes em alguns aminoácidos essenciais. A lisina, é um destes aminoácidos, que por ser um dos componentes essenciais da dieta humana e de animais monogástricos o torna especialmente importante do ponto de vista científico e tecnológico.

1. ORIGEM, DESENVOLVIMENTO E FUNÇÃO DO ENDOSPERMA:

O estudo do desenvolvimento do endosperma é importante para a caracterização dos diferentes tipos celulares deste tecido de armazenamento, permitindo também estabelecer a correlação entre expressão de genes e proteínas ao longo do desenvolvimento das diferentes estruturas celulares do endosperma e da semente como um todo.

1.1. Origem e desenvolvimento:

O envolvimento de cada uma das duas células espermáticas do tubo polínico em eventos de fusão separados com as células ovo e central caracteriza o processo de fertilização dupla, um evento descoberto há um século e que só ocorre em plantas superiores (Figura 1) (Nawaschin, 1898; Guinard, 1899; revisado por Russell, 1992). Este processo dá início à formação do embrião e do endosperma. A fusão de um núcleo espermático com a célula-ovo, origina o zigoto que se desenvolverá no embrião. Ao mesmo tempo, o núcleo primário do endosperma é formado pela fusão do segundo núcleo espermático com um núcleo polar e a posterior fusão desta com o segundo núcleo polar da célula central (Randolph, 1936). Este processo resulta na formação de um tecido triplóide, o endosperma. Discute-se duas hipóteses a respeito do processo de fertilização dupla. A primeira postula que o endosperma deriva de um embrião gêmeo "altruísta", que se desenvolveria em corpo de armazenamento. Na segunda, o endosperma resultaria de uma extensão no desenvolvimento do gametófito feminino, que seria iniciada quando a célula central fosse fertilizada pelo segundo núcleo espermático (revisadas em Friedman, 1994). A primeira hipótese é corroborada por evidências descobertas no gênero *Ephedra*, que representa um elo evolutivo

entre gimnospermas e angiospermas. A dupla fertilização nestas plantas resulta em embriões gêmeos, sugerindo que o endosperma seria originado a partir de um deles (Friedman, 1994). A dupla fertilização confere ao endosperma as vantagens da heterose, assim como pode ser também um importante mecanismo na manutenção de diferenças epigenéticas que determinam o desenvolvimento do endosperma e do embrião, baseado nos diferentes níveis de ploidia destes tecidos (revisado em Lopes e Larkins, 1993).

O nível de ploidia do endosperma é determinante para o seu desenvolvimento, como demonstrado através do uso do mutante *ig/ig* (gametófito indeterminado), onde não há controle preciso do número de núcleos na célula central (Lin, 1984). Os resultados indicaram que apenas endospermas de constituição (♀:♂) 2x:1x e 4x:2x possuem desenvolvimento normal, enquanto que o endosperma de constituição 3x:1x tem subdesenvolvimento, e todas as outras constituições fazem com que o endosperma seja abortado (Lin, 1984). Estas observações confirmaram a predição de Sarkar e Coe (1971) de que a triploidia é uma condição essencial para o desenvolvimento do endosperma e a inferência de que a relação 2:1 entre cromossomos maternos e paternos é essencial neste tecido (Nishiyama e Inomata, 1966). Estes resultados indicam claramente que dosagem de genes é um fator crucial para o desenvolvimento do endosperma.

O milho apresenta o padrão nuclear mais comum de desenvolvimento do endosperma. Segundo este padrão, o núcleo primário do endosperma se divide entre 2 e 4 horas após completada a fusão e os núcleos resultantes migram para a parte basal do saco embrionário, adjacente ao zigoto (Figura 1). Até cerca de 12 horas após a singamia, ou 36 a 48 horas após a polinização, a segunda e a terceira divisões nucleares ocorrem e os 4 ou 8 núcleos resultantes ficam distribuídos na periferia da zona micropilar do saco embrionário (Figura 1). O endosperma continua o processo intermitente de divisões mitóticas sem citocinese por um período de 72 a 84 horas após a polinização. À medida que as divisões mitóticas ocorrem, os núcleos migram

em direção às antípodas, porém permanecendo sempre organizados na periferia do endosperma (Figura 1). A partir do estágio em que estão presentes de 128 a 256 núcleos livres, há o início da celularização, que também se inicia pela extremidade micropilar do saco embrionário, avançando em direção às antípodas, partindo da periferia para o centro do endosperma incipiente (Figura 1). Aos 4 dias após a polinização (DAP) o endosperma está completamente celurizado, excetuando-se a região adjacente às antípodas (Figura 1)(Randolph, 1936; Vijayaraghavan e Prabhakar, 1984).

O endosperma se desenvolve muito rapidamente em direção ao interior do tecido nucelar, iniciando uma rápida elongação entre 2 e 3 DAP. As antípodas continuam no ápice do endosperma e parecem desempenhar um papel importante para o crescimento do endosperma por entre o tecido nucelar. Conforme o endosperma aumenta de tamanho, o tecido nucelar é digerido para permitir tal crescimento (Figura 2)(Randolph, 1936). O endosperma até 8 ou 9 DAP é essencialmente indiferenciado e consiste de células parenquimatosas de tamanho e forma variados (Figura 3). Não há uma camada epidérmica especializada e as divisões celulares ocorrem por todo o tecido. Gradualmente, as divisões celulares tendem a se concentrar na periferia do endosperma e o crescimento da região interna é determinado pelo aumento de volume das células (Figura 3)(Randolph, 1936). Entre 12 e 16 DAP, muitas camadas de células são responsáveis pelas divisões celulares na região periférica. As células da camada epidérmica sofrem seguidas divisões periclinais, formando verdadeiras colunas celulares dirigidas da periferia para o centro do tecido. A partir de 17 DAP são raras as divisões celulares na epiderme. A partir deste estágio, esta camada celular única pode ser chamada de aleurona e as células adjacentes, de sub-aleurona. Por outro lado, divisões celulares periclinais podem ser observadas na sub-aleurona até 45 - 48 DAP (figura 3)(Randolph, 1936).

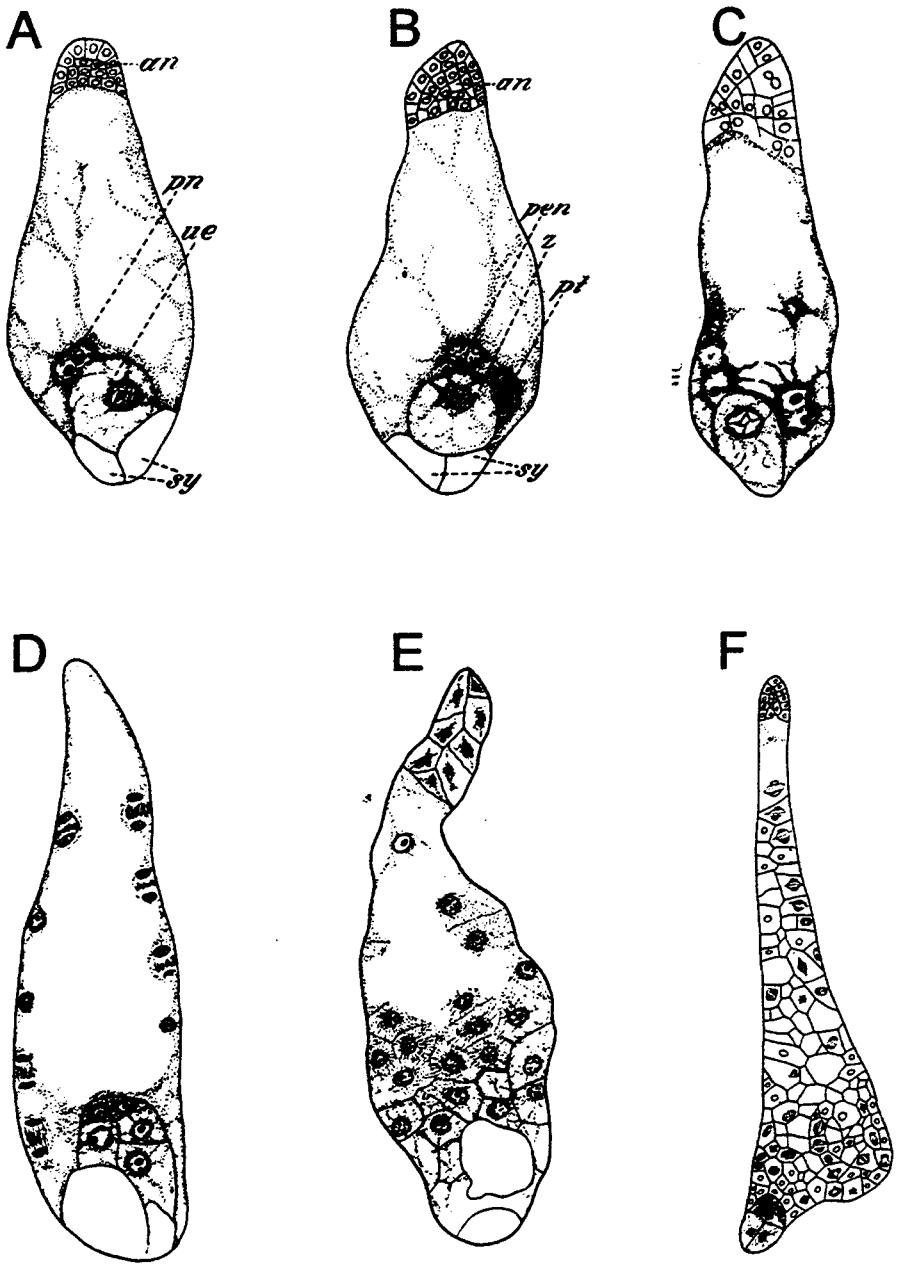

Figura 1. Saco embrionário antes e depois da fertilização e estágios iniciais do desenvolvimento do endosperma. **A**. Secção radial longitudinal do saco embrionário antes da fertilização: *an*, antípodas; *pn*, núcleo polar ainda não fusionado; *ue*, ovo não fertilizado; *sy*, sinérgides. **B**. Secção radial longitudinal do saco embrionário após a fertilização: *an*, antípodas; *pen*, núcleo primário do endosperma; *z*, zigoto (ovo fertilizado); *pt*, resquícios do tubo polínico; *sy*, sinérgides. **C**. Secção longitudinal do saco embrionário 36 horas após a polinização, mostrando o zigoto e o endosperma no estágio de oito núcleos livre. **D**. Secção longitudinal mostrando o endosperma durante divisão nuclear entre o estágio de 128 para 256 núcleos livre 72 horas após a polinização. **E**. Secção oblíqua-longitudinal de um endosperma passando do estágio de núcleo livre para a fase celular 84 horas após a polinização. **F**. Secção longitudinal média 4 dias após a polinização, mostrando o endosperma quase todo celular. Adaptado de Randolph, 1936.

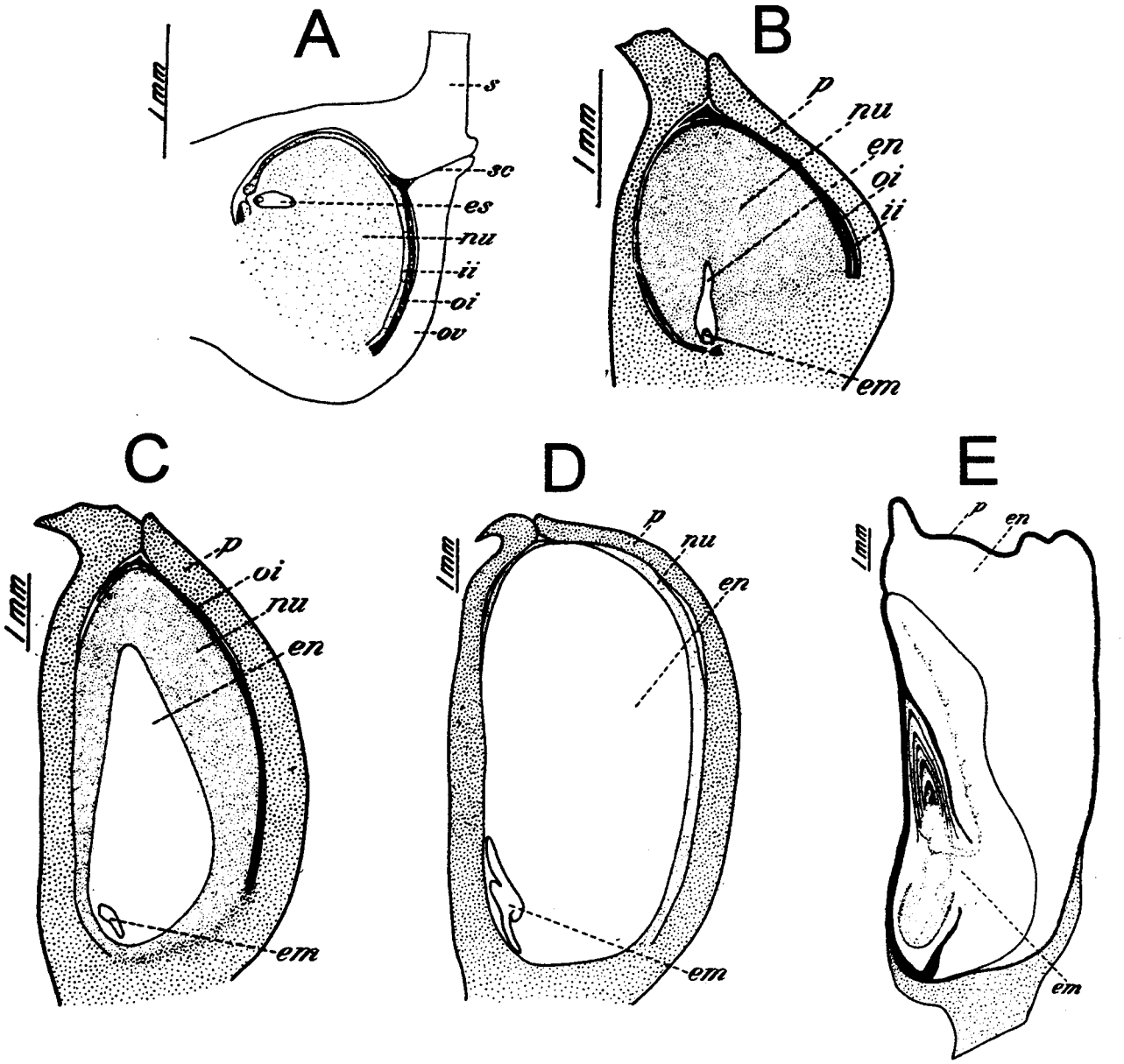

Figura 2. Estágios de transformação do pistilo em cariópse. **A.** Secção radial longitudinal do ovário maduro. **B.** 4 dias após a polinização; o embrião e o endosperma já iniciaram o desenvolvimento, mas a estrutura do ovário é essencialmente a mesma do momento da fertilização. **C.** 10 dias após a polinização; o cariópse jovem aumentou consideravelmente de tamanho. Os integumentos estão desaparecendo e o endosperma está tomando rapidamente o lugar do tecido nucelar. **D.** 18 dias após a polinização; o cariópse continua a aumentar de tamanho. Os integumentos virtualmente desapareceram e o tecido nucelar está nos estágios finais, antes de desaparecer por completo. **E.** Semente morfologicamente madura 45 dias após a polinização, consistindo essencialmente do embrião, endosperma e pericarpo. A camada de aleurona na periferia do endosperma é denotada como uma linha pontilhada justo abaixo do pericarpo. P, pericarpo; nu, tecido nucelar; oi, integumento externo; ii, integumento interno; en, endosperma; em, embrião; ov, ovário; sc, canal estilar; s, estilete; es, saco embrionário. Adaptado de Randolph, 1936.

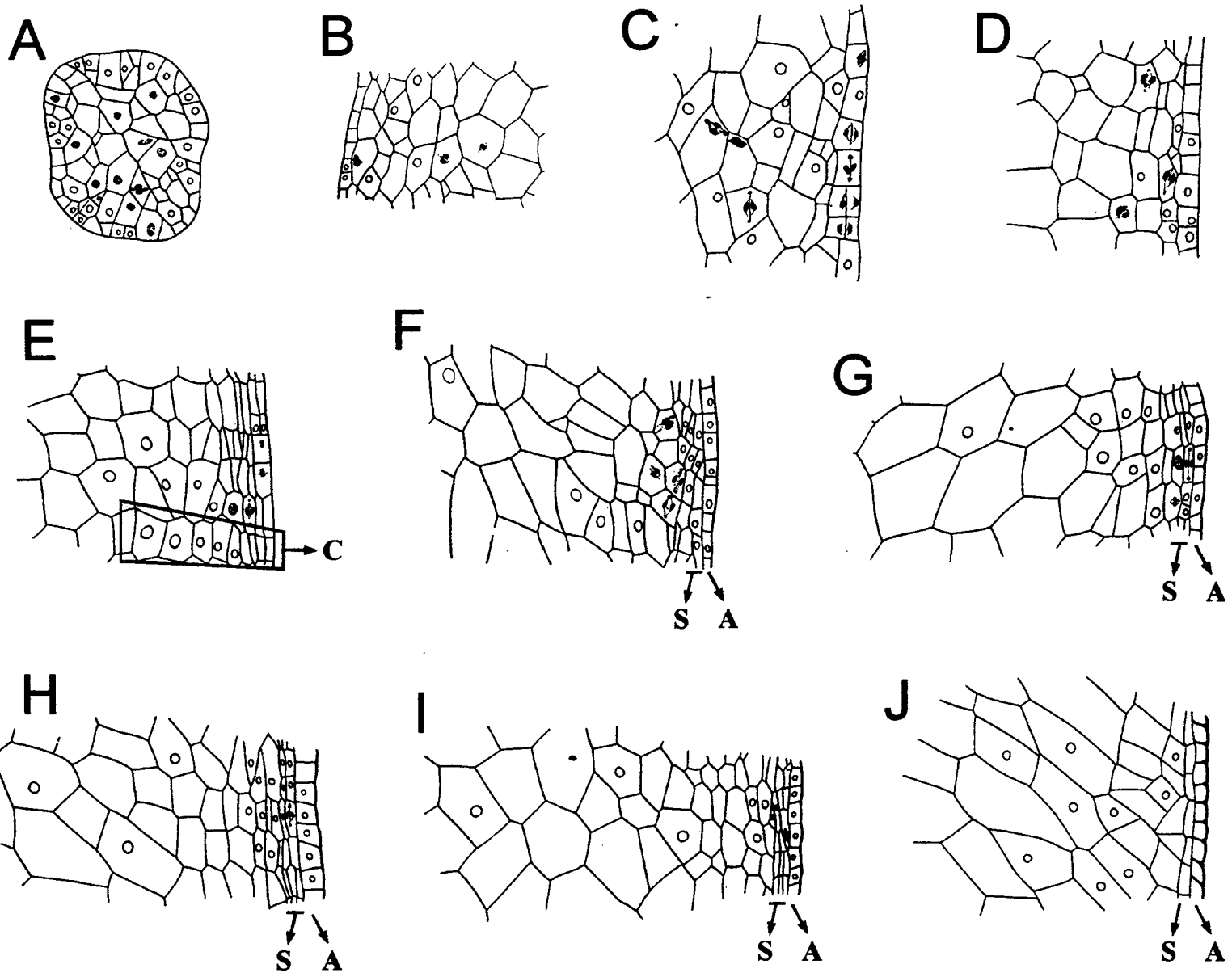

Figura 3. Estrutura do desenvolvimento do endosperma em intervalos de 6 a 55 dias após a polinização, mostrando o crescimento e o progresso da diferenciação. **A.** Secção mediana do endosperma aos 6 DAP. **B.** 9 DAP. **C.** 11 DAP. **D.** 16 DAP. **E.** 18 DAP. **F.** 21 DAP. **G.** 26 DAP. **H.** 36 DAP. **I.** 48 DAP. **J.** 55 DAP. Durante este período do desenvolvimento, a epiderme do endosperma é formada, a qual também é chamada de camada de aleurona após os 18 DAP. c, colunas de células; a, camada de aleurona; s, camada de subaleurona. Adaptado de Randolph, 1936.

A aleurona é um tecido formado por células morfológica e funcionalmente distintas das outras células do endosperma. Após a maturação da semente, apenas estas células continuam vivas e, durante a germinação, são induzidas à síntese de enzimas hidrolíticas por um estímulo mediado por ácido giberélico produzido pelo embrião. Estas enzimas catalisam a degradação das macromoléculas de reserva, tais como: amido, proteínas, outros polissacarídeos e DNA que foram acumulados no endosperma (Fincher e Stone, 1986; Fincher, 1989). Já a sub-aleurona aparentemente é responsável pelo processo de divisão celular após 18 DAP e parece ser o local onde ocorre o início da deposição de proteínas e amido. A deposição de proteínas e amido ocorre como um gradiente, da região de sub-aleurona para o interior do endosperma, o que se reflete no padrão de maturação e diferenciação celular (Lending e Larkins, 1989).

Durante o desenvolvimento do endosperma ocorre um aumento considerável no conteúdo de DNA nuclear, que se dá através de duplicações cromossômicas endonucleares sem mitose. No milho, entre 10 e 20 DAP, o conteúdo de DNA passa de 3 vezes o conteúdo do genoma haplóide para até mais de 600 vezes. O papel da endoreduplicação ainda não foi completamente estabelecido. Uma hipótese é que este fenômeno serviria para o armazenamento de nucleotídeos a serem utilizados posteriormente no desenvolvimento da plântula. Entretanto também tem sido sugerido que este processo leva à amplificação de genes envolvidos na síntese de proteínas de reserva e de amido. Porém, estes genes não são amplificados preferencialmente, i.e., o genoma todo é copiado em ciclos repetidos de replicação (Lopes e Larkins, 1993; Müller et al., 1995), indicando que este suposto mecanismo de amplificação seria ineficiente ou que o aumento do número de cópias possui uma função adicional, como estoque de nucleotídeos.

Muitos aspectos da diferenciação do endosperma ainda não são compreendidos. Até o momento não foram identificadas as moléculas ou eventos que ativam os controles bioquímicos sobre polaridade, divisão, formato e tipo celular, bem como aqueles controlando a poliploidização do genoma ou

mesmo o acúmulo de nutrientes no endosperma. O desenvolvimento da semente resulta das interações entre o endosperma, o embrião e os tecidos maternais (Lopes e Larkins, 1993). Algumas questões ainda permanecem: "Que tipo de trocas bioquímicas ocorrem entre endosperma, embrião e os tecidos maternais, e como estas trocas afetam o desenvolvimento integrado destes tecidos?" "Existe um programa genético que determina a forma, a função, e a integração entre estes tecidos?" (Lopes e Larkins, 1993). Os trabalhos de Kermicle e Alleman (1990) apontam para diferenças na atividade dos genomas oriundos das partes materna e paterna, sugerindo a possibilidade de que muitos aspectos do desenvolvimento do endosperma, bem como sua interação com o embrião e os tecidos maternais sejam definidos por *impriting* genético diferencial.

Ainda, analisando cerca de 285 diferentes mutantes *dek* (defective kernel), Neuffer e Sheridan (1980) observaram que a maioria destes *loci* afeta o embrião e o endosperma e que apenas uma pequena parte deles seria específico do endosperma. Assim, um grande número de genes específicos da semente seria expresso no endosperma e no embrião, mesmo que estes tecidos sejam muito diferentes dos pontos de vista morfológico e fisiológico.

1.2. Função do endosperma – armazenamento de energia e nutrientes

O endosperma parece possuir função diferenciada em monocotiledôneas e dicotiledôneas. No primeiro grupo, possui a função de armazenar as reservas de nutrientes para a germinação da semente e o estabelecimento da nova planta. Entretanto, nas dicotiledôneas, o endosperma também é considerado como um meio que propicia a embriogênese, nutrindo o embrião desde os estágios iniciais e auxiliando no seu posicionamento na semente. Na maioria das dicotiledôneas, o endosperma é inteiramente assimilado durante a embriogênese, sendo os cotilédones os tecidos de armazenamento de nutrientes para a germinação (Lopes e Larkins, 1993).

1.2.a. Carbohidratos

Nos cereais, as reservas de carbohidratos do endosperma são fundamentais para prover ao embrião uma fonte de energia e carbono até que a plântula possa realizar fotossíntese. Nestas espécies, como na maioria das plantas, o amido é o composto de armazenamento de carbohidratos. O amido é sintetizado a partir da sacarose, tão logo esta alcança o endosperma em desenvolvimento, e é, concomitantemente, depositado na forma de grânulos (revisado em Lopes e Larkins, 1993). No endosperma de milho, a transcrição dos genes responsáveis por enzimas chaves da via de síntese de amido é máxima entre 14 e 22 DAP e seu padrão de expressão parece ser coordenadamente regulado com a síntese de proteínas de reserva (Giroux et al., 1994). Apesar de existirem evidências para a regulação transcricional dos genes envolvidos nesta via, até o momento não foi identificado qualquer fator regulatório. Dentre as várias mutações que afetam o metabolismo de carbohidratos na semente de milho, até o momento nenhuma delas foi associada a tais fatores (Lopes e Larkins, 1993). Por outro lado, é possível que esta via seja diretamente regulada pelo fluxo de fotossintatos para o endosperma através de um mecanismo de indução via sacarose. Açúcares como glicose, frutose e sacarose podem induzir a expressão gênica da patatina em batata (Park, 1992), bem como agir como moduladores da interação fonte e dreno na folha de milho e como retro-reguladores da expressão gênica no aparelho fotossintetizador (Foyer, 1988; Sheen, 1990).

1.2.b. Proteínas de reserva:

As sementes dos cereais constituem a maior fonte de proteínas para a nutrição animal e humana, apesar de suas frações protéicas constituírem apenas 10% do peso seco da semente (Payne, 1983). O endosperma contém um variedade de proteínas. Algumas possuem funções metabólicas e estruturais, tais como enzimas, proteínas nucleares e componentes da parede

celular e da membrana citoplasmática. Outras, presentes em grandes quantidades, possuem função de reserva.

As proteínas de reserva tem como única função armazenar nitrogênio, carbono e enxofre para a germinação. Elas se caracterizam por uma composição peculiar de aminoácidos, sendo ricas em asparagina, glutamina e arginina ou prolina, mas deficientes em lisina, treonina e triptofano nos cereais, e em cisteína, metionina e triptofano, nas leguminosas. Elas são geralmente encontradas em vacúolos especializados ou em vesículas do retículo endoplasmático rugoso (Pernollet e Mossé, 1983; Higgins, 1984).

As proteínas de reserva podem ser caracterizadas em dois grandes grupos. O primeiro, consiste de globulinas e albuminas que estão presentes em todas as espécies com sementes. O outro grupo, exclusivo dos cereais, é o das prolaminas. Em milho, onde são chamadas de zeínas, as prolaminas representam 50-60% da proteína total do endosperma (revisado em Shotwell e Larkins, 1989).

As zeínas são sintetizadas por polissomos ligados à membrana do retículo endoplasmático rugoso e transportadas para seu lúmem, que aumenta de tamanho e se fissiona para formar os corpúsculos protéicos (Larkins e Dalby, 1975; Larkins e Hurkman, 1978). O acúmulo de zeínas começa logo após a queda do índice mitótico do endosperma, quando suas células iniciam o processo de poliploidização (Feix e Quayle, 1993), e a taxa máxima de biossíntese acontece geralmente ao final da terceira semana após a polinização, coincidindo com a fase de expansão das células do endosperma (Pernollet, 1985). Isto parece refletir o acúmulo dos transcritos de zeínas, que cresce exponencialmente a partir de 10 DAP para alcançar um máximo em torno dos 20 DAP, decrescendo até a maturação da semente (Marks et al., 1985). A biossíntese destas proteínas parece ser controlada principalmente na transcrição, observando-se, nos diferentes estágios de desenvolvimento do endosperma, uma estreita correlação entre a taxa de síntese de cada classe e os níveis do seu respectivo RNA mensageiro (Higgins, 1984; Kodrzycki et al., 1989; Feix e Quayle, 1993). Em decorrência disto, as pesquisas a respeito dos

mecanismos que regulam a expressão das zeínas estão direcionadas principalmente para a identificação de seqüências de DNA atuando em *cis* e fatores nucleares atuando em *trans* no controle da transcrição dos genes destas proteínas de reserva.

O estudo da taxa de acumulação das proteínas de reserva e de suas quantidades finais em diferentes mutantes fenotípicos de semente permitiram a descoberta de vários genes que regulam a biossíntese destes polipeptídeos. Mutações nestes genes reguladores têm drásticos efeitos quantitativos e qualitativos no perfil das prolaminas da semente e, freqüentemente, são também acompanhadas por significativas mudanças em algumas das outras frações protéicas. Em milho, este último fato geralmente acarreta um vantajoso aumento no conteúdo de lisina no endosperma, às custas de um decréscimo no conteúdo protéico total e na queda de produtividade (revisado por Lopes e Larkins, 1993).

Várias mutações que afetam a síntese de zeínas podem estar associadas a loci reguladores. Nos mutantes *opaco2* (*o2*) e *defective endosperm B30* (*De-B30*) ocorre preferencialmente uma redução na quantidade das proteínas de reserva da classe das zeínas de 22 kDa. Em *opaco7* (*o7*) há redução nos níveis das zeínas de 19 kDa, enquanto que nos mutantes *floury2* (*fl2*), *mucronate* (*Mc*) e *opaco6* (*o6*) ocorre supressão na síntese de todas as classes com a mesma intensidade. Foi constatado que o menor acúmulo de polipeptídeos de zeínas nestes mutantes é resultante de uma menor população de seus mRNA nos endospermas *o2*, *o7* e *fl2*, ou de problemas com a tradução do mRNA em proteínas nos mutantes *o6*, *Mc* e *De-B30* (Soave e Salamini, 1984; Motto et al., 1989). A mutação *o2* afeta significativamente a transcrição da α- e β-zeína, mas as β-zeínas são afetadas a um nível mais brando que as α-zeínas de 22 kDa (Kodrzycki et al., 1989).

2. O REGULADOR DE TRANSCRIÇÃO Opaco2:

A mutação homozigota recessiva *o2* confere um aspecto opaco à semente madura, que normalmente seria translúcida. Ela causa uma diminuição de 50 a 70% na síntese de zeínas, e um acréscimo no teor de lisina e triptofano, resultando na melhoria das qualidades nutritivas da semente (Mertz et al., 1964; Delhaye e Landry, 1986). Nas sementes *o2*, a taxa de degradação de lisina é menor do que a encontrada nas sementes normais (Sodek e Wilson, 1971). Vários outros aspectos do metabolismo da semente são também modificados no endosperma mutante em comparação com o normal: ocorre um acréscimo na atividade de RNase (Wilson e Alexander, 1967) e os conteúdos de várias proteínas e enzimas relacionadas ao metabolismo de nitrogênio e açúcares são alterados (Habben et al., 1993; Giroux et al., 1994; Galusci et al, 1996; Damerval e Guilloux, 1998). No mutante *o2* foram ainda encontradas alterações nos níveis de carbohidratos (Murphy e Dalby, 1971), maior susceptibilidade a patógenos (Loesch et al., 1976), redução na protandria (Gupta, 1979), alterações na atividade fotossintetizante das plântulas (Morot-Gaudry et al., 1979) e aumento na concentração de amônia (Misra e Oaks, 1981). A baixa resistência ao ataque de insetos e patógenos (Loesch et al., 1976; Gupta et al., 1970) pode estar associada, nas sementes *o2*, a um decréscimo significativo nos níveis da albumina b-32, que tem atividade de inativação de ribossomos (Bass et al., 1992). A mutação *o2* afeta ainda os níveis da proteína b-70, análoga à "heat shock protein" hsp70 que se acumula na superfície do corpúsculo protéico e pode atuar como "chaperonina", auxiliando no empacotamento das proteínas durante a sua formação (Marocco et al. 1991).

O gene *O2* codifica uma proteína pertencente à classe "basic domain/leucine zipper" (bZIP) dos fatores reguladores da transcrição (Hartings et al., 1989; Schmidt et al., 1990), cuja expressão se inicia especificamente no endosperma em torno de 11 DAP e continua a acumular-se até 30 DAP (Gallusci et al., 1994). O gene *O2* foi o primeiro gene regulador para o qual se demonstrou uma função no controle da expressão dos genes de proteínas de

reserva (Schmidt et al., 1992). Recentemente, determinou-se que o fator *O2* controla a expressão do gene para a forma citosólica da enzima piruvato ortofosfato diquinase-1 (cyPPDK-1) no endosperma do milho (Maddaloni et al., 1996).

2.1. O endosperma *opaco2* apresenta alto conteúdo de lisina

Aparentemente, a deficiência dos cereais em lisina é reflexo da deficiência de resíduos deste aminoácido nas zeínas, a principal classe de proteínas de reserva do milho. O milho *opaco2* foi originalmente identificado no intuito selecionar genótipos ricos em lisina (Mertz et al., 1964). As vantagens nutritivas deste tipo de semente logo foram notadas. Ratos alimentados com uma dieta composta de milho *opaco2*, minerais e vitaminas ganhavam peso quase quatro vezes mais rapidamente do que aqueles que recebiam apenas milho normal (Mertz et al., 1965). Crianças sofrendo de deficiência crônica de proteínas devido a sua dieta a base de milho comum foram curadas com a substituição por milho *opaco2* (Harpstead, 1971).

Nas sementes de milho em desenvolvimento, o conteúdo de lisina é influenciado por seis componentes: a taxa de sua síntese, a taxa da sua translocação, o teor de aminoácidos livres, o teor de lisina presente em zeínas e o teor de lisina em outras proteínas e a degradação do aminoácido. O teor de aminoácidos livres aumenta de duas a cinco vezes no endosperma *o2* em relação ao selvagem (Misra et al., 1975) mas apenas 5% do conteúdo total de lisina no endosperma é lisina não-protéica (Mehta et al., 1979). Assim, este componente contribui relativamente pouco para os níveis elevados deste aminoácido na semente. É característico das zeínas a quase total ausência de resíduos de lisina (Dalby e Tsai, 1975) e, assim, a redução na síntese destas proteínas também contribui para o aumento na percentagem de lisina no endosperma. Isto ocorre devido ao aumento da síntese de outras proteínas ricas em lisina. Por exemplo, no endosperma *opaco2*, a maior parte da lisina se

origina da fração de não-zeínas - as albuminas, globulinas e glutelinas - que contém em média 5% de resíduos de lisina (Murphy e Dalby, 1971).

O aumento na concentração de lisina pode estar associado, em parte, com a baixa taxa de degradação deste aminoácido no endosperma mutante (Sodek e Wilson, 1970; Arruda e Silva, 1983). Analisando as frações protéicas e a composição de aminoácidos de endospermas do tipo normal e de mutantes simples e duplo, Azevedo et al. (1990) observaram um aumento na síntese de proteínas ricas em lisina no mutante duplo *o2o2Ask1Ask1*. Este aumento foi atribuído a uma maior disponibilidade de lisina originada da síntese aumentada deste aminoácido, devido à presença de uma aspartato quinase, enzima da via da síntese de lisina, menos sensível a retroinibição por lisina (codificada pelo gene *Ask1*), e à reduzida atividade de degradação, determinada por *o2*.

A lisina é sintetizada nas plantas superiores pela via do diaminopimelato, um ramo específico da via biosintética dos aminoácidos derivados do aspartato (Bryan, 1980; Azevedo et al., 1997). Nesta via, o aspartato é o precursor inicial para a produção de lisina, além de metionina e treonina, através de outras ramificações (Figura 4). Uma série de enzimas são alostericamente retroinibidas pelos aminoácidos produzidos pelas respectivas ramificações da via. A atividade da aspartato quinase (AK), a primeira enzima da via, é inibida por lisina e treonina. A lisina também inibe a atividade da dihidrodipicolinato sintase (DHDPS), enquanto que a treonina inibe a homosserina desidrogenase (HSD). Ou seja, lisina e treonina regulam sua própria síntese por inibição da atividade da primeira enzima da via do aspartato e da enzima que leva exclusivamente a sua produção (Figura 4). A DHDPS é muito mais sensível à inibição por lisina do que a AK (Galili, 1995), o que a torna particularmente importante na regulação da síntese de lisina.

Embora ocorra síntese de lisina no endosperma de milho em desenvolvimento (Sodek, 1976), quantidades substanciais (5% do total) deste aminoácido são translocados de outros tecidos para o endosperma durante o desenvolvimento (Arruda e Silva, 1979). Por exemplo, o aminoácido mais translocado é a glutamina (de 18 a 30%), enquanto que a metionina é pouco

translocada (de 0.8 a 1.7%), indicando que este aminoácido deve ser sintetizado no endosperma. Já a prolina não é translocada, implicando que este aminoácido deve ser obrigatoriamente sintetizado no endosperma, para adequar o "*pool*" de aminoácidos para a síntese de zeínas (proteínas de reserva ricas em prolina) (Arruda e Silva, 1979). As zeínas também são pobres em lisina, indicando que a demanda por este aminoácido para a síntese proteíca deve ser baixa (Esen, 1986; Shewry e Tatham, 1990; Dalby e Tsay, 1975).

Mutantes de cevada e milho, com AK insensível a retro-inibição pela lisina não acumularam este aminoácido no endosperma, mas sim treonina (Bright et al., 1982; Diedrick, 1990; Hibbert e Green, 1982; Muahlbauer *et al.,* 1994). Isto, por sua vez, poderia ser atribuído à retro-inibição da DHDPS. Entretanto mutantes contendo DHDPS insensível a lisina também não mostraram aumentos significativos no conteúdo de lisina no endosperma (Negrutiu et al., 1994), e mesmo mutantes duplos AK/DHDPS insensíveis a lisina não mostraram superprodução de lisina (Frankard et al., 1991). Mesmo plantas transgênicas de tabaco e milho expressando genes bacterianos que codificam formas de AK e DHDPS insensíveis a retro-inibição pela lisina também não a acumularam em sementes e no endosperma, apesar de terem acumulado treonina e metionina (Karchi et al., 1993; Mazur et al., 1999). Já plantas de canola e soja acumularam lisina após terem sido transformadas com genes codificadores de formas de AK e DHDPS insensíveis à retroinibição por lisina No entanto, estas plantas apresentaram alterações fenotípicas nas folhas jovens e alta atividade de degradação de lisina nas sementes (Karchi et al., 1994; Falco et al., 1995), além de acumular produtos do catabolismo de lisina – sacaropina em soja e ácido α-amino adípico em canola (Falco et al., 1995). Já plantas transgênicas de milho expressando genes codificadores de formas enzimáticas insensíveis a retroinibição por lisina no embrião apresentaram acúmulo de lisina sem a detecção de produtos do catabolismo deste aminoácido (Mazur et al., 1999).

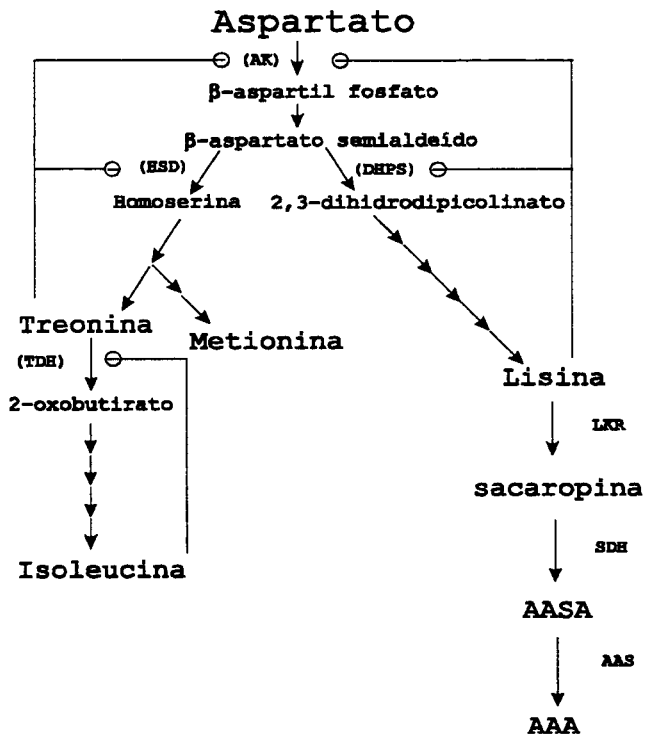

Figura 4. Via metabólica do aspartato. Os pontos de regulação negativa estão indicados pelas retas com círculo e sinal negativo. **AK** - Aspartato quinase; **HSD** - Homoserina desidrogenase; **DHDPS** - dihidrodipicolinato sintase; **TDH** - Treonina desidrogenase; **LKR** - Lisina cetoglutarato redutase; **SDH** - Sacaropina desidrogenase; **AAS** - Ácido α-aminoadípico sintase; **AASA** - Ácido α-aminoadípico-δ-semialdeído; **AAA** - Ácido α-aminoadípico (Modificado a partir de Bryan, 1990).

O catabolismo de lisina desempenha um papel importante no mecanismo de controle dos níveis de lisina livre no endosperma de milho (Arruda e Silva, 1979; Silva e Arruda, 1979). Ao longo do desenvolvimento da semente de milho, a quantidade de lisina encontrada na seiva do pedúnculo da espiga e na região pedicelo-plascento-chalazal são superiores à encontrada no endosperma (Arruda e Silva, 1979; Arruda e da Silva, 1983; Lyznik et al., 1982). A quantidade de lisina translocada é aproximadamente duas a três vezes maior que a necessária para a síntese de proteínas (Arruda e da Silva, 1983). Dessa maneira, espera-se um acúmulo de lisina livre no endosperma. Contudo, este não é o caso. O conteúdo total de lisina é cerca de 1.5% e a

concentração de lisina livre é mantida baixa durante todo o desenvolvimento para não inibir a síntese de metionina (Figura4)(Arruda e da Silva, 1983). Estas observações indicam que a concentração de lisina livre é primariamente influenciada pelo seu catabolismo e não apenas pelo mecanismo de retro-inibição de sua síntese.

3. CATABOLISMO DE LISINA

O catabolismo da lisina em plantas foi demonstrado com experimentos utilizando ^{14}C-lisina em trigo, cevada e milho. Nestes ensaios, a radioatividade foi incorporada em ácido α-amino adípico e ácido glutâmico, indicando que a lisina é catabolizada via sacaropina (Figura 4)(Nigan e McConnell, 1963; Sodek e Wilson, 1970; Brandt, 1975; Moller, 1976). De fato, foi demonstrado que a lisina-cetoglutarato redutase (LKR), e a sacaropina desidrogenase (SDH), respectivamente a primeira e a segunda atividade enzimática envolvidas na degradação de lisina pela via da sacaropina, possuem alta atividade no endosperma de milho (Arruda et al. 1982; Arruda e da Silva, 1983; Gonçalves-Butruille et al. 1996, Gaziola et al. 1999) e em arroz em desenvolvimento (Gaziola et al. 1997). Já em animais, o estudo de disfunções hereditárias no metabolismo de lisina contribuiu para a elucidação da via catabólica deste aminoácido. Pacientes com altas concentrações de lisina e sacaropina na urina e no sangue evidenciaram a existência da atividade das enzimas LKR e SDH (Woody, 1964; Ghadimi et al., 1965), as quais foram isoladas posteriormente por Hutzler & Dancis (1968; 1970).

O primeiro passo da degradação de lisina é a condensação de lisina e α-cetoglutarato em sacaropina pela atividade de LKR. O passo subseqüente de degradação envolve a hidrólise da sacaropina em ácido α-aminoadípico-δ-semialdeído (AASA) e ácido glutâmico pela SDH (Figura 4). Estas atividades residem em um único polipeptídio em animais e plantas (Markovitz *et al.*, 1984; Gonçalves-Butruille *et al.*, 1996). Ao contrário, em leveduras, as enzimas LKR e SDH estão em polipeptídios separados, e suas atividades catalisam os dois

últimos passos de uma via de biossíntese de lisina (Jones e Broquist, 1965 e 1966; Saunders e Broquist, 1966).

A LKR e a SDH de plantas, animais e leveduras possuem algumas propriedades similares como pH ótimo neutro para a atividade de LKR e pH básico para a atividade de SDH (Gonçalves-Butruille *et al.*, 1996; Saunders e Broquist, 1966; Arruda *et al.*, 1982; Fjellstedt e Robinson, 1975a e 1975b). Porém muitos aspectos físicos e propriedades regulatórias são diferentes. Em animais, a forma nativa da LKR-SDH é um tetrâmero de 460 kDa, constituído de subunidades de 115 kDa (Markovitz e Chuang, 1987; Fjellstedt e Robinson, 1975b). Já em plantas, a forma nativa possui 260 kDa e é constituída por dois monômeros de 125 kDa (Gonçalves-Butruille *et al.*, 1995). Ao contrário, em fungos e leveduras, as enzimas LKR e SDH são monômeros de 49 kDa e 73 kDa respectivamente (Ramos *et al.*, 1988). Em leveduras estas atividades são codificadas pelos genes *Lys1* e *Lys9*, respectivamente.

A expressão destes genes é reprimida a nível transcricional pelo aumento da concentração de lisina, causando queda nas atividades de LKR e SDH (Ramos *et al.*, 1988; Borell *et al.*, 1984). Os genes *Lys1* e *Lys9* e outros dois genes, *Lys2* e *Lys5*, são induzidos a nível transcricional pelo produto do gene *Lys14* quando associado ao intermediário ácido α-aminoadípico-δ-semialdeído (AASA) (Feller *et al.*, 1994). Já em plantas e animais, a superprodução ou a injeção de lisina aumentam a atividade da LKR/SDH em sementes de tabaco e em fígado de rato (Foster *et al.*, 1993; Karchi *et al.*, 1994; Papes et al., 1999). Estas observações indicam que a própria lisina, ou algum produto do seu catabolismo, induz a sua degradação.

Por outro lado, é importante destacar que a proteína Opaco2 complementa a mutação *gcn4* em leveduras. A proteína GCN4 é um fator de transcrição que regula a expressão de genes envolvidos na biossíntese de aminoácidos, incluindo aqueles da via de biossíntese de lisina (Hinnesbusch, 1988). Curiosamente, o gene *Lys14* apresenta em sua região promotora dois sítios de ligação do fator de transcrição *GCN4* (Feller *et al.*, 1994). Estas

evidências sugerem a existência de mecanismos regulatórios e/ou fatores semelhantes na via biosintética de lisina em leveduras e na via do catabolismo deste aminoácido em plantas e animais. Estes possíveis mecanismos e/ou fatores ainda são desconhecidos.

APRESENTAÇÃO DOS TRABALHOS E OBJETIVOS

O corpo desta tese está constituído por três trabalhos, um dos quais já publicado, outro no prelo e um terceiro submetido à publicação. Os trabalhos tratam da regulação da enzima lisina cetoglutarato redutase/sacaropina desidrogenase.

Trabalho 1:

Kemper EL, Cord-Neto G, Capella AN, Gonçalves-Butruille M, Azevedo RA e Arruda P. **Structure and regulation of the bifunctional enzyme lysine-oxoglutarate reductase-saccharopine dehydrogenase in maize.** *Eur. J. Biochem.* 253 (1998), 720-729.

A natureza de uma enzima bifuncional é desperta curiosidade. Neste trabalho demonstramos que a atividade da LKR é modulada por cálcio. Em seguida verificamos que a atividade da LKR também é modulada por força iônica, solventes orgânicos e magnésio. Entretanto a atividade de SDH não foi alterada por nenhum destes moduladores. Esta aparente independência levou-nos a utilizar experimentos de proteólise limitada para comprovar a independência dos domínios na enzima inteira. Curiosamente, verificamos que a atividade de LKR é inibida por frações contendo o domínio SDH separado. Objetivos deste trabalho:

1. Caracterizar a indução da atividade de LKR e SDH por cálcio, magnésio, força iônica e solventes orgânicos.
2. Separar as atividades de LKR e SDH por proteólise limitada e cromatografia. Caracterizar a influência do processo de proteólise limitada nas atividades de LKR e SDH

3. Estudar se os domínios isolados de LKR são modulados ou não por cálcio e força iônica, assim como a influência do domínio isolado de SDH sobre a atividade do domínio isolado de LKR.

Trabalho 2:

Kemper EL, Cord-Neto G, Papes F, Martinez Moraes KC, Leite A e Arruda P. **The Role of Opaque2 on the Control of Lysine Degrading Activities in Developing Maize Endosperm.** *Plant Cell*, no prelo.

Dados anteriores do grupo mostraram que a atividade de LKR e a síntese de zeínas possuem o mesmo padrão temporal de expressão no endosperma de milho. Estes estudos demonstraram também haver queda da atividade de LKR no endosperma opaco2, onde sabidamente, ocorre queda da expressão de algumas classes de zeínas. O fator transcricional Opaco2 controla diretamente a expressão de algumas zeínas. Neste trabalho, os objetivos foram:

1. Isolar e caracterizar o gene que codifica a enzima LKR/SDH.
2. Determinar o padrão de expressão do gene codificador da LKR/SDH.
3. Determinar o padrão temporal da deposição da enzima LKR/SDH e atividade de LKR e de SDH.
4. Determinar o local da expressão de LKR/SDH na semente de milho e em qual compartimento sub-celular a enzima está localizada.

Observação: Este trabalho foi desenvolvido em colaboração com Germano Cord-Neto e parte dos resultados deste trabalho já foram apresentados na sua tese de doutorado. Neste trabalho fui responsável pelos resultados apresentados nas Figuras 3, 6A, 6B, 7 e 8.

Trabalho 3:

Kemper EL, Papes F, da Silva AC, Leite A e Arruda P. **The activity of lysine-oxoglutarate reductase in maize is modulated by a Ca^{2+}-mediated dimerization and lysine-dependent phosphorylation.** *J. Biol. Chem.*, submetido.

No trabalho anterior verificamos que as atividades de LKR e SDH são reduzidas no endosperma *opaco2* em comparação com o normal. Entretanto a redução na atividade da LKR é muito mais acentuada que a queda da atividade da SDH. Como o número de moléculas responsável por cada atividade é o mesmo (já que a enzima é bifuncional), é de se esperar que alguma modificação pós traducional pode ser responsável pela queda acentuada da atividade de LKR no mutante *opaco2*. Além disto, estudamos com maior detalhe qual é o efeito do cálcio na atividade de LKR. Neste trabalho os objetivos foram:

1. Estudar a natureza da possível modificação pós traducional sofrida pela LKR.
2. Uma vez descoberta a fosforilação como sendo o agente de alteração da atividade de LKR, caracterizou-se o efeito da fosforilação nas atividades de LKR e SDH.
3. Determinar se a fosforilação altera o padrão de dimerização da LKR/SDH.
4. Determinar a natureza da modulação da atividade de LKR por Ca^{2+}.

Eur. J. Biochem. 253, 720–729 (1998)

Structure and regulation of the bifunctional enzyme lysine-oxoglutarate reductase-saccharopine dehydrogenase in maize

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The lysine-oxoglutarate reductase (LOR) domain of the bifunctional enzyme lysine-oxoglutarate reductase-saccharopine dehydrogenase (LOR/SDH) from maize endosperm was shown to be activated by Ca^{2+}, high salt concentration, organic solvents and Mg^{2+}. The Ca^{2+}-dependent enhancement of LOR activity was inhibited by the calmodulin antagonists *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and calmidazolium. Limited proteolysis was used to assess the structure/function relationship of the enzyme. Digestion with elastase separated the bifunctional 125-kDa polypeptide into two polypeptides of 65 kDa and 57 kDa, containing the functional domains of LOR and SDH, respectively. Proteolysis did not affect SDH activity, while LOR showed a time-dependent and protease-concentration-dependent inactivation followed by reactivation. Prolonged digestion or increasing amounts of elastase produced a complex pattern of limit polypeptides derived from additional cleavage sites within the 65-kDa (LOR) and 57-kDa (SDH) domains. The SDH-containing polypeptides inhibited the enzymatic activity of LOR-containing polypeptides. When separated from the SDH domain by limited proteolysis and ion-exchange chromatography, the LOR domain retained its Ca^{2+} activation property, but was no longer activated by high salt concentrations. These results suggest that the LOR activity of the native enzyme is normally inhibited such that after modulation, the enzyme undergoes a conformational alteration to expose the catalytic domain for substrate binding.

Keywords: lysine-oxoglutarate reductase; saccharopine dehydrogenase; lysine catabolism; calcium activation; limited proteolysis.

The saccharopine pathway is utilized for the biosynthesis or degradation of lysine in several organisms. In plants and mammals, lysine is catabolized through this pathway via two reactions catalyzed by a bifunctional enzyme containing the activities of lysine-oxoglutarate reductase (LOR) (Eqn 1) and saccharopine dehydrogenase (SDH) (Eqn 2) [1, 2]. The net result of the two reactions resembles a transaminase reaction in which the α-amino group of lysine is transferred to 2-oxoglutarate to form glutamic acid. In yeast and fungi, these two activities correspond to the last steps of the biosynthetic route for the formation of lysine and reside in two separate proteins [3–5].

$$\text{lysine} + \text{2-oxoglutarate} + \text{NAD(P)H} + \text{H}^+ \leftrightarrow \text{saccharopine} + \text{NAD(P)}^+ + \text{H}_2\text{O} \quad (1)$$

$$\text{saccharopine} + \text{NAD(P)}^+ + \text{H}_2\text{O} \leftrightarrow \alpha\text{-aminoadipic-}\delta\text{-semialdehyde} + \text{glutamic acid} + \text{NAD(P)H} + \text{H}^+ . \quad (2)$$

Although LOR and SDH from plants, mammals, yeast and fungi share several common properties such as a neutral pH optimum for LOR and a basic pH optimum for SDH [2, 5–7], many other aspects of their physical and regulatory properties are different. The native form of the mammalian LOR/SDH is a 460-kDa homotetramer [1, 8], while the maize enzyme is a 260-kDa homodimer [2]. Limited proteolysis of the LOR/SDH from bovine liver and maize resulted in the separation of LOR from SDH, indicating that the two activities reside in functionally independent domains of the bifunctional enzymes [1, 2]. This bifunctional structure contrasts with the LOR and SDH from yeast and fungi, which are independent activities residing in monomers of 49 kDa (LOR) and 73 kDa (SDH) [4, 5] encoded by the genes *Lys1* and *Lys9*, respectively [9].

In yeast cells, an increase in the free lysine concentration decreases the activities of LOR and SDH trough repression of the genes *Lys1* and *Lys9* [9, 10]. In contrast, *Lys1* and *Lys9*, along with two other genes, *Lys2* and *Lys5*, are induced at the transcriptional level by the lysine precursor α-aminoadipic-δ-semialdehyde. This compound has been shown to modulate the transcriptional factor encoded by the gene *Lys14* that controls the transcription of *Lys1*, *Lys9*, *Lys2* and *Lys5* [11]. This complex regulatory system controls the level of lysine synthesis in yeast. In contrast, in plants and mammals, lysine induces its own degradation. Overproduction and/or treatment with exogenous

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Abbreviations. W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; CSPD, disodium 3-{4-methoxyspiro [1,2-dioxethane-3,2′-(5′-chloro)tricyclo(3.3.1.1^{3,7})decan]-4-yl}phenyl phosphate; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; LOR, lysine-oxoglutarate reductase; SDH, saccharopine dehydrogenase; GAD, glutamate decarboxylase; ChAT, choline acetyltransferase.

Enzymes. Saccharopine dehydrogenase (NAD$^+$, L-lysine-forming) (EC 1.5.1.8); Saccharopine dehydrogenase (NADP$^+$, L-glutamate-forming) (EC 1.5.1.9); glutamate decarboxylase (EC 4.1.1.15); elastase (EC 3.4.21.36).

lysine dramatically increase the activity of LOR in rat liver mitochondria [12] and in developing tobacco seeds [13]. In rats, the induction of LOR by excess lysine is mediated by Ca^{2+} [14] whereas in tobacco seeds it is mediated by Ca^{2+} and protein phosphorilation [15].

The level of LOR activity in the endosperm of the *opaque-2* maize mutant is reduced 2–3-fold compared with the normal endosperm [16]. The Opaque-2 protein, an endosperm-specific basic domain–leucine-zipper transcription factor that controls the transcription of several endosperm protein genes [17–19], complements the *gcn4* yeast mutation [20]. GCN4 is a general yeast transcription factor involved in regulating the expression of several genes encoding amino acid biosynthetic enzymes, including those of lysine biosynthesis [11, 21]. These findings suggest the existence of a parallel regulatory mechanism shared by common compounds and/or factors in the lysine biosynthetic route of yeast and in the lysine catabolic pathway of plants and mammals.

In this work, we demonstrate that Ca^{2+} and ionic strength activate the LOR domain of the bifunctional enzyme LOR/SDH from maize endosperm. We used limited proteolysis to assess the structure/function relationship of the maize enzyme and obtained evidence for the presence of an inhibitory domain regulating the LOR activity.

MATERIALS AND METHODS

Chemicals. L-Saccharopine, poly(ethylene glycol) 8000, NADPH, NAD, dithiothreitol, glycine, Tris-base, EDTA, EGTA, elastase (type IV, from porcine pancreas), Tween 20, phenylmethylsulfonyl fluoride ($PhMeSO_2F$), DEAE Sepharose and benzamidine were obtained from Sigma. Disodium 3-{4-methoxyspiro [1,2-dioxethane-3,2′-(5′-chloro)tricyclo(3.3.1.1^{3,7})decan]-4-yl}phenyl phosphate (CSPD) was obtained from Tropix.

Plant material. The commercial maize (*Zea mays* L.) hybrid F-352 from Agroceres was used. The plants were grown in the field and self-pollinated, and the ears were harvested 17 days after pollination and stored frozen at −70°C.

Enzyme purification. LOR/SDH was purified from immature maize endosperms as described by Gonçalves-Butruile et al. [2], with minor modifications. The following steps were performed at 4°C. The tissues were homogenized with buffer A (25 mM sodium phosphate, pH 7.4, 1 mM dithiothreitol, 1 mM EDTA and 5 mM benzamidine). The homogenate was centrifuged at 20000 *g* for 10 min and the supernatant was brought to pH 5.5 by addition of NaH_2PO_4. Poly(ethylene glycol) 8000 (50% mass/vol.) was added to the homogenate to a final concentration of 7.5%. The mixture was centrifuged at 20000 *g* for 10 min and the supernatant was adjusted to 15% (mass/vol.) polyethylene glycol and centrifuged at 20000 *g* for 10 min. The pellet was resuspended in buffer B (50 mM Tris/HCl, pH 8.5, 1 mM dithiothreitol and 1 mM EDTA) and dialyzed overnight against the same buffer. The dialyzed enzyme was applied to a DEAE-Sepharose column (2.5 cm×40 cm) previously equilibrated with buffer B. The enzyme was eluted from the column with a linear gradient of 0 to 0.5 M NaCl in buffer B. The fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20000 *g* for 10 min. The pellet was resuspended in buffer B, dialyzed against the same buffer, and applied to a Protein-Pak Q 8HR (Waters) column. The enzyme was eluted from the column with a linear gradient of 0 to 0.5 M NaCl in buffer B. The fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20000 *g* for 10 min. The pellet was resuspended in buffer B and applied to a Superdex 200 HR (Pharmacia) column previously equilibrated with buffer C (buffer B containing 0.3 M NaCl). The enzyme was eluted from the Superdex column with buffer C and stored at −70°C.

Antibody preparation. Purified LOR/SDH was separated by SDS/PAGE on a 7% gel. After electrophoresis the gel was washed three times with cold distilled water for 10 min and the protein stained with cold 250 mM KCl. The acrylamide band containing the purified LOR/SDH was cut out of the gel, crushed, and mixed (1:1) with incomplete Freund's adjuvant. 4 ml of the mixture, corresponding to approximately 300 μg protein, was injected into a rabbit. The rabbit was given a booster injection 4 weeks later. The rabbit was bled 2 weeks after the last injection. After removal of the clot, the serum was divided into aliquots and stored at −20°C.

Polyacrylamide gel electrophoresis. Discontinuous PAGE was performed at pH 8.5 in 7% slab gels. After electrophoresis, the gels were developed for LOR and SDH activities as described by Gonçalves-Butruille et al. [2]. SDS/PAGE was performed in 7%, 10% or 6% to 18% gradient slab gels according to Laemmli [22]. After electrophoresis, the gels were stained with 0.27% (mass/vol.) Coomassie brilliant blue R-250 in methanol/acetic acid/distilled water (54:12.5:33.5, by vol.) and destained with methanol/acetic acid (30:10, by vol.).

Immunoblotting. Immunoblotting was performed as described by Timmons and Dunbar [23] and Gallagher [24]. After electrophoresis, the gels were soaked in 25 mM Tris-base solution containing 190 mM glycine and 20% methanol for 10 min, and the proteins then electrotransferred to nylon membranes (Hybond N, Amersham) in a semidry blotting apparatus (Pharmacia). The membranes were blocked overnight at 4°C in 20 mM Tris, pH 7.4, containing 137 mM NaCl, 0.1% (by vol.) Tween 20, and 5% (mass/vol.) non-fat dry milk and then incubated with anti-LOR polyclonal antibodies. After incubation with anti-(rabbit IgG)-alkaline phosphatase conjugate, the membranes were incubated for 20 min in the dark in a developing mixture containing 100 mM Tris/HCl, pH 9.5, 100 mM NaCl and a 1:1000 solution of CSPD (Tropix). The resulting bands were detected by autoradiography.

Separation of maize LOR and SDH domains by limited proteolysis. Aliquots of LOR/SDH partially purified by chromatography on DEAE-Sepharose were incubated with elastase (Sigma). The enzyme/protease ratios and the conditions for proteolysis are indicated in the figure legends. The reaction was stopped by the addition of $PhMeSO_2F$ at a final concentration of 2 mM. The digest was applied to a Protein-Pak Q 8HR column (Waters) previously equilibrated with buffer B. The column was washed with buffer B and then eluted with a linear gradient of 0 to 0.5 M NaCl in buffer B. The fractions containing the separated LOR and SDH domains were used for enzyme assays.

Enzyme assays. LOR activity (Eqn 1) was measured spectrophotometrically in the direction of NADPH to $NADP^+$ at 30°C. The reaction mixture had a final volume of 0.3 ml and contained 20 mM L-lysine, 10 mM 2-oxoglutaric acid (neutralized to pH 7.0 with potassium hydroxide), 0.1 mM NADPH, various concentrations of Tris/HCl buffer, pH 7.4, and 0.04–0.1 mg of protein. SDH activity (Eqn 2) was also measured spectrophotometrically by following the reduction of NAD^+ to NADH at 30°C in a 0.3-ml reaction mixture containing 2 mM L-saccharopine, 2 mM NAD^+ and various concentrations of Tris/HCl, pH 8.5. Activators and/or inhibitors were added to the assay mixtures as indicated in the figure and table legends. The oxidation of NADPH and reduction of NAD^+ were monitored at 340 nm in a DU-65 Beckman spectrophotometer. 1 U enzyme activity was defined as 1 nmol NADPH oxidized or NAD^+ reduced/min at 30°C. The protein concentration in the enzyme

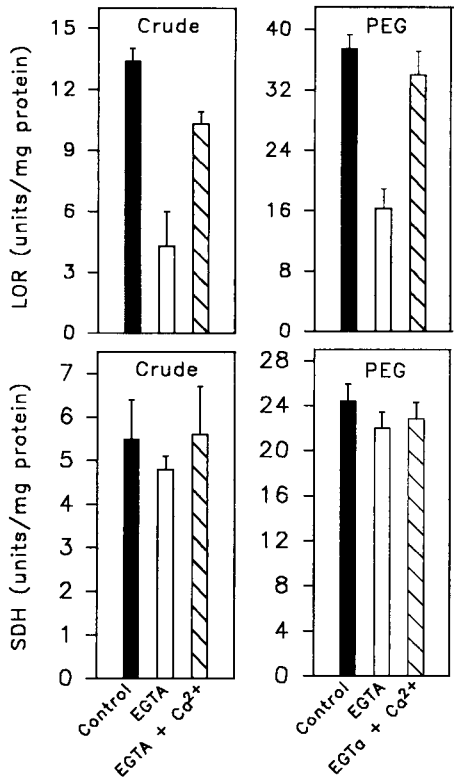

Fig. 1. Effect of EGTA on the LOR and SDH activities of crude and poly(ethylene glycol) fractionated extracts from maize endosperm. Crude and poly(ethylene glycol)-fractionated preparations of LOR/SDH were incubated at pH 7.4 (LOR) or 8.5 (SDH) in 50 mM Tris/HCl, 1.6 mM EGTA (EGTA) or 1.6 mM EGTA plus 1.6 mM $CaCl_2$ (EGTA + Ca^{2+}). The assays were performed at least in duplicate. The bars indicate the standard deviation. PEG, poly(ethylene glycol).

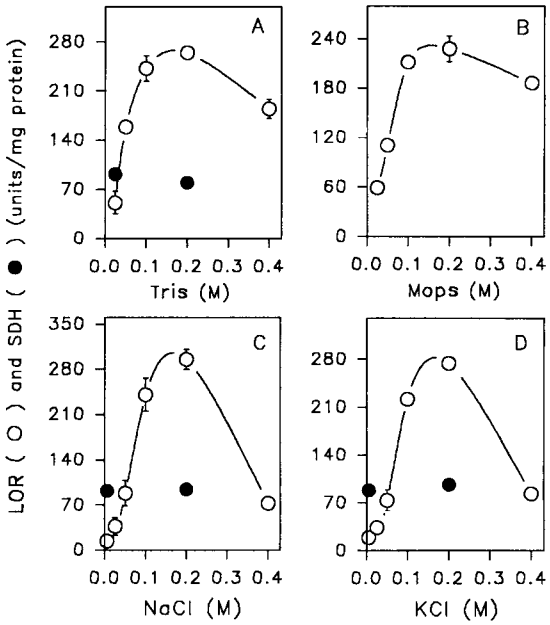

Fig. 2. Effect of buffer and salt concentrations on the LOR and SDH activities of maize endosperm. Aliquots of partially purified enzyme were assayed in the presence of varying concentrations of buffers and salts. (A) Tris/HCl, pH 7.4, (LOR) and 8.5 (SDH). (B) Mops/Tris, pH 7.4. (C) NaCl in 25 mM Tris/HCl, pH 7.4 (LOR) and 8.5 (SDH). (D) KCl in 25 mM Tris/HCl, pH 7.4 (LOR) and 8.5 (SDH). Each point is the average of at least duplicate assays. The bars indicate the standard deviation.

extracts was determined by the method of Bradford [25] using the Bio-Rad protein assay dye reagent.

RESULTS

LOR but not SDH activity is inhibited by EGTA in crude extracts and poly(ethylene glycol) precipitates of immature maize endosperm. The addition of 1.6 mM EGTA to the assay mixture decreased the LOR activity by 70% in the crude extract but had no significant effect on SDH activity (Fig. 1). The addition of 1.6 mM $CaCl_2$ to the assay mixture containing 1.6 mM EGTA (free Ca^{2+} concentration ≈2.3 μM) restored LOR activity almost to the control levels and, as expected, did not affect SDH activity (Fig. 1). A preliminary purification of the maize LOR/SDH by poly(ethylene glycol) fractionation [2] did not modify the enzyme response to the addition of EGTA and EGTA plus Ca^{2+} (Fig. 1).

LOR but not SDH is activated by high ionic strength. The LOR activity of a partially purified preparation obtained after chromatography on DEAE-Sepharose increased sixfold when the concentration of Tris/HCl in the assay mixture was increased from 25 mM to 200 mM; SDH activity was not affected by the buffer concentration (Fig. 2A). To verify whether the effect of Tris reflected its reduced buffering capacity at low concentration and neutral pH, LOR activity was assayed in a Mops-Tris buffer, which has a greater buffering capacity at pH 7.4. Again LOR activity increased fivefold when the Mops-Tris concentration was increased from 25 mM to 200 mM (Fig. 2B). Confirmation that LOR activity was affected by ionic strength, and not by the buffering capacity of the buffers utilized, was obtained when the enzyme was assayed in a medium containing 25 mM Tris/HCl, pH 7.4, plus increasing amounts of NaCl or KCl. The activity of LOR but not SDH increased 15-fold when the salt concentration was raised from 0 to 200 mM (Fig. 2C and D, respectively). The effect of salt concentration on LOR activity could result, at least in part, from a decreased water activity in the assay medium which could induce conformational modifications in the LOR domain of the enzyme. To verify the effect of lowering the water activity, LOR was assayed in the presence of organic solvents (Table 1). As can be seen, poly(ethylene glycol) 8000 and ethyleneglycol increased the LOR activity to levels corresponding to about 30% of that observed for 200 mM Tris/HCl buffer.

Table 1. Effect of organic solvents on maize LOR activity. Aliquots of partially purified enzyme were assayed in 25 mM Tris/HCl, pH 7.4, plus different organic solvents at the indicated concentrations.

Experiment	Relative activity
	%
Tris/HCl 25 mM	6.8 ± 1.9
Tris/HCl 200 mM	100.0 ± 8.3
Tris/HCl 25 mM + dimethylsolfoxide 30%	4.5 ± 1.2
Tris/HCl 25 mM + poly(ethylene glycol)-8000 30%	30.2 ± 1.8
Tris/HCl 25 mM + ethyleneglycol 30%	27.5 ± 3.2

The Ca^{2+}-dependent LOR activity is inhibited by calmodulin antagonists. To examine the nature of the Ca^{2+} activation of maize LOR, a partially purified preparation obtained after chro-

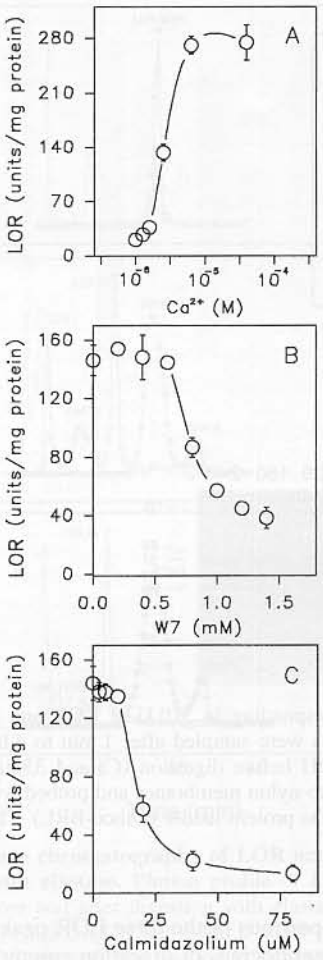

Fig. 3. Activation of maize endosperm LOR activity by free Ca^{2+} and inhibition by calmodulin antagonists. Aliquots of partially purified enzyme were assayed in the presence of varying concentrations of free Ca^{2+} and calmodulin antagonists. (A) LOR activity in 25 mM Tris/HCl, pH 7.4, plus 1 mM EGTA and different concentrations of $CaCl_2$. (B) LOR activity in 25 mM Tris/HCl, pH 7.4, plus 30 μM free Ca^{2+} and different concentrations of W7. (C) LOR activity in 25 mM Tris/HCl, pH 7.4, plus 30 μM free Ca^{2+} and different concentrations of calmidazolium. Each point is the average of at least duplicate assays. The bars indicate the standard deviation.

Table 2. Effect of divalent cations on maize LOR activity. Aliquots of partially purified enzyme were assayed for LOR activity in 25 mM Tris/HCl, pH 7.4, plus different divalent cations.

Experiment	Relative activity
	%
Tris/HCl 25 mM	6.8 ± 1.9
+ Ca^{2+} 30 μM	100.0 ± 10.3
+ Co^{2+} 5 mM	5.8 ± 2.1
+ Co^{2+} 20 mM	6.5 ± 1.8
+ Zn^{2+} 5 mM	4.2 ± 1.7
+ Zn^{2+} 20 mM	5.4 ± 1.8
+ Mg^{2+} 5 mM	47.6 ± 11.2
+ Mg^{2+} 20 mM	97.9 ± 11.6

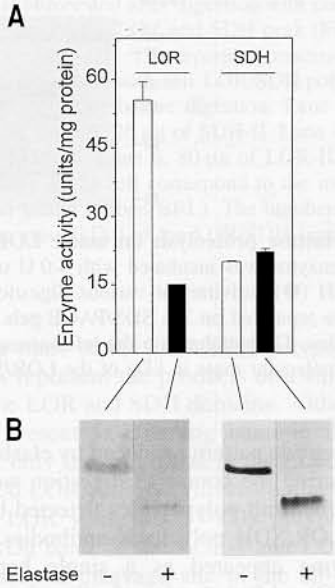

Fig. 4. Separation of LOR and SDH activities by limited proteolysis. An aliquot corresponding to 16 U LOR and 2 U of SDH of partially purified enzyme was incubated with 1.4 U of elastase for 5 min at room temperature. (A) Activities of LOR and SDH measured before (−) and after (+) digestion. (B) Aliquots of native and digested enzyme were run on 7% non-denaturing polyacrylamide gels. After electrophoresis, the gels were developed for LOR and SDH activities.

matography on DEAE-Sepharose was assayed in the presence of 25 mM Tris/HCl, pH 7.4, plus 1 mM EGTA and increasing concentrations of $CaCl_2$ calculated to provide free Ca^{2+} concentrations in the micromolar range. Under these conditions, LOR activity increased 15-fold when the Ca^{2+} concentration was increased from 0 to 50 μM (Fig. 3A). Of the other divalent cations tested, only Mg^{2+} at millimolar concentrations activated LOR activity (Table 2).

Two non-related calmodulin antagonists, W7 and calmidazolium, were tested for their ability to inhibit the Ca^{2+}-dependent LOR activity. W7 at a concentration of 1.5 mM and calmidazolium at a concentration of 40 μM almost completely inhibited the Ca^{2+}-dependent LOR activity (Fig. 3B and C, respectively).

Limited proteolysis separates the LOR and SDH activities. To elucidate the mechanisms of activation by Ca^{2+} and high salt concentrations, the LOR and SDH domains were separated by proteolysis. We have shown previously that incubation of purified maize LOR/SDH with elastase separates LOR from SDH, indicating the presence of elastase-sensitive sites between the two enzymatic domains of the native polypeptide [2]. However, LOR activity was much lower than that of SDH following such separation, suggesting the presence of additional protease-sensitive sites in the LOR domain [2]. A partially purified enzyme was incubated with elastase and the activities of LOR and SDH measured spectrophotometrically and developed in non-denaturing PAGE gels. Digestion with elastase decreased the LOR activity 3.5-fold but had no significant effect on SDH activity (Fig. 4A). The elastase treatment separated the two activities and, as expected, the activity of LOR in the non-denaturing gel was much lower than that of SDH (Fig. 4B).

To determine the elastase digestion pattern and its effect on LOR and SDH activities, the partially purified preparation was incubated with elastase and the activities of LOR and SDH measured over a 4-h period. Digestion with elastase did not significantly change SDH activity, while LOR activity decreased to less than 10% of the undigested enzyme activity within 20 min of proteolysis (Fig. 5A). Surprisingly, the activity of LOR recovered after 50 min elastase treatment to reach 70% of that of the native enzyme after 4 h of digestion (Fig. 5A). To verify the

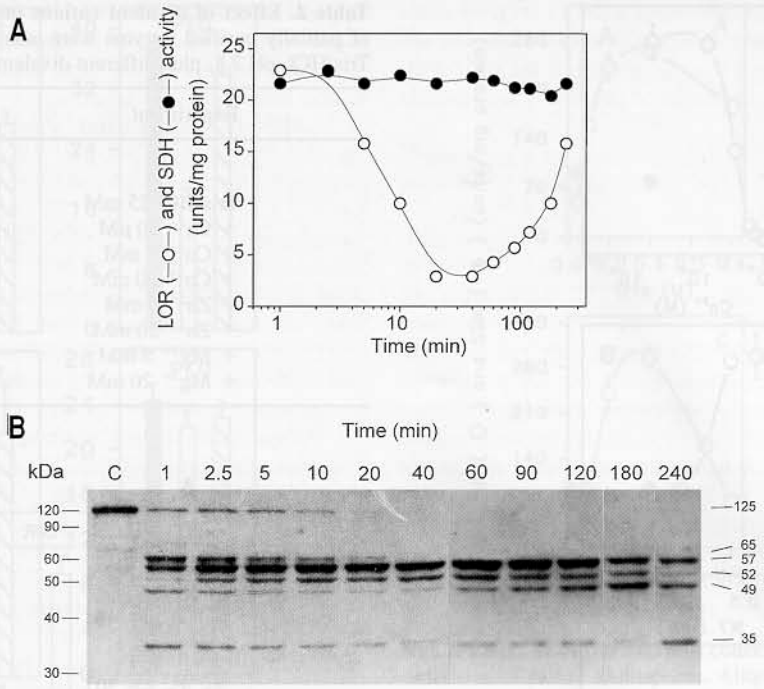

Fig. 5. Effect of elastase proteolysis on maize LOR and SDH activities. An aliquot corresponding to 310 U of LOR and 210 U of SDH of partially purified enzyme was incubated with 4.0 U of elastase at room temperature. Aliquots were sampled after 1 min to 4 h of digestion. (A) LOR (○) and SDH (●) activities at various digestion times. (B) 30 µg protein of LOR/SDH before digestion (C) and 35 µg protein for each digestion time were separated on 7% SDS/PAGE gels. The separated proteins were blotted onto nylon membranes and probed with anti-LOR/SDH polyclonal antibodies. The numbers on the left correspond to the molecular mass of the 10-kDa protein ladder (Gibco-BRL). The numbers on the right indicate the molecular mass in kDa of the LOR/SDH-limit polypeptides.

LOR/SDH polypeptide pattern produced by elastolytic digestion, samples taken during the course of digestion were resolved by SDS/PAGE and the limit polypeptides detected by immunoblotting using anti-LOR/SDH polyclonal antibodies (Fig. 5B). The undigested enzyme appeared as a single band of 125 kDa (Fig. 5B, lane C). After 1 min of digestion, the 125-kDa polypeptide was almost completely cleaved into two major bands of 65 kDa and 57 kDa and into three minor bands of 52, 49 and 35 kDa. The proportions of the five bands changed during the course of proteolysis. The 65-kDa band decreased as digestion progressed and was no longer detectable after 20 min. The 57-kDa and 52-kDa bands remained constant from 2.5 min to 180 min, and then decreased from 180 min to 240 min of digestion. The 49-kDa band decreased slightly during the first 40 min of treatment and then increased substantially from 40 min to 180 min of digestion. The 35-kDa band remained constant during the entire course of digestion.

Elastase hydrolyses C-terminal peptide bonds at uncharged non-aromatic amino acid side chains like Ala, Leu, Ile, Gly and Ser. Analysis of the *Arabidopsis* LOR/SDH protein sequence [26] revealed that those cleavage sites are present in a large number over the entire protein sequence. This makes difficult to associate the elastolytic LOR/SDH polypeptides with domains in the *Arabidopsis* LOR/SDH protein.

Separation of limit polypeptides by ion exchange chromatography. To identify the polypeptides corresponding to LOR and SDH activities, partially purified bifunctional enzyme was incubated with elastase and the digest applied to a Protein-Pak Q 8HR (Waters) column. The LOR and SDH peaks of the undigested enzyme co-eluted at approximately 250 mM NaCl (Fig. 6A). Digestion with elastase resulted in three LOR peaks (LOR-I, LOR-II and LOR-III) and two SDH peaks (SDH-I and SDH-II; Fig. 6B and C) that eluted with 75–300 mM NaCl. Although the proportions of the three LOR peaks varied perhaps because of different degrees of digestion among the experiments (Fig. 5B), it is noteworthy that the activity of LOR-III, which accounted for more than 90% of the activity of the three LOR peaks, was twice the total LOR activity applied to the column (Table 3). The increase in LOR-III activity was easily seen by comparison with SDH-I activity (since SDH activity was not affected by elastase digestion, this enzyme may be used as an internal control). The amount of SDH-I, which contained over 85% of the activity of the two SDH peaks, represented 50% of the total SDH activity applied to the column (Table 3).

To determine the polypeptide pattern of each LOR and SDH peak, 10 µl of the most active fractions of each peak were resolved by SDS/PAGE and the limit polypeptides were detected by immunoblotting using anti-LOR/SDH polyclonal antibodies (Fig. 7). Two lanes, corresponding to the undigested (Fig. 7, lane 1) and the digested enzyme (Fig. 7, lane 2), were included as controls. Lane 2 of Fig. 7, shows that the digest applied to the column corresponded, approximately, to the 240-min digestion shown in Fig. 5B. The SDH-II peak (Fig. 7, lane 3) contained predominantly bands of 65, 57 and 52 kDa, with very faint bands of 49, 35 and 19 kDa. The SDH-I peak (Fig. 7, lane 4) contained predominantly bands of 52 and 35 kDa and a less abundant group of polypeptides ranging from 19 kDa to 49 kDa. The major difference between the SDH-I and SDH-II peaks was the large proportion in the SDH-II peak of 52-kDa and 35-kDa bands and the absence of 65-kDa and 57-kDa bands. The bands observed in the three LOR peaks were very faint compared with the bands detected in the SDH peaks. LOR-III (Fig. 7, lane 5) contained bands of 65, 26 and 20 kDa, LOR-II (Fig. 7, lane 6) contained predominantly a 49-kDa band along with a very weak 20-kDa band, and LOR-I contained only a 49-kDa band. Since digestion for 1 min (Fig. 5B, lane 1) produced predominantly bands of 65 kDa and 57 kDa whose sum (122 kDa) corresponds

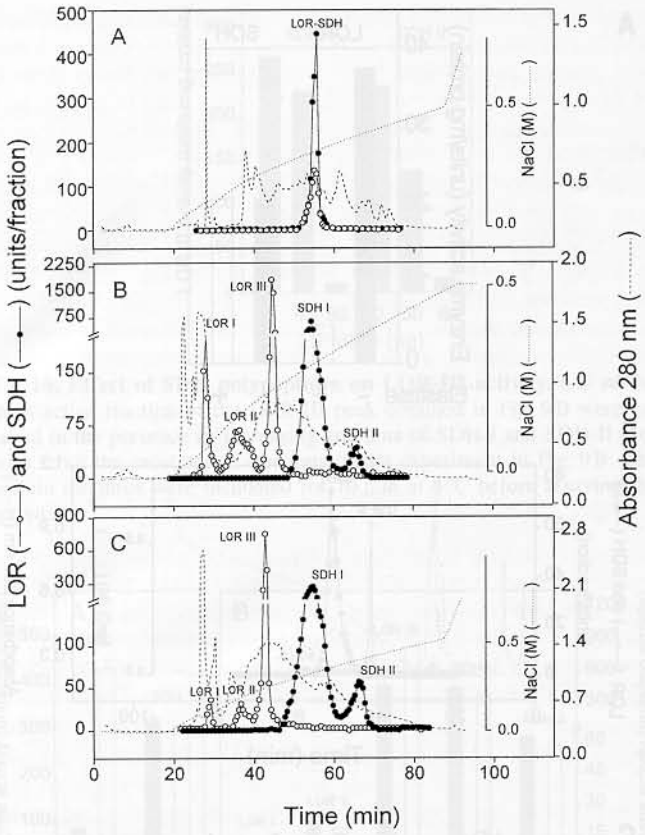

Fig. 6. Ion-exchange chromatography of LOR and SDH before and after digestion with elastase. Elution profile of LOR (○) and SDH (●) activities before and after digestion with elastase. (A) An aliquot corresponding to 11500 U LOR and 5400 U SDH of partially purified enzyme on DEAE-Sepharose was applied to a Protein-Pak Q 8HR column. (B) An aliquot corresponding to 2200 U LOR and 5400 U SDH was digested with 16.8 U elastase for 30 min at 4°C. (C) An aliquot corresponding to 4000 U LOR and 5100 U SDH was digested with 33.1 U elastase for 30 min at 4°C and the digest was applied to a Protein-Pak Q 8HR column. The columns were eluted with a linear gradient of 0 to 0.5 M NaCl in buffer B at a flow rate of 1.5 ml/min. Fractions of 0.75 ml were collected and assayed for LOR and SDH activities.

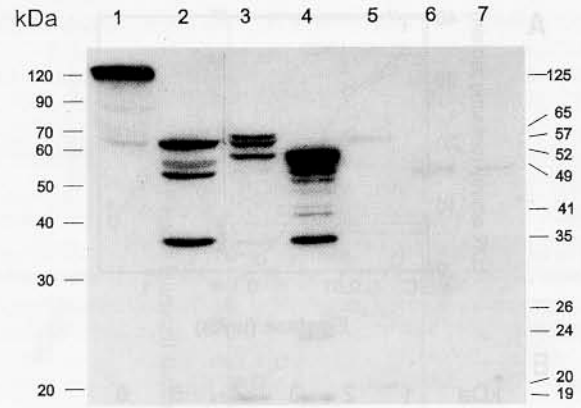

Fig. 7. Identification of the polypeptides corresponding to the LOR and SDH peaks separated by ion-exchange chromatography. Aliquots of LOR/SDH before and after digestion with elastase and from the most active fractions of each LOR and SDH peak (Fig. 6B) were separated on 10% SDS/PAGE gels. The separated proteins were blotted onto nylon membranes and probed with anti-LOR/SDH polyclonal antibodies. Lane 1, 35 µg of LOR/SDH before digestion. Lane 2, 90 µg of LOR/SDH after digestion. Lane 3, 26 µg of SDH-II. Lane 4, 28 µg of SDH-I. Lane 5, 40 µg of LOR-III. Lane 6, 80 µg of LOR-II. Lane 7, 44 µg of LOR-I. The numbers on the left correspond to the molecular masses of the 10-kDa protein ladder (Gibco-BRL). The numbers on the right indicate the molecular mass (kDa) of the LOR/SDH-limit polypeptides.

to the molecular mass of the LOR/SDH polypeptide, it is likely that these bands represent the products of a single cleavage site that separates the LOR and SDH domains. Although the 65-kDa polypeptide is present as a strong band in the SDH-II peak (Fig. 7, lane 3), only the faint band in the LOR-III peak (Fig. 7, lane 5) possessed LOR activity. Therefore, the 65-kDa polypeptide represents LOR while the 57-kDa polypeptide represents SDH. The 49-kDa band of the LOR-I and LOR-II peaks must derive from a second cleavage site in the 65-kDa polypeptide whereas the 52-kDa and 35-kDa bands of the SDH-I peak, probably derive from additional cleave sites in the 57-kDa polypeptide. The large proportion of 65-kDa and 49-kDa polypeptides present in the SDH-II and SDH-I peaks (Fig. 7, lanes 3 and 4),

Table 3. Purification of undigested and proteolytic digested LOR and SDH from immature maize endosperm. The data correspond to the profile shown in Fig. 6B.

Fraction	LOR					SDH			
	total protein	total activity	H-Q/DEAE ratio	specific activity	H-Q/DEAE ratio	total activity	H-Q/DEAE ratio	specific activity	H-Q/DEAE ratio
	mg	U	%	U/mg	-fold	U	%	U/mg	-fold
Crude extract	6542	31336		4.8		7646		1.2	
Poly(ethylene glycol) fractionation	1380	10361		7.5		10801		7.8	
Undigested									
DEAE-Sepharose	276.5	11533		41.7		5374		19.4	
Protein-Pak Q	7.6	9677	83.9	1273.3	30.5	3460	64.4	455.3	23.5
Elastolytic digested									
DEAE-Sepharose	254.7	2177		8.6		5423		21.3	
Protein-Pak Q									
LOR I	2.8	544	25.0	197.9	23.1				
LOR II	4.2	546.7	25.1	130.2	15.2				
LOR III	2.5	3639	167.1	1455.5	170.2				
SDH I	15.6					2745	50.6	176	8.3
SDH II	6.4					218.7	4.0	34.2	1.6

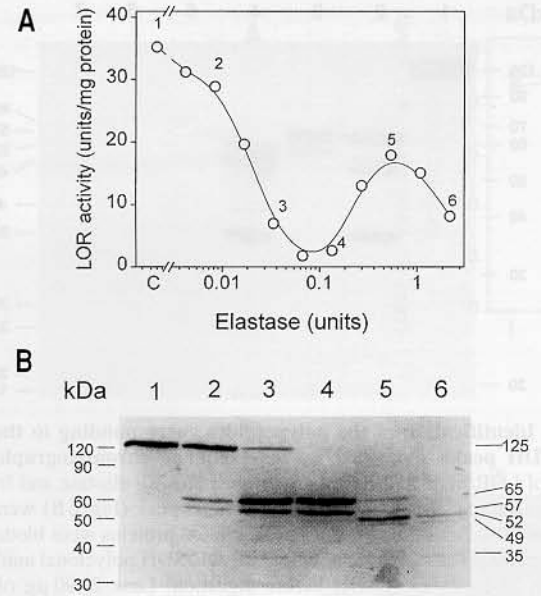

Fig. 8. Effect of elastase concentration on LOR activity. Aliquots corresponding to 7 U of LOR of partially purified enzyme were incubated with 0.004−2.16 U elastase for 10 min at room temperature. (A) Aliquots of each digest were used to determine LOR activity. (B) Aliquots corresponding to 64 μg of protein of each digest were subjected to discontinuous electrophoresis in 6% to 18% gradient SDS/PAGE gels. The separated proteins were blotted onto nylon membranes and probed with anti-LOR/SDH polyclonal antibodies. Lane 1, 13 μg of LOR/SDH before digestion. Lane 2, incubation with 0.016 U elastase. Lane 3, incubation with 0.034 U elastase. Lane 4, incubation with 0.138 U elastase. Lane 5, incubation with 0.54 U elastase. Lane 6, incubation with 2.16 U elastase. The numbers on the left correspond to the molecular masses of the 10-kDa protein ladder (Gibco-BRL). The numbers on the right indicate the molecular mass (kDa) of the LOR/SDH-limit polypeptides.

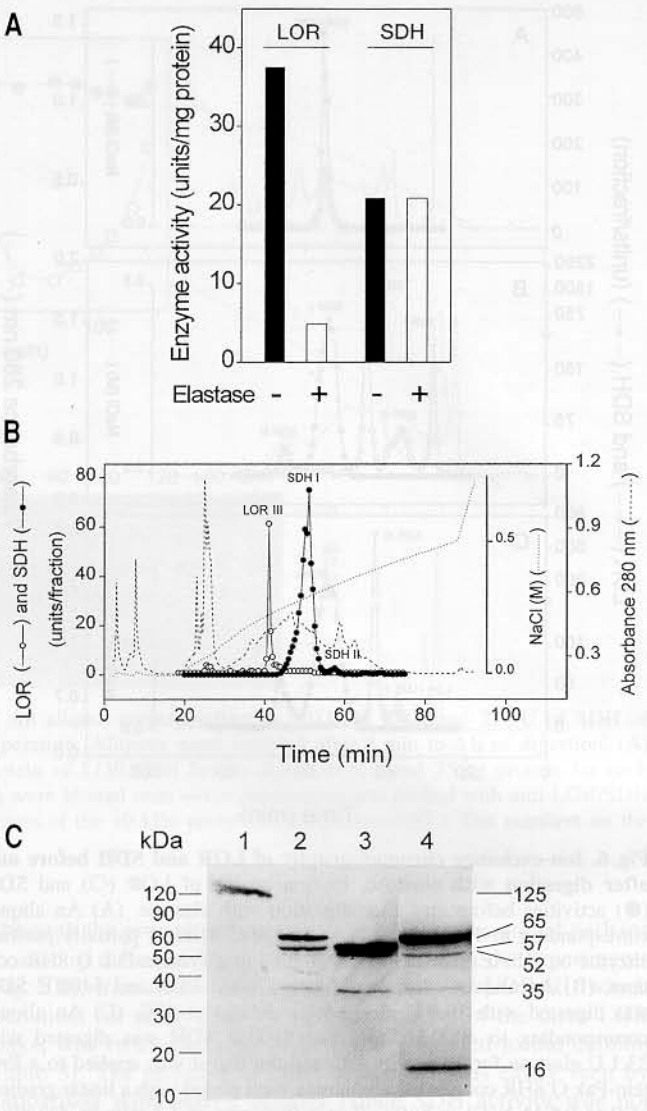

Fig. 9. Separation of the 65-kDa (LOR) and 57-kDa (SDH) domains by limited proteolysis. An aliquot corresponding to 3000 U LOR and 1670 U SDH was digested with an elastase/enzyme ratio equal to that in lane 4 of Fig. 8 for 10 min at room temperature. (A) Activities of LOR and SDH before (−) and after (+) digestion. (B) The digest was applied to a Protein-Pak Q 8HR column and the column was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer B at a flow rate of 1.5 ml/min. Fractions of 0.75 ml were collected and assayed for LOR (○) and SDH (●) activities. 1 U enzyme activity was defined as 1 nmol of NADPH oxidized or NAD^+ reduced/min at 30°C. (C) Equivalent aliquots of the most active fractions of LOR-III and SDH-I were run on 6% to 18% gradient SDS/PAGE gels. The separated proteins were blotted onto nylon membranes and probed with anti-LOR/SDH polyclonal antibodies. Lane 1, 32 μg of native enzyme. Lane 2, 32 μg of the digest. Lane 3, 10 μg of SDH-I. Lane 4, 23 μg of LOR-III. The numbers on the left correspond to the molecular masses of the 10-kDa protein ladder (Gibco-BRL). The numbers on the right indicate the molecular mass (kDa) of the LOR/SDH-limit polypeptides.

respectively, suggested that their LOR activities could be inhibited by the SDH polypeptides.

To determine the amount of protease necessary to cleave, only the highly elastase-sensitive site that separates the LOR and SDH domains, aliquots of partially purified LOR/SDH were incubated with varying amounts of elastase for 10 min at room temperature. This digestion (Fig. 8A) resulted in an inhibition followed by reactivation of LOR activity similar to that observed in Fig. 5. LOR activity decreased to less than 10% of the undigested enzyme activity when the amount of elastase was increased from 0.008 U to 0.064 U (Fig. 8A). Increasing the quantity of elastase from 0.14 U to 0.54 U resulted in a recovery of LOR activity whereas a further increment again decreased the LOR activity, probably as a result of extensive digestion of the active LOR polypeptides (Fig. 8A). Aliquots of the digests were resolved by SDS/PAGE and the limit polypeptides detected by immunoblotting using anti-LOR/SDH polyclonal antibodies (Fig. 8B). Digestion of LOR/SDH with very small amounts of elastase resulted predominantly in the 65-kDa (LOR) and 57-kDa (SDH) polypeptides (Fig. 8B, lanes 2 to 4). Increasing the amount of elastase (Fig. 8B, lane 5) reduced the 65-kDa LOR to a very faint band, similar to that observed in Fig. 7. A digestion using an elastase/enzyme proportion that resulted in the polypeptide pattern shown in lane 4 of Fig. 8B was prepared. This digest showed the expected reduction in LOR activity (Fig. 9A) and, when separated on a Protein-Pak Q 8HR (Waters) column, produced predominantly LOR-III and SDH-I peaks (Fig. 9B). The separation of LOR-III and SDH-I by SDS/PAGE followed by immunoblotting with anti-LOR/SDH polyclonal antibodies (Fig. 9C) revealed the presence of mainly the 65-kDa polypeptide in the LOR-III peak (Fig. 9C, lane 4) and the 57-kDa polypeptide in the SDH-I peak (Fig. 9C, lane 3). These results confirmed that the polypeptides of 65 kDa and 57 kDa corresponded to the LOR and SDH activities, respectively, as already deduced from the results in Figs 5 and 7. However, two major differences were observed when Fig. 9C was compared with Fig. 7. The amount of the 65-kDa LOR band was equiva-

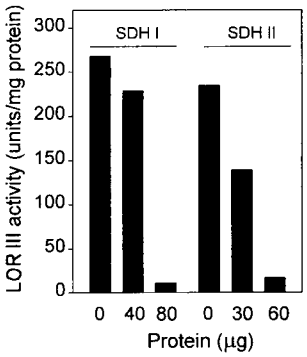

Fig. 10. Effect of SDH polypeptides on LOR-III activity. 6 U of the most active fraction of the LOR-III peak obtained in Fig. 9B were assayed in the presence of increasing amounts of SDH-I and SDH-II proteins from the most active fractions of the experiment in Fig. 9B. The protein mixtures were incubated for 10 min at 4°C before assaying the activities.

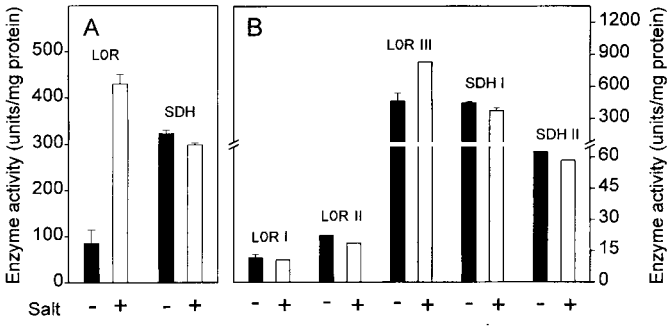

Fig. 11. Effect of high ionic strength on the activities of the proteolytically separated LOR and SDH domains. Aliquots of the most active fractions of each of the LOR and SDH peaks (Fig. 6B) were assayed in 25 mM Tris/HCl, pH 7.4, with or without 155 mM NaCl. Each point is the average of at least duplicate assays. The bars indicate the standard deviation.

lent to that of the 57-kDa SDH band (the activity of the LOR-III peak was nearly the same as that of the SDH-I peak, whereas in the Fig. 6 profiles, LOR-III activity was twofold higher than that of SDH-I), and a relatively strong band of 16 kDa was now seen in the LOR-III peak (Fig. 9C, lane 4).

To assess whether the polypeptides of the SDH-I and SDH-II peaks could inhibit LOR activity, aliquots of the LOR-III peak were assayed for LOR activity in the presence of varying protein amounts of the SDH-I and SDH-II peaks. As expected, both SDH-I and SDH-II almost completely inhibited the LOR-III activity (Fig. 10).

LOR-limit polypeptides are no longer activated by high salt concentrations. Aliquots of the undigested enzyme and of the most active fractions of the LOR and SDH peaks shown in Fig. 6A and B were assayed in the absence or presence of 155 mM NaCl. For the native enzyme, the high salt concentration produced a fivefold increase in LOR activity without affecting SDH activity (Fig. 11A). None of the three LOR peaks was significantly affected by a high salt concentration and, as expected, the SDH peaks remained unchanged (Fig. 11B).

LOR-limit polypeptides are activated by Ca^{2+}. Aliquots of the most active fractions of the three LOR peaks (Fig. 6B) were assayed in the presence of varying amounts of free Ca^{2+}. The activities of all three LOR peaks increased 2–3-fold in the presence of free Ca^{2+}, although clearly two activation constants were detected: one of high-affinity in the range of 100 nM, and a second, similar to that of the undigested enzyme, in the range of 100 µM (Fig. 12; compare Fig. 3A).

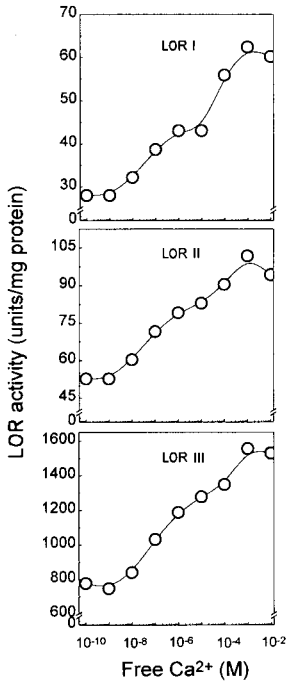

Fig. 12. Effect of Ca^{2+} on the activities of the proteolytically separated LOR domains. Aliquots of the most active fractions of each LOR peak (Fig. 6B) were assayed in 25 mM Tris/HCl, pH 7.4, containing varying concentrations of free Ca^{2+}.

DISCUSSION

Little is known about the physiological role of LOR/SDH and their reaction products in eukaryotes. The presence of LOR and SDH activities in a bifunctional polypeptide [1, 2] may be important for the rapid channeling of lysine into glutamic acid as a mechanism regulating the free lysine level and/or of producing compounds that may play a role in eukaryotic cell metabolism.

Since LOR and SDH are activities of a bifunctional polypeptide, SDH activity can be used as an ideal internal control for studying the modulation of LOR activity because non-specific effects should affect both activities, whereas specific effects should affect one or the other activity. In all experiments where LOR activity responded to a modulator, SDH activity was not affected.

To study the effect of modulators and/or limited proteolysis on LOR and SDH activities, we utilized highly enriched LOR/SDH preparations obtained after chromatography on DEAE-Sepharose or Protein Pak Q 8HR columns. This was done because the enzymatic activity, mainly that of LOR, is lost in highly purified preparations of maize LOR/SDH [2], so that it would not be possible to assay the effect of modulators or proteolysis on the enzyme activity using a highly purified enzyme. To identify the proteolytic products of LOR/SDH, we raised polyclonal antibodies against a highly purified maize LOR/SDH, and used these to detect the peptides in a partial purified LOR/SDH preparations in which the enzyme activities are much more stable (data not shown). We did not observe endogenous proteolytic activity in these preparations (see Fig. 5B, lane C; Fig. 8B, lane 1 and Fig. 9C, lane 1). Therefore the polypeptides observed

when extracts were incubated with elastase are likely to be the products of the added protease.

The enzymatic activity of the LOR domain of maize LOR/SDH was activated by Ca^{2+}. Furthermore, the inhibition of LOR activity by EGTA in crude extracts and poly(ethylene glycol) precipitates was only observed at low buffer concentrations, suggesting that a high ionic strength was sufficient to activate the enzyme. The effect of high ionic strength was clearly demonstrated by varying the salt and buffer concentrations of the assay mixture. In all cases, LOR activity increased with the increasing ionic strength of the medium, indicating that the modulation was not an effect of monovalent cations. Organic solvents, at concentrations that decreased the water activity, also enhanced LOR activity. The observation that the LOR domain separated by proteolysis retained its sensitivity to activation by Ca^{2+}, but was no longer activated by a high ionic strength, suggests the existence of different mechanisms for LOR activity modulation.

The activation of LOR by Ca^{2+} or ionic strength may result from a stabilizing effect of the dimeric form of the enzyme. Increasing salt concentrations provide the conditions for oligomerization of certain cytosolic and membrane-bound enzymes [27]. However, it is not clear why the stabilizing effect of high salt concentrations or Ca^{2+} did not affect the contiguous domain responsible for SDH activity. It is also likely that Ca^{2+}, ionic strength and organic solvents induce conformational changes in the LOR domain of the enzyme, exposing hydrophobic plates on the surface of the protein, as has been shown for calmodulin and other Ca^{2+}-binding proteins [28]. The Ca^{2+}-dependent enhancement of LOR activity was inhibited by two structurally different calmodulin antagonists, W7 and calmidazolium, but we were unable to detect the binding of calmodulin to LOR (data not shown). Similarly, we were unable to detect binding of Ca^{2+} to LOR/SDH (data not shown).

The bifunctional LOR/SDH polypeptide possessed a highly elastase-sensitive site, the cleavage of which yielded polypeptides of 65 kDa and 57 kDa, which contained the functional domains of LOR and SDH, respectively. The LOR/SDH from bovine liver is also cleaved by elastase into two limit polypeptides of 63 kDa and 49 kDa, which contain the LOR and SDH domains, respectively [1]. Thus, apparently the maize and bovine enzymes possess an elastase-sensitive site at similar positions. However, while the bovine enzyme activities did not change with elastase treatment [1], the maize LOR activity was drastically affected by proteolysis. The inactivation followed by reactivation after a few hours of digestion or with increasing amounts of elastase (Figs 5A and 8A) most likely reflect complex interactions between the limit polypeptides. One possibility is that since the active form of the native enzyme is a dimer [2], dimerization could also be a strict condition for the activities of the proteolytically separated functional domains. Native bovine LOR/SDH is a tetramer and its isolated LOR domain behaves as a tetramer during gel filtration [1]. In the maize LOR/SDH digest, the polypeptides containing the LOR and SDH domains could interact with each other to form LOR-limit/SDH-limit heterodimers. However, these heterodimers must be inactive for LOR. The only active LOR appear to be homodimers formed by two LOR-limit polypeptides. Thus, the large proportions of the 49-kDa and 65-kDa LOR polypeptides that eluted with the SDH-I and SDH-II peaks, respectively, resulted from the formation of LOR-limit-SDH-limit heterodimers (Fig. 7). Other LOR polypeptides resulting from the formation of LOR-limit/SDH-limit heterodimers can also be seen in the SDH-I peak (Fig. 7, lane 4). Why such LOR and SDH polypeptide interactions did not affect SDH activity during the digestion is unclear (Fig. 5). A possible explanation may be that SDH activity does not depend on dimerization, or that SDH-limit/SDH-limit homodimers could form even in the presence of bound LOR polypeptide(s). Another possibility is that the SDH polypeptide(s) contain a protein domain that inhibits LOR activity. This inhibitory domain of SDH polypeptides could interact strongly with LOR polypeptides, allowing the co-elution of bound LOR and SDH polypeptides from the Protein-Pak Q 8HR column. Following a few hours digestion or incubation with increasing amounts of elastase, this inhibitory domain could be removed, resulting in the reactivation of LOR activity (Figs 5 and 8). The experiments in Figs 8–10 support this hypothesis by indicating that SDH polypeptides inhibited LOR-III activity.

As shown in lane 5 of Fig. 7, only a very small amount of LOR-III polypeptide was responsible for the twofold increase in activity seen in relation to the total activity of the digest applied to the column (Fig. 6B and C and Table 3). Since most of the LOR polypeptides eluted with the SDH polypeptides, it is likely that the total LOR activity could be increased several-fold if all the LOR polypeptides were in the form represented in lane 5 of Fig. 7. When digestion was controlled to produce almost exclusively the 65-kDa (LOR) and 57-kDa (SDH) kDa polypeptides (note that in this digest the LOR activity was strongly inhibited; Fig. 9A), both polypeptides were present in equimolar amounts, but the activity of LOR did not increase in relation to the SDH-I activity. Therefore it is likely that in this case the LOR activity was partially inhibited, and it suggests that LOR is activated by proteolysis. Enzymatic activation by limited proteolysis has been shown for many enzymes [29–33], including choline acetyltransferase (ChAT) which is activated fivefold by limited proteolysis [29]. High salt concentrations also activate native but not the proteolytically activated ChAT [29]. Similar results were observed for maize LOR. High salt levels were observed to stimulate by several-fold the LOR activity of native enzyme, but not the proteolytically separated LOR polypeptides (Figs 2 and 11). An inhibitory domain has been found for the calmodulin-binding glutamate decarboxylase (GAD) from plants [34]. After binding Ca^{2+}-calmodulin to its inhibitory domain, the catalytic center of GAD is released for substrate binding. Deletion of this inhibitory domain from a recombinant GAD produced a fully active enzyme insensitive to modulation by Ca^{2+}-calmodulin *in vitro* [34–36]. LOR was activated by Ca^{2+} in the native enzyme complex and in the proteolytically separated LOR polypeptides (Figs 3A and 12). Thus, it is possible that, as for GAD [35], Ca^{2+} activation of LOR activity results from the derepression of an inhibitory domain so that *in vivo*, Ca^{2+} may be the most important physiological modulator of LOR activity. Analysis of the *Arabidopsis* LOR/SDH protein sequence [26] revealed an EF-hand-like sequence between residues 384–397 of the LOR domain. Thus if this sequence is conserved in the maize LOR/SDH protein, it could be a possible Ca^{2+} binding site, although we did not detect direct binding of Ca^{2+} to the protein using experimental conditions similar to that described for other Ca^{2+} binding proteins (data not shown). However, it is possible that post-translational protein modifications, such as phosphorylation, are also required for Ca^{2+} binding. Phosphorylation has being shown to modulate LOR activity in soybean [37]. Limited proteolysis is unlikely to have any physiological role *in vivo* since we have never detected significant amounts of the proteolytic products of the LOR/SDH complex in crude extracts from immature maize endosperm.

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The Role of Opaque2 in the Control of Lysine-Degrading Activities in Developing Maize Endosperm

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We have isolated a cDNA clone, designated *ZLKRSDH*, encoding the bifunctional enzyme lysine–ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) from maize. The predicted polypeptide has an N-terminal LKR domain and a C-terminal SDH domain that are similar to the yeast LYS1 and LYS9 monofunctional proteins, respectively. The maize LKR/SDH protein is located in the cytoplasm of subaleurone endosperm cell layers. Transcripts and polypeptides as well as enzyme activities showed an upregulation and downregulation during endosperm development. The developmental expression of *ZLKRSDH* was examined in normal and *opaque2* seeds. In the mutant endosperm, mRNA levels were reduced by >90%, with concomitant reductions in polypeptide levels and LKR/SDH activity. These results suggest that lysine levels in the endosperm are likely to be controlled at the transcriptional level by the Opaque2 transcription factor.

INTRODUCTION

Since the discovery of the high-lysine *opaque2* (*o2*) maize mutant (Mertz et al., 1964), research efforts have been directed toward understanding the biochemical and molecular mechanisms leading to the increase in lysine content in the endosperm. Studies conducted during the past 30 years revealed that the homozygous *o2* mutation causes an ~70% reduction in zein content and affects the content of a number of proteins and enzymes related to nitrogen and sugar metabolism in the maize endosperm (Habben et al., 1993; Giroux et al., 1994; Gallusci et al., 1996). The cloning of the *O2* gene revealed that it encodes a basic leucine zipper protein transcription factor involved in the transcriptional control of the zein genes, the b-32 ribosome-inactivating protein, and cytoplasmic pyruvate orthophosphate dikinase (cyPPDK), an enzyme involved in carbon partitioning (Lohmer et al., 1991; Bass et al., 1992; Schmidt et al., 1992; Cord Neto et al., 1995; Gallusci et al., 1996). Lysine catabolism also is affected in the *o2* mutant. ^{14}C-lysine feeding experiments revealed that lysine was converted to glutamic acid and proline but to a much lesser extent in *o2* than in normal endosperm (Sodek and Wilson, 1970).

Despite the observation of extensive lysine degradation in maize seeds (Sodek and Wilson, 1970; da Silva and Arruda, 1979), there have been several attempts to establish positive selection strategies to isolate mutants with aspartate kinase (AK) insensitivity to feedback inhibition caused by lysine. AK is the first regulatory enzyme of the aspartate pathway, which leads to the biosynthesis of lysine, methionine, threonine, and isoleucine. Thus, the derepression of AK would result in an overproduction of these amino acids (reviewed in Azevedo et al., 1997). AK-insensitive mutants that overproduce threonine have been described in barley (Bright et al., 1982), maize (Diedrick et al., 1990), and tobacco (Frankard et al., 1992), but they exhibit only marginal effects on lysine accumulation. The absence of lysine overproduction in these mutants was attributed to the second regulatory enzyme, dihydrodipicolinate synthase (DHDPS), which is the first enzyme of the lysine branch of the aspartate pathway. This enzyme is much more sensitive to lysine feedback inhibition than is AK (reviewed in Azevedo et al., 1997). A few experiments succeeded in isolating lysine-insensitive DHDPS mutants, but the seeds from these mutants did not show a significant increase in lysine (Negrutiu et al., 1984). AK/DHDPS–insensitive double mutants did not have a significant increase in lysine content either (Frankard et al., 1991).

Recently, transgenic tobacco and canola plants expressing lysine-insensitive AK or DHDPS or both enzymes were produced. These transgenic plants showed threonine overproduction and appreciable free lysine increments in young leaves and seeds. These mutants, however, displayed severe

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phenotypic alterations (Karchi et al., 1993; Shaul and Galili, 1993; Falco et al., 1995; Pederson et al., 1996), and lysine overproduction was followed by increased lysine degradation (Karchi et al., 1994; Falco et al., 1995).

We have postulated that lysine catabolism is an important mechanism for the control of free lysine levels in maize endosperm cells (Arruda and da Silva, 1979; da Silva and Arruda, 1979). Lysine degradation in maize endosperm is catalyzed by lysine–ketoglutarate reductase (LKR; Arruda et al., 1982). During kernel development, LKR activity is coordinated with nitrogen input and zein protein synthesis (Arruda and da Silva, 1983). Further analysis of the developmental pattern of LKR activity in normal and *o2* endosperm showed that enzyme activity is decreased in the mutant, suggesting that reduced lysine degradation in the *o2* endosperm could be due to a lower content of LKR polypeptides (Brochetto-Braga et al., 1992).

Maize LKR was purified to homogeneity and demonstrated to be associated with saccharopine dehydrogenase (SDH) in a bifunctional 125-kD polypeptide (Gonçalves-Butruille et al., 1996), similar to the previously isolated mammalian enzyme (Markovitz and Chuang, 1987). The complete genomic and cDNA sequences recently reported for the Arabidopsis LKR/SDH gene confirm the bifunctional nature of the enzyme (Epelbaum et al., 1997; Tang et al., 1997).

To further study the influence of the *o2* mutation on lysine accumulation in the developing maize endosperm, we cloned the gene encoding the maize bifunctional LKR/SDH (designated *ZLKRSDH* for *Zea mays* LKR and SDH) and examined its spatial and temporal patterns of expression. We found that the maize LKR/SDH is a cytosolic enzyme encoded by a single gene that is highly expressed in the subaleurone cell layers of the distal part of the developing endosperm. In the *o2* mutant, *LKR/SDH* mRNA and protein quantities are severely reduced, and the expression pattern during kernel development is markedly modified.

RESULTS

Isolation of a cDNA Encoding the Maize LKR/SDH Bifunctional Enzyme

LKR/SDH was purified to homogeneity from 17–days after pollination (DAP) maize endosperm, and the N-terminal sequences of four tryptic peptides were used to design a set of 21- to 23-mer degenerate oligonucleotides. These primers and the cDNA produced from reverse-transcribed 17-DAP total endosperm RNA were used in reverse transcription–polymerase chain reactions (RT-PCRs). A 1.2-kb DNA fragment was amplified and confirmed to be part of the *ZLKRSDH* gene by comparing its sequence with the nucleotide sequences of the yeast *Lys1* and *Lys9* genes, which encode LKR and SDH, respectively. This fragment was used as a probe to screen an immature endosperm cDNA library (Aukerman et al., 1991). The nucleotide sequences from 20 positive clones were analyzed, but they either contained mostly the SDH domain or were chimeric at their 5′ ends. To obtain a full-length clone, we isolated the 5′ end of the cDNA by using rapid amplification of cDNA ends (RACE), and the 3.5-kb cDNA was further amplified using primers annealing to the 5′ and 3′ untranslated regions.

As shown in Figure 1A, the amplified cDNA encodes an open reading frame of 1064 amino acids, which predicts a protein of 116.8 kD, close to the maize 125-kD polypeptide observed after SDS-PAGE (Gonçalves-Butruille et al., 1996). The 5′ untranslated region is 178 bp long and presents two in-frame ATG codons. The first one does not present a good initiation context and is followed by a stop codon. On the other hand, the second ATG codon is in a favorable context for translation initiation (Luehrsen and Walbot, 1994). The 3′ untranslated region is 136 bp long and has two putative polyadenylation signals (AATAAA) located 93 and 102 bp downstream from the TAG stop codon (data not shown). Residues H-110, K-113, and R-146 of the maize LKR domain (Figure 1B) are conserved in all LKR sequences obtained to date. These residues have been shown to be essential for substrate binding in the yeast LYS1 protein (Ogawa et al., 1979; Fujioka et al., 1980; Ogawa and Fujioka, 1980; Fujioka and Takata, 1981).

Figure 2 shows a schematic representation of similarities between the predicted maize polypeptide and other known monofunctional and bifunctional LKR/SDH enzymes. A SDH C-terminal domain sharing 42% similarity with the yeast LYS9 protein and an N-terminal domain sharing 27% similarity with the yeast LYS1 protein constitute the bifunctional maize enzyme. Similarities to the related mouse (F. Papes, E.L. Kemper, G. Cord Neto, F. Langone, and P. Arruda, submitted manuscript), *Caenorhabditis elegans*, and Arabidopsis proteins are significantly higher (44, 46, and 72%, respectively). The interdomain region (Figure 1A), which is ~106 residues long in maize, is 57% identical to the corresponding Arabidopsis sequence. Interestingly, this region seems to be absent in *C. elegans* and mouse LKR/SDH proteins (Figure 2).

Comparisons of primary protein structures also reveal several conserved motifs (Figure 2). The initiation ATG and stop codons, as well as some motifs with a high degree of similarity, appear at comparable sites in the open reading frame of all compared proteins.

To confirm the structure of the LKR/SDH bifunctional polypeptide, we separated the LKR and SDH domains by limited proteolysis (Figure 3). A partially purified preparation of LKR/SDH protein was chromatographed on an ion exchange column (Figure 3A) or digested with elastase before chromatography (Figure 3B). The most active fractions of LKR and SDH peaks from Figure 3B were immunoblotted with anti–LKR/SDH polyclonal antibodies (Figure 3C). Functional isolated LKR and SDH domains were recovered, confirming the bifunctional enzyme structure (Gonçalves-Butruile et al., 1996; Kemper et al., 1998).

A

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   1 MGSAATEGNDTLLGNGVVGILAETCNMWER
  31 RAPLTPSHCARLLLGGGKNGPRVNRIIVQP
  61 STRRIHHDAQYEDAGCEISEDLSECGLIIG
  91 IKQPKLQMILSDRAYAFFSHTHKAQKENMP
 121 LLDKILEERVSLFDYELIVGDDGKRSLAFG
 151 KFAGRAGLIDFLHGLGQRYLSLGYSTPFLS
 181 LGQSHMYPSLAAAKAAVIVVAEEIATFGLP
 211 SGICPIVFVFTGVGNVSQGAQEIFKLLPHT
 241 FVDAEKLPEIFQARNLSKQSQSTKRVFQLY
 271 GCVVTSRDMVSHKDPTRQFDKGDYYAHPEH
 301 YTPVFHERIAPYASVIVNCMYWEKRFPPLL
 331 NMDQLQQLMETGCPLVGVCDITCDIGGSIE
 361 FINKSTSIERPFFRYDPSKNSYHDDMEGAG
 391 VVCLAVDILPTEFSKEASQHFGNILSRLVA
 421 SLASVKQPAELPSYLRRACIAHAGRLTPLY
 451 EYIPRMRNTMIDLAPAKTNPLPDKKYSTLV
 481 SLSGHLFDKFLINEALDIIETAGGSFHLVR
 511 CEVGQSTDDMSYSELEVGADDTATLDKIID
 541 SLTSLANEHGGDHDAGQEIELALKIGKVNE
 571 YETDVTIDKGGPKILILGAGRVCRPAAEFL
 601 ASYPDICTYGVDDHDADQIHVIVASLYQKD
 631 AEETVDGIENTTATQLDVADIGSLSDLVSQ
 661 VEVVISLLPASFHAAIAGVCIELKKHMVTA
 691 SYVDESMSNLSQAAKDAGVTILCEMGLDPG
 721 IDHLMSMKMIDEAHARKGKIKAFTSYCGGL
 751 PSPAAANNPLAYKFSWNPAGALRSGKNPAV
 781 YKFLGETIHVDGHNLYESAKRLRLRELPAF
 811 ALEHLPNRNSLIYGDLYGISKEASTIYRAT
 841 LRYEGFSEIMVTLSKTGFFDAANHPLLQDT
 871 SRPTYKGFLDELLNNISTINTDLDIEASGG
 901 YDDDLIARLLKLGCCKNKEIAVKTVKTIKF
 931 LGLHEETQIPKGCSSPFDVICQRMEQRMAY
 961 GHNEQDMVLLHHEVEVEYPDGQPAEKHQAT
 991 LLEFGKVENGRSTTAMALTVGIPAAIGALL
1021 LLKNKVQTKGVIRPLQPEIYVPALEILESS
1051 GIKLVEKVETKFPE
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B

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ZEA  FFSHTHKAQKENMPLLDKILEERVSLFDYELIVGDDGKRSLAFG
ATH  FFSHTHKAQKENMPLLDKILSERVTLCDYELIVGDHGKRLLAFG
CEL  FFSHTIKAQQDNMEMLDTILQRNIRLLDYEKICDDKGKRLVMFG
CAL  QFAHCYKDQAGWQDVLKRFPQGNGILYDLEFLENDQGRRVAAFG
YLI  QFAHCYKDQGGWKDVLSRFPAGNGTLYDLEFLEDDNGRRVAAFG
SCE  QFAHCYKDQAGWQNVLMRFIKGHGTLYDLEFLENDQGRRVAAFG
      * *  * *     *         * * *   * * *   **
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Figure 1. Predicted Sequence of the *ZLKRSDH* cDNA–Encoded Protein.

(A) The protein sequence encoded by the 3.5-kb *ZLKRSDH* cDNA is shown. Underlined residues highlight the interdomain region. Conserved residues shown in **(B)** are in boldface. The GenBank accession number is AF003551.

(B) Alignment of the LKR subdomain that is homologous to the putative substrate binding site previously assigned to the yeast LYS1 protein. GenBank accession numbers are listed in the legend to Figure 2. The essential conserved residues H-110, K-113, and R-146 of the maize protein are shown in boldface characters. Identical residues are marked by asterisks. ZEA, *Z. mays*; ATH, *Arabidopsis thaliana*; CEL, *C. elegans*; CAL, *Candida albicans*; YLI, *Yarrowia lipolytica*; SCE, *Saccharomyces cerevisiae.*

ZLKRSDH Is Present in Maize and Related Species

We investigated the presence of the *ZLKRSDH* gene in maize and related cereals. The 1.2-kb RT-PCR DNA fragment, comprising part of the LKR domain, the interdomain region, and part of the SDH domain (residues 444 through 849; Figure 1), was hybridized to genomic DNA from maize, Coix, and sorghum. As shown in Figure 4, both BamHI and EcoRV digests gave single, strong hybridizing bands, suggesting that *ZLKRSDH* is likely to be present as a single-copy gene in *Andropogoneae* sp.

Analysis of *ZLKRSDH* mRNA Levels in Different Maize Tissues

Total RNA extracted from roots, leaves, coleoptiles, embryos, and endosperm was hybridized with either LKR- or SDH-specific probes. As shown in Figure 5, *ZLKRSDH* mRNA was detected mainly in the endosperm. No signal was detected in embryos, and only marginal amounts of expression were detected in roots, leaves, and coleoptiles. Five different transcripts were detected in 20-DAP developing endosperm with both probes. Along with the major 3.5-kb band, which corresponds to >90% of the total hybridizing mRNA, alternate transcripts of 4.1, 2.6, 2.4, and 1.5 kb were detected. The 4.1- and 3.5-kb transcripts were detected equally by both probes. The 2.4- and 2.6-kb species seemed to contain mainly SDH sequences (Figure 5B), because they hybridized only weakly with the LKR-specific probe (Figure 5A), whereas the 1.5-kb transcript seemed to contain only LKR sequences (Figure 5A). The nature and function of these alternate transcripts are not known, but they certainly do not encode monofunctional LKR or SDH enzymes because in the results shown in Figure 3, only one polypeptide product was detected in the endosperm by the anti–LKR/SDH polyclonal antibodies. This polypeptide is able to recognize with similar efficacy both the LKR and SDH domains.

Developmental Expression of *ZLKRSDH* in Normal and *o2* Mutant Endosperm

We showed previously that LKR activity is reduced two- to threefold in the *o2* endosperm (Brochetto-Braga et al., 1992). To test whether this lower enzyme activity was due to

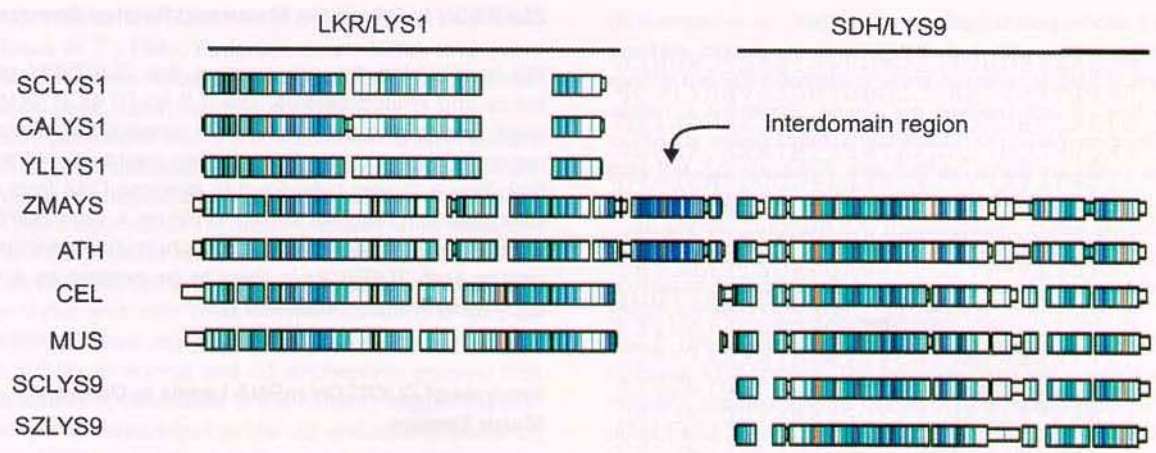

Figure 2. Schematic Diagram of Similarities among Monofunctional and Bifunctional LKR/SDH Polypeptides from Yeast, Plants, and Animals.

The predicted amino acid sequences for monofunctional saccharopine dehydrogenase (LYS1, lysine forming) and saccharopine dehydrogenase (LYS9, glutamate forming) from yeast were aligned to plant, nematode, and mammalian bifunctional LKR/SDH polypeptides. Sequence alignments and similarity analysis were performed using the MACAW program applying the BLOSUM 62 matrix. The similarity at a given position is designated by the following color codes: red, >90%; blue, 50 to 90%; cyan, 10 to 49%; and white, <10%. Similarity at a given position means the percentage of sequences being compared that match the consensus sequence. The LKR and SDH domains, corresponding to the yeast LYS1 and LYS9 proteins, respectively, as well as the unique interdomain region present in plant bifunctional polypeptides, are indicated. Blank spaces indicate sequence gaps introduced to maximize the alignment. Nomenclature and GenBank accession numbers are as follows: SCLYS1, *S. cerevisiae* (P38998); CALYS1, *C. albicans* (P43065); YLLYS1, *Y. lipolytica* (P38997); ZMAYS, *Z. mays* (AF003551); ATH, *A. thaliana* (U95759); CEL, *C. elegans* (AF038615); MUS, *Mus musculus* (AJ224761); SCLYS9, *S. cerevisiae* (P38999); and SZLYS9, *Schizosaccharomyces pombe* (AL022244).

reduced expression of the *ZLKRSDH* gene, we assayed normal and *o2* seeds, harvested at 10, 15, 20, 25, 30, 35, and 40 DAP, for enzymatic activities and analyzed them by RNA gel blotting and immunoblotting using anti–LKR/SDH polyclonal antibodies (Kemper et al., 1998).

As shown in Figure 6, transcripts and polypeptides as well as enzyme activities showed an upregulation and a downregulation during endosperm development. In normal endosperm, LKR and SDH activities and LKR/SDH protein levels reached a maximum at 20 DAP, whereas in the mutant endosperm, maximum polypeptide levels and enzyme activities were observed at 25 DAP. *ZLKRSDH* mRNA amounts in normal endosperm peaked at 15 DAP, whereas in *o2* endosperm, the mRNA amount peaked at 20 to 25 DAP.

At 20 DAP, LKR and SDH activities in the mutant were reduced to ~2 and 18%, respectively, of the values in normal seeds (Figure 6A). This was accompanied by an ~90% reduction in the amounts of transcript and polypeptide (Figures 6B and 6C). At 25 DAP, the polypeptide amount increased in the mutant in comparison to the wild type, with a concomitant increase in LKR and SDH activities to ~9 and 50%, respectively.

The correlation between enzyme activities and quantitative immunoblot band intensities, for both normal and *o2* endosperm, was 94% for SDH and 81% for LKR activity (data not shown). This indicates that decreased enzyme activity is due to reduced polypeptide levels resulting from a reduced transcript accumulation from the *ZLKRSDH* gene in the mutant endosperm.

LKR/SDH Is Located in the Cytosol of Subaleurone Cell Layers

Histochemical staining for SDH activity was used to locate the LKR/SDH protein within the endosperm. SDH activity was detected in the peripheral distal part of the endosperm (Figures 7A and 7B). An immunological study of LKR/SDH was conducted to further characterize the cells currently expressing the bifunctional enzyme (Figures 7C to 7F). The immunolabeled region was similar to that stained for SDH activity. Expression of LKR/SDH is most intense in the subaleurone layers and surrounding the nuclei and starch grains. In cells far away from the subaleurone cell layers, the immunostaining was weak and tended to be near the peripheral cell regions, adjacent to cell walls. No immunolabeling was detected in the aleurone layer.

Subcellular fractionation was used to determine the cell compartmentalization of LKR/SDH. As shown in Figure 8, LKR and SDH activities were only detected in the cytosol, whereas the activities of the marker enzymes, cytochrome *c* oxidase and cytochrome *c* reductase, were observed in the endoplasmatic reticulum and mitochondria, respectively.

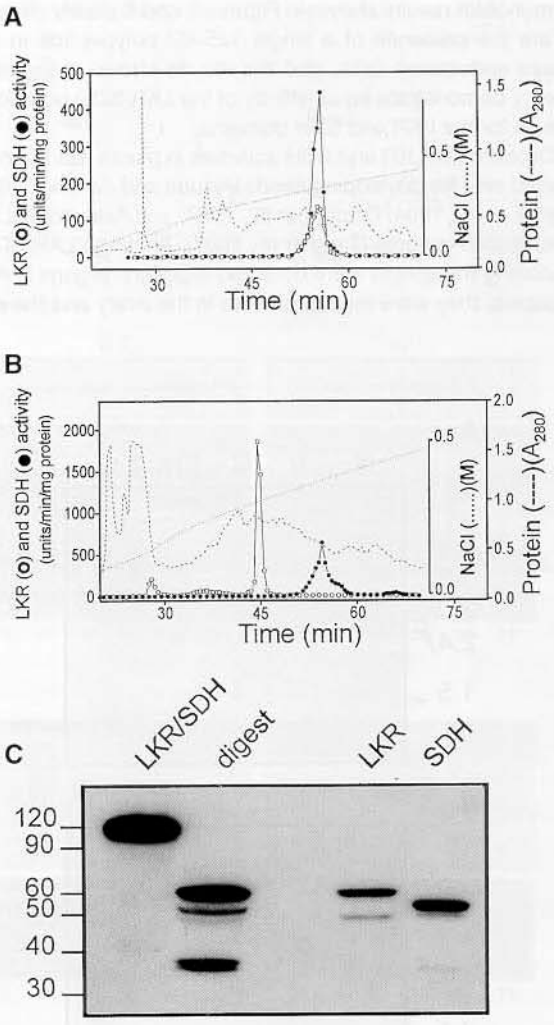

Figure 3. Delimitation of Maize Bifunctional LKR and SDH Domains.

(A) and **(B)** Elution profiles for LKR and SDH activities before **(A)** and after **(B)** digestion with elastase. A partially purified DEAE-Sepharose preparation of LKR/SDH was digested with elastase, applied to a Protein-Pak Q 8HR column, and eluted with a linear gradient of 0 to 5 M NaCl in buffer B at a flow rate of 1.5 mL/min (see Methods for further details). Fractions of 0.75 mL were collected and assayed for LKR and SDH activities. Absorbance at 280 nm is indicated at right.

(C) Immunoblot of native and elastase-digested LKR/SDH. Equivalent aliquots of the most active fractions of LKR and SDH peaks were applied to a 6 to 18% gradient SDS–polyacrylamide gel. The separated proteins were blotted onto nylon membranes and incubated with anti–LKR/SDH polyclonal antibodies. Numbers at left correspond to the molecular mass markers of the 10-kD protein ladder (Gibco BRL). The lane labeled LKR/SDH contains native enzyme; digest, elastase digestion products; LKR, LKR peak; SDH, SDH peak.

DISCUSSION

The biochemical and molecular mechanisms by which the *o2* mutation increases lysine content in the endosperm have been investigated during the past 30 years. Both lysine synthesis and degradation as well as its incorporation into lysine-rich proteins should contribute to the final content of this amino acid in the seed. In this report, we describe the isolation of a maize cDNA clone encoding the lysine-degrading enzyme LKR/SDH and present evidence on how lysine levels may be controlled in the endosperm. A conspicuous correlation between lysine-degrading activities and transcriptional regulation by the Opaque2 transcription factor was observed. LKR/SDH seems to be located within cells in which zein protein is being actively synthesized.

The Bifunctional Maize LKR/SDH Enzyme

A cDNA encoding the bifunctional enzyme LKR/SDH was isolated from the immature maize endosperm mRNA pool. The cDNA predicts a 117-kD protein (Figure 1) bearing distinct N- and C-terminal domains identified, respectively, as LKR and SDH due to similarities to yeast monofunctional enzymes (SDH lysine– and glutamate-forming enzymes encoded by the *Lys1* and *Lys9* genes, respectively). Further confirmation of the identity of the maize cDNA was obtained by comparing sequences from similar genes recently isolated from Arabidopsis (Epelbaum et al., 1997; Tang et al., 1997) and mouse (F. Papes, E.L. Kemper, G. Cord Neto, F. Langone, and P. Arruda, submitted manuscript).

In plants and mammals, the saccharopine pathway is used for lysine catabolism, whereas in yeast and other fungi, this pathway is used in the reverse order for lysine biosynthesis. Either bifunctional (maize, soybean, Arabidopsis, bovine, murine, and human) or monofunctional (yeast, rat, and Arabidopsis) polypeptides operate in this pathway. As illustrated in Figure 2, the amino acid sequences from these enzymes share a high degree of similarity. Nevertheless, there are marked differences between bifunctional LKR/SDH polypeptides from plants and animals.

First, the enzyme seems to be expressed in different cell compartments. The mammalian LKR and SDH enzymes have been located in the mitochondrial fraction (Markovitz et al., 1984; Ameen et al., 1987; Blemings et al., 1994), whereas the maize enzyme seems to be located in the cytosol (Figure 8). It is interesting that neither the predicted maize LKR/SDH (Figure 1) nor the Arabidopsis protein (Epelbaum et al., 1997) contains putative mitochondrial or chloroplast targeting sequences.

Second, the interdomain region is present in plants but seems to be absent from the animal enzymes (Figure 2). This region is 57% identical in maize and Arabidopsis, but its functional role remains unknown. Limited proteolysis studies with the maize LKR/SDH suggest that it might be involved in

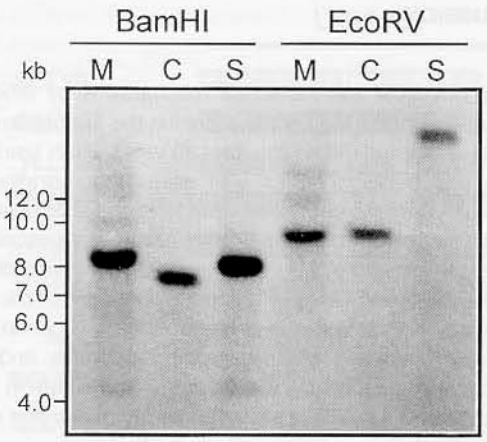

Figure 4. DNA Gel Blot Analysis of *ZLKRSDH* in Maize, Coix, and Sorghum.

Ten micrograms of DNA isolated from maize, Coix, and sorghum was digested with BamHI and EcoRV, resolved in a 1% agarose gel, transferred onto a nylon membrane, and hybridized under high-stringency conditions with the LKR/SDH 1.2-kb RT-PCR cDNA fragment as a probe. Restriction fragments hybridizing to the probe are present in maize (M), Coix (C), and sorghum (S). The migration of molecular length markers is indicated at left in kilobases.

the modulation of LKR activity (Kemper et al., 1998). Other evidence for the role of the interdomain region on the modulation of LKR activity came from studies of the effect of Ca^{2+} and ionic strength on maize and bovine enzymes. Maize LKR is activated by Ca^{2+} and high salt concentrations, whereas bovine LKR is insensitive to these modulators (Kemper et al., 1998).

Tissue-Specific and Developmental Expression of *ZLKRSDH*

RNA gel blot analysis of total RNA from 20-DAP endosperm revealed multiple hybridizing bands ranging in size from 1.5 to 4.1 kb (Figure 5). The 3.5-kb transcript, which accounts for >90% of total *ZLKRSDH* transcribed mRNA, has the expected size of the isolated cDNA shown in Figure 1. The alternate transcripts may originate from a single gene, and whether they have any functional relevance is still unknown. Tang et al. (1997) have shown that Arabidopsis cells contain mRNA species, encoding bifunctional LKR/SDH and monofunctional SDH, likely to be transcribed from a single gene. Bifunctional LKR/SDH and monofunctional SDH also were observed in mouse (F. Papes, E.L. Kemper, G. Cord Neto, F. Langone, and P. Arruda, submitted manuscript). In maize, however, the sole protein product detected corresponds to a single protein band of 125 kD exhibiting both LKR and SDH activities (Brochetto-Braga et al., 1992; Gonçalves-Butruille et al., 1996; Kemper et al., 1998). In addition, the immunoblot results shown in Figures 3 and 6 clearly demonstrate the presence of a single 125-kD polypeptide in the maize endosperm cells, and the results shown in Figure 3 clearly demonstrate equal affinity of the LKR/SDH polyclonal serum for the LKR and SDH domains.

Detection of LKR and SDH activities in plants has been reported only for developing seeds (Arruda and da Silva, 1983; Karchi et al., 1994; Gaziola et al., 1997) and Arabidopsis cell suspension cultures (Tang et al., 1997). Although LKR/SDH–encoding transcripts were detected in several organs in Arabidopsis, they were most abundant in the ovary and the em-

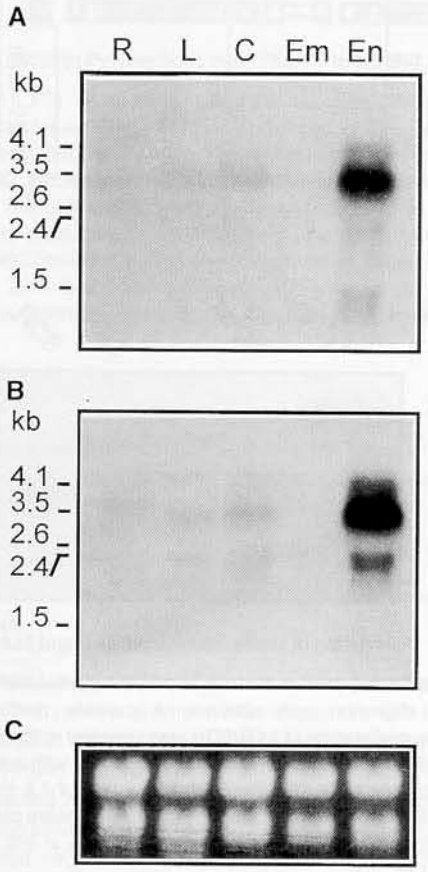

Figure 5. RNA Gel Blot Analysis of LKR/SDH–Endcoding mRNA in Different Maize Tissues.

(A) and **(B)** Twenty micrograms of total RNA from roots (R), leaves (L), coleoptiles (C), embryos (Em), and 20-DAP normal endosperm (En) was fractionated in a 1% agarose gel containing 0.66 M formaldehyde, transferred to a nylon membrane, and hybridized with an LKR domain probe **(A)**. After 48 hr of exposure, the membrane was stripped and hybridized against an SDH domain probe **(B)**. The lengths of the detected LKR/SDH transcripts are shown at left in kilobases. A better resolution of the 2.4- and 2.6-kb bands is shown in Figure 6C.

(C) Loading control performed by ethidium bromide staining.

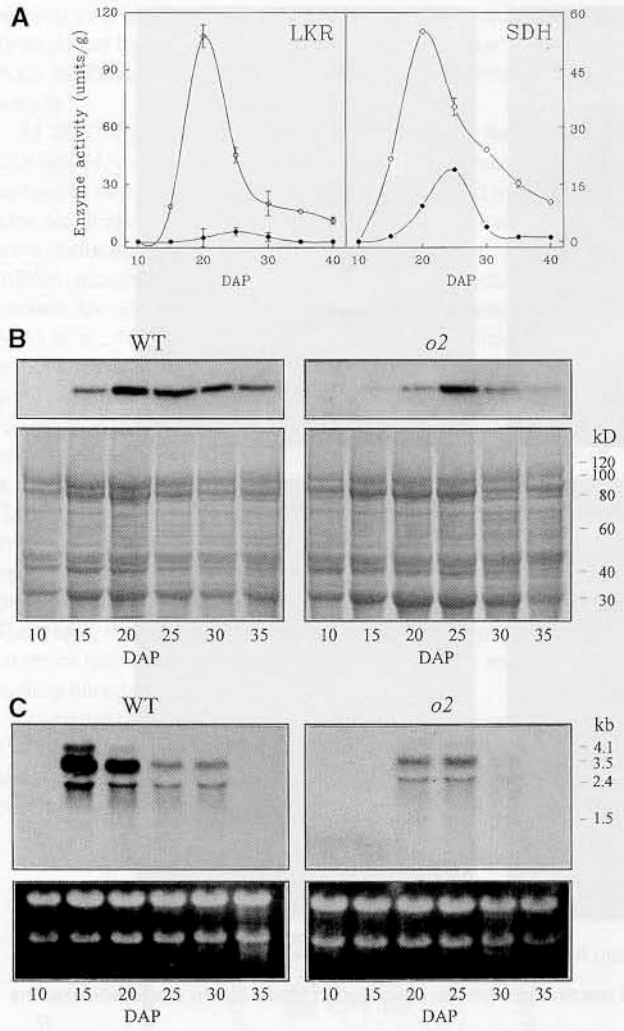

Figure 6. Developmental Expression of LKR/SDH in Wild-Type and *o2* Endosperm.

(A) Temporal patterns of LKR and SDH activities in normal and *o2* endosperm. Seeds of wild type (open circles) and *o2* (closed circles) were harvested from 10 to 40 DAP at 5-day intervals. Equal amounts of endosperm were homogenized in buffer A and fractionated with ammonium sulfate. The fractions collected between 35 and 60% saturation were assayed for LKR and SDH activities. Each point is the average of duplicate assays, and the bar indicates the standard deviation.

(B) Immunoblot showing LKR/SDH protein temporal profile in normal and *o2* endosperms. Aliquots of wild-type (WT) and *o2* ammonium sulfate–fractionated extracts from **(A)** were dialyzed against buffer A, separated in a 7% SDS–polyacrylamide gel, blotted onto nylon membranes, and incubated with anti–LKR/SDH polyclonal antibodies at a 1:10,000 (v/v) dilution. At top are the immunoblots, and at bottom are the protein loading controls stained with Coomassie blue. Numbers at right correspond to the molecular mass markers of the 10-kD protein ladder (Gibco BRL).

(C) *ZLKRSDH* gene expression during development of normal and *o2* endosperm. Fifteen-microgram samples of total RNA extracted from the same lots of wild-type (WT) and *o2* endosperm shown in **(A)** were fractionated in a formaldehyde-containing 1.0% agarose gel, transferred to a nylon membrane, and hybridized with the 1.2-kb RT-PCR cDNA fragment as a probe. The lengths of the detected LKR/SDH–encoding transcripts are shown at right. Loading controls, representing an ethidium bromide–stained gel, are shown at bottom.

bryo (Tang et al., 1997). In maize, *ZLKRSDH* mRNA is abundant in the endosperm but is completely absent in the embryo and scarcely detectable in roots, leaves, and coleoptiles (Figure 5). Enzymatic activity, indeed, was not detected in those organs. Furthermore, endosperm specific activity is ~30-fold higher in maize than in soybean and common bean (data not shown), suggesting that LKR/SDH may play an important physiological role in maize endosperm.

Histochemical and immunological assays revealed that LKR/SDH is highly expressed in the subaleurone cell layers (Figure 7). This region comprises cells that are actively expressing zein genes at a high rate (Dolfini et al., 1992). These cells probably have a low lysine requirement because zein proteins, which represent >70% of total endosperm protein, are devoid of lysine residues. Thus, we suggest that LKR/SDH activity is important to regulate lysine levels in this narrow area of the endosperm.

In addition, it could be possible that some product arising from lysine degradation could regulate cellular and developmental processes operating in subaleurone cells. Interestingly, it has been suggested that lysine degradation may have an influence on the growth of the mammalian brain, because LKR is highly active during embryonic rat brain development (Rao et al., 1992).

Effects of the *o2* Mutation on *ZLKRSDH* mRNA Amounts

Altered lysine catabolism (Sodek and Wilson, 1970; Arruda and da Silva, 1979) may be one of the mechanisms by which the *o2* mutation creates a high-lysine maize phenotype. Most of the lysine in the endosperm seems to originate from lysine-rich proteins, which are more abundant in *o2* than in normal endosperm (Habben et al., 1993). Synthesis of lysine-rich proteins presumes the existence of increased availability of free lysine to be incorporated. We previously observed increased contents of lysine-rich proteins in the double homozygous mutant *o2 Ask1* (Azevedo et al., 1990). This was attributed to increased lysine availability due to the feedback-insensitive AK encoded by *Ask1* and to reduced lysine degradation determined by *o2*.

The amount of LKR activity in the *o2* endosperm is reduced in comparison to the wild type (Brochetto-Braga et al., 1992). In this work, we calculated LKR and SDH activities

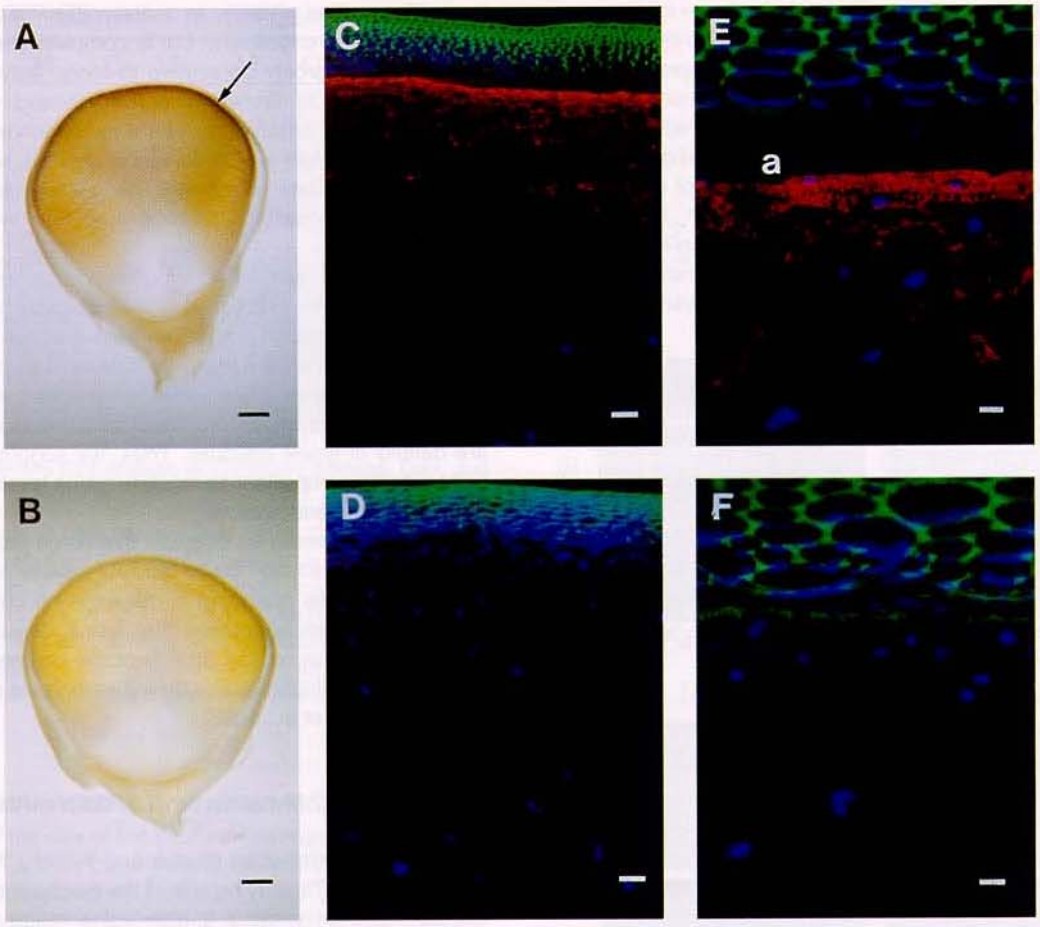

Figure 7. Localization of LKR/SDH in Normal Endosperm through SDH in Situ Activity and Immunocytochemistry.

(A) Seeds 17 DAP were longitudinally sectioned and incubated in an SDH reaction mixture, as described in Methods. An arrow indicates the positive colorimetric reaction for SDH activity.

(B) Endosperm of a tissue section incubated in the absence of saccharopine, the substrate for the SDH reaction.

(C) Immunolocalization of LKR/SDH from endosperm sections visualized by using a confocal laser scanning microscope. A positive reaction is shown in red. The images include nuclei stained blue by 4′,6-diamdino-2-phenylindole. The autofluorescence of the cell walls appears in green and in blue. Immunolabeling was most intense in the subaleurone cell layers. The immunocytochemical reaction for LKR/SDH was barely detected in cells farther away from the first several subaleurone cell layers.

(D) and **(F)** Tissue sections incubated with nonimmune serum followed by treatment with the secondary antibody.

(E) Higher magnification of **(C)**. LKR/SDH labeling is found around the starch granules and tended to be at the cell periphery, close to the cell wall in cells far away from the subaleurone. a, aleurone layer.

Bars in **(A)** and **(B)** = 1 mm; bars in **(C)** and **(D)** = 50 μm; bars in **(E)** and **(F)** = 12.8 μm.

per amount of endosperm and found 36-fold and fourfold reductions in LKR and SDH activities, respectively, in the mutant endosperm (Figure 6A). Between 15 and 20 DAP, this decrease correlates well with reduced amounts of both the LKR/SDH polypeptide (Figure 6B) and the *ZLKRSDH* mRNA (Figure 6C).

Interestingly, in 25-DAP *o2* endosperm, LKR/SDH protein accumulated to levels comparable to those in the normal endosperm, but LKR and SDH activities did not reach normal levels. In addition, the high accumulation of the LKR/SDH polypeptide in the 25-DAP *o2* endosperm was not accompained by a proportional accumulation of *ZLKRSDH* mRNA, suggesting post-transcriptional regulation possibly due to stabilization of *ZLKRSDH* mRNA by translation (Gallie et al., 1991; Sullivan and Green, 1993; Jacobson and Peltz, 1996; Petracek et al., 1998). LKR activity seems to be differentially modulated in *o2* when compared with the normal endosperm. The enzyme is hypophosphorylated in *o2*, probably due to the absence of a casein kinase II in the mutant endosperm. Curiously, casein kinase II was able to restore LKR

activity in a lysine-dependent manner, after in vitro dephosphorylation by alkaline phosphatase (E.L. Kemper, F. Papes, A.C. da Silva, A. Leite, and P. Arruda, submitted manuscript).

At 20 DAP, the *o2* mutation causes a 90% reduction in *ZLKRSDH* steady state mRNA levels. This effect was observed in two near-isogenic mutant lines derived from alleles *Oh43o2* (Figure 6) and *W64Ao2* (data not shown). The severe reduction caused by the *o2* mutation on *ZLKRSDH* mRNA accumulation is comparable to the reduction observed for α-zein in the same genetic background (Cord Neto et al., 1995). The O2 protein is a transcriptional activator of the basic domain/leucine-zipper family that is specifically expressed in the nonaleuronic endosperm and recognizes different binding sites in the promoters of several endosperm-specific genes (Lohmer et al., 1991; Varagona et al., 1991; Schmidt et al., 1992; Cord Neto et al., 1995; Gallusci et al., 1996). Recent evidence suggests that O2 is involved in the coordinated regulation of storage protein synthesis and nitrogen and sugar metabolism during seed development (Giroux et al., 1994; Gallusci et al., 1996). Therefore, the involvement of O2 in the transcriptional regulation of lysine catabolism is in keeping with its role of controlling nitrogen metabolism in the seed.

O2 regulates cyPPDK, in an expression pattern similar to that observed for LKR/SDH. cyPPDK is localized in the subaleurone cell layers, with a weak signal spreading toward the central endosperm cells (Gallusci et al., 1996). The endosperm region, where LKR/SDH and cyPPDK are localized, is also the region actively expressing zein genes and the O2 protein (Varagona et al., 1991; Dolfini et al., 1992). This observation correlates with the hypothesis of transcriptional control of LKR/SDH by O2 protein and supports the idea of a general control of amino acid metabolism and protein synthesis by O2.

In yeast, the GCN4 factor is one of the major determinants of the regulatory system termed general control of nitrogen (GCN). In this organism, the synthesis of lysine and many other amino acids is coordinately regulated by nutritional conditions (Hinnebusch, 1988). In addition, it has been demonstrated that the GCN4-like box may be a key element in regulating the response of a barley storage protein to nitrogen (Müller and Knudsen, 1993). Also, the prolamin composition of maize seeds seems to be dictated by nitrogen status (Singletary et al., 1990). The effects of the *o2* mutation on enzymes participating in amino acid metabolism and carbon partitioning, coupled with structural (Lohmer et al., 1993) and functional (Mauri et al., 1993) similarities between O2 and the yeast GCN4 factor, suggest that the O2 protein may be involved in similar general amino acid control in the maize endosperm.

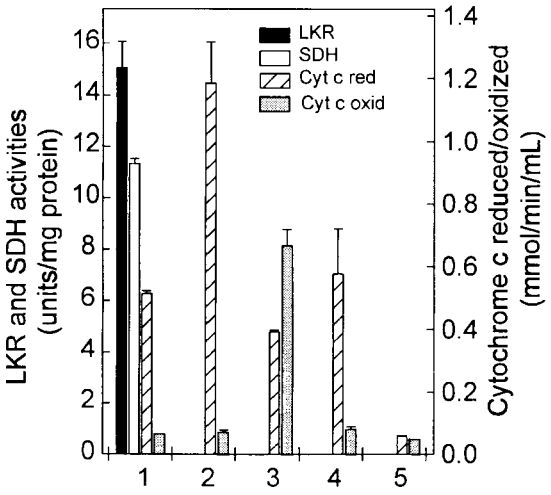

Figure 8. Subcellular Localization of LKR/SDH.

A homogenate from 20-DAP endosperm was separated by sucrose gradient centrifugation, and the fractions corresponding to the cytoplasm (1), endoplasmic reticulum (2), mitochondria (3), peroxisomes (4), and protein bodies (5) were assayed for LKR and SDH activities. Marker enzymes cytochrome *c* oxidase (Cyt c oxid) and cytochrome *c* reductase (Cyt c red) were assayed for resolution of organellar components.

METHODS

Plant Material

The commercial maize (*Zea mays* L.) hybrid F-352 from Agroceres SA (São Paulo, Brazil) was used. To obtain near-isogenic lines exhibiting normal phenotypes and the phenotype of the *opaque2* (*o2*) mutant, we crossed either *Oh43o2* or *W64Ao2* mutant lines to the F-352 hybrid. The resulting F_1 progeny was self-pollinated, and normal seeds from ears segregating for normal and *o2*-conferred phenotypes were planted. Plants were self-pollinated, and again normal seeds from ears segregating for normal and *o2*-conferred phenotypes were planted. Six successive rounds of self-pollination were performed. After the sixth generation, seeds from homozygous normal and *o2* ears were planted, and the resulting ears were collected for the experiments. Ears were harvested either at 17 days after pollination (DAP) or at distinct developmental stages and stored frozen at −70°C.

Roots, leaves, and coleoptiles from 4-day-old seedlings, grown in vermiculite under controlled conditions, were used for the isolation of total RNA.

Enzyme Purification

Lysine–ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) was purified from 17-DAP F-352 hybrid endosperms, as described by Gonçalves-Butruille et al. (1996), with minor modifications. All steps were performed at 4°C. Tissues were homogenized in buffer A (25 mM sodium phosphate, pH 7.4, 1 mM DTT, 1 mM EDTA, and 5 mM benzamidine) and centrifuged at 20,000*g* for 10 min, and the supernatant was brought to pH 5.5 by adding solid NaH_2PO_4.

Polyethylene glycol 8000 at 50% (w/v) was added to the homogenate to obtain a final concentration of 7.5%. The mixture was centrifuged at 20,000*g* for 10 min, and the supernatant was brought to a 15% (w/v) polyethylene glycol concentration and centrifuged again at 20,000*g* for 10 min. The pellet was resuspended in buffer B (50 mM Tris-HCl, pH 8.5, 1 mM DTT, and 1 mM EDTA) and dialyzed overnight against the same buffer. The dialyzed sample was applied to a DEAE-Sepharose column (2.5 × 40 cm) previously equilibrated with buffer B. The enzyme was eluted from the column with a linear gradient from 0 to 0.5 M NaCl in buffer B. Fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20,000*g* for 10 min. The pellet was resuspended in buffer B, dialyzed against the same buffer, and applied to a Protein-Pak Q 8HR (Waters, Milford, MA) column. The enzyme was eluted from the column with a linear gradient from 0 to 0.5 M NaCl in buffer B. Fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20,000*g* for 10 min. The pellet was resuspended in buffer B and applied to a Superdex 200 HR (Pharmacia) column previously equilibrated with buffer C (buffer B containing 0.3 M NaCl). The enzyme was eluted from the Superdex column with buffer C and stored at −70°C.

N-Terminal Protein Sequencing

The purified LKR/SDH was separated in by SDS-PAGE in a 7% gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R 250 and sent to the protein sequencing facility of the Weizmann Institute of Science (Rehovot, Israel). The protein band was eluted from the gel and digested with trypsin, and the major peaks were sequenced. Four internal peptide sequences were obtained: (1) GLIDFLHGL, (2) RYEGFSEIMVTLS, (3) RLTPLYEYI, and (4) RELPAFALEHLPNR.

cDNA Cloning

Cloning of a full-length maize LKR/SDH–encoding cDNA was completed by using a combination of three procedures: (1) reverse transcription–polymerase chain reaction (RT-PCR), (2) cDNA library screening, and (3) 5′ rapid amplification of cDNA ends (5′ RACE).

After protein sequencing, a set of degenerate oligonucleotides was synthesized based on the tryptic peptide sequences and used in RT-PCR experiments. One microgram of total RNA extracted from 17-DAP endosperm was used in an RT reaction by using an RT-PCR kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. A 1.2-kb cDNA fragment was amplified by subsequent PCR reactions (2 μL of template cDNA, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate mix, 100 pmol of each primer, and 2.5 units of *Taq* DNA polymerase in a final volume of 50 μL) by using primers derived from the above-described peptide sequences 2 (5′-ISWIARIGTIACCATDATTTC-3′) and 3 (5′-CTIACICCICTITAYGARTATAT-3′). S (G or C), W (A or T), R (A or G), D (A, G, or T), and Y (C or T) designate IUB codes for variable nucleotide sites, and I denotes inosine.

Amplification was performed on a thermal cycler (model 480; Perkin-Elmer) as follows: 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 42°C, 2 min at 72°C; and 5 min at 72°C. After sequencing, the 1.2-kb amplified cDNA fragment was confirmed to be part of the maize LKR/SDH–encoding gene by sequence comparison to the yeast *Lys1* and *Lys9* genes. This fragment then was used as a probe to screen a maize endosperm cDNA library constructed from RNA extracted from 25-DAP seeds of the R-802 inbreed line (Aukerman et al., 1991). Twenty clones were isolated and sequenced, but all of them were incomplete. The 5′ cDNA end was cloned by using the RACE system (Gibco BRL), according to the manufacturer's instructions, with the primers designed on the basis of the cDNA sequences (5′-TATCAAATAGGTGCCCAC-3′ and 5′-GTGTGGGAAAAGAAGGCGTA-3′).

The full-length maize *ZLKRSDH* cDNA clone was finally isolated from F-352 total RNA extracted from 17-DAP endosperms. Primers annealing to the 5′ untranslated region (5′-TTCAACTCTCCACTTTCTCAACCA-3′) and 3′ untranslated region (5′-CTCGTCCGTCTCCGTTTCCGTC-3′) of the cDNA were used to obtain a 3.5-kb complete cDNA in RT-PCR reactions catalyzed by Pfu polymerase (Stratagene) according to the manufacturer's instructions. This fragment subsequently was cloned into pBluescript KS+ and sequenced in an automatic ABI377 DNA sequencer (Perkin-Elmer).

DNA Gel Blot Analysis

Genomic DNA was extracted from maize, Coix, and sorghum seedlings as described by Rivin et al. (1982). Ten micrograms of genomic DNA was digested to completion with BamHI or EcoRV. The digests were ethanol precipitated and loaded onto a 0.7% agarose gel, blotted onto a nylon membrane (Hybond-N; Amersham), and hybridized with the ^{32}P-labeled (Megaprime DNA labeling system; Amersham) 1.2-kb RT-PCR fragment. Hybridization was conducted at 65°C in SSPE buffer (5 × SSPE [1 × SSPE is 150 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4], 5 × Denhardt's solution [1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA], and 0.5% SDS), and washes were performed twice at room temperature in 2 × SSPE 0.1% SDS solution for 20 min and repeated twice at 65°C in 0.1 × SSPE 0.1% SDS. Autoradiography was performed at −70°C for 72 hr by using intensifying screens.

Total RNA Isolation and RNA Gel Blot Analysis

Total RNA used in both RT reactions or RNA gel blot analysis was extracted from developing seeds and seedling tissues, according to the procedures described by Prescott and Martin (1987). For RNA gel blot experiments, 20 μg of total RNA was electrophoresed in 1.0 or 1.5% agarose–formaldehyde gels (0.66 M formaldehyde, 50 mM Mops). After electrophoresis, gels were stained with ethidium bromide as a loading control. The RNA then was transferred from the gel onto a nylon membrane (Hybond-N^+; Amersham). The probes used were either the 1.2-kb RT-PCR fragment or specific LKR or SDH domain probes. The LKR probe consisted of a 0.55-kb cDNA fragment corresponding to the region spanning residues 96 through 284, and the SDH probe (0.49 kb) encompassed residues 652 to 814 (Figure 1). DNA labeling, hybridization, and washing were as described above for the DNA gel blots. For quantitative experiments, preflashed x-ray films were exposed to the radioactive membrane, according to the manufacturer's instructions (Amersham). Relative amounts of hybridized mRNA were analyzed through scanning with a laser densitometer (LKB UltroScan XL, Bromma, Sweden).

Immunoblotting

Immunoblotting was performed as described by Kemper et al. (1998), with minor modifications. After electrophoresis, the gel was

soaked in 25 mM Tris-base, 190 mM glycine, and 20% methanol for 10 min. Proteins were transferred to nylon membranes (Hybond-N; Amersham) in a semidry electroblotting apparatus (Pharmacia). Membranes were blocked overnight at 4°C in 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20, and 5% (w/v) nonfat dry milk and then incubated with anti-LKR polyclonal antibodies. After incubation with anti–rabbit IgG alkaline phosphatase conjugate, the membranes were incubated for 20 min in the dark in a developing mixture containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and a 1:1000 solution of CSPD (Tropix, Bedford, MA). Bands were detected by exposure on a preflashed chemiluminescence-sensitive film (Amersham) and quantified by using laser densitometry.

Histochemical Staining

Histochemical staining of SDH activity on maize kernel sections was based on the gel staining reaction described by Gonçalves-Butruille et al. (1996). Seventeen-DAP maize kernels were longitudinally hand sectioned every 2 mm with a razor blade. Sections were fixed in 4% formalin, pH 7.0, for 30 min, rinsed 10 times in water over 18 hr at 4°C to remove endogenous substrates, and then incubated for 10 min at room temperature in a reaction mixture containing 2 mM saccharopine, 1 mM NAD, 0.5% nitro blue tetrazolium, 0.1 mM phenazine methasulfate, and 100 mM Tris-HCl, pH 8.5, with rocking. Control sections were incubated in the absence of saccharopine. The reaction was stopped by rinsing the sections in double distilled water.

Immunohistochemistry

Indirect immunofluorescence and confocal laser scanning microscopy were used to visualize LKR/SDH in frozen sections of maize endosperm. Seeds at 17 DAP were dissected from cobs, immersed in ice-cold PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, and 0.02% [w/v] of sodium azide) containing 4% (w/v) paraformaldeyde and 0.1 M sucrose, and cut into 2- to 3-mm-thick longitudinal slices. After overnight fixation at 4°C, the slices were rinsed three times in PBS containing 0.5 M sucrose for 20 min each and frozen in liquid nitrogen. The frozen slices were mounted in specimen supports with Cryo-embedding compound (Microm Laborgerate, Waldorf, Germany) and sectioned at 16 μm in a Micron cryostat (Microm Laborgerate). Sections were collected on albumin-coated slides, postfixed with acetone for 15 min, and air dried.

Sections were blocked with 2% (w/v) BSA and 0.3% (v/v) Tween 20 in PBS (PBS-T-B) for 30 min in a 200-mL coupling jar before incubation with the primary antibody. Sections then were incubated with a polyclonal antibody raised in rabbit against LKR/SDH (Kemper et al., 1998) diluted at 1:200 (v/v) in PBS-T-B overnight at room temperature in a 50-mL coupling jar. The control consisted of incubation with an unrelated rabbit serum at the same dilution. The slides were washed three times for 20 min in PBS-T in a 200-mL coupling jar. Sections were covered with a rhodamine-conjugated goat anti–rabbit immunoglobulin (Calbiochem-Novabiochem, La Jolla, CA) diluted 1:30 (v/v) in PBS-T-B and incubated for 1 hr at room temperature. After washing, the sections were counterstained with 4′,6-diamidino-2-phenylindole (0.8 μg/mL VectaShield; Vector Laboratories, Burlingame, CA). Sections were analyzed by using a confocal laser scanning microscope (model MRC 1024UV; Bio-Rad Life Sciences, Richmond, CA). Filter sets for rhodamine (immunolabeling), fluorescein (natural cell wall fluorescence), and 4′,6-diamidino-2-phenylindole (nuclear staining plus natural cell wall fluorescence) were used. Immunolabeled and control sections were analyzed using the same laser power, iris, and gain settings as their experimental counterparts. This treatment was performed twice with kernels from different field seasons. Photographs were taken using Focus Graphics (Focus Graphics Inc., Plymouth, MN) equipment with Kodak ISO 100 film and processed commercially.

Subcellular Localization of the LKR/SDH Enzyme

Subcellular fractionation was conducted according to Habben et al. (1993), with the following modifications. Buffer A contained 10 mM Hepes, pH 7.5, 1 mM EDTA, 10 mM KCl, 200 mM sucrose, 1 mM DTT, and 5 mM benzamidine. After centrifugation in a discontinuous sucrose gradient, the interface fractions were collected by lateral puncture through the centrifuge tube wall and assayed for LKR and SDH activities. The activities of the marker enzymes cytochrome *c* oxidase and cytochrome *c* reductase were assayed for resolution of organellar components, as described by Tolbert (1974) and Larkins and Hurkman (1978).

Separation of Maize LKR and SDH Domains by Limited Proteolysis

Aliquots of LKR/SDH partially purified by chromatography on DEAE-Sepharose were incubated with elastase (Sigma). Enzyme/protease proportions and proteolysis conditions are indicated in the legend to Figure 3. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM. The digest was applied to a Protein-Pak Q 8HR column (Waters) previously equilibrated with buffer B. The column was washed with buffer B and then eluted with a linear gradient from 0 to 0.5 M NaCl in buffer B. The fractions containing separated LKR and SDH domains were used for enzyme assays and immunobloting analysis.

Enzyme Assays

LKR and SDH activities were measured spectrophotometrically by following the oxidation of NADPH and reduction of NAD^+, respectively, at 30°C. LKR assays contained 20 mM L-lysine, 10 mM α-ketoglutaric acid (neutralized to pH 7.0 with potassium hydroxide), 0.1 mM NADPH, 150 mM Tris-HCl buffer, pH 7.4, and 0.04 to 0.1 mg of protein in a final volume of 0.3 mL. SDH assays contained 2 mM L-saccharopine, 2 mM NAD^+, and 100 mM Tris-HCl buffer, pH 8.5, in a final volume of 0.3 mL. Oxidation of NADPH and reduction of NAD^+ were monitored at 340 nm in a spectrophotometer (model DU-65; Beckman). One unit of enzyme activity is defined as 1 nmol of NADPH oxidized or NAD^+ reduced per min at 30°C. The protein concentration in the enzyme extracts was determined by the method of Bradford (1976), using the Bio-Rad protein assay dye reagent.

Computer Analysis

Amino acid sequence alignments and similarity analysis were performed using the MACAW (Schuler et al., 1991) and BOXSHADE (FTP downloaded from Vax0.biomed.uni-koeln.de) programs.

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Lysine-oxoglutarate reductase activity in maize endosperm is modulated by a Ca^{2+}-mediated dimerization and lysine-dependent phosphorylation*

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Running title: Regulation of lysine degradation in maize

SUMMARY

Lysine-oxoglutarate reductase and saccharopine dehydrogenase are enzymatic activities that catalyze the first two steps of lysine degradation in higher eukaryotes. In maize, these enzymatic activities reside on a single bifunctional polypeptide, the native form of which is a dimeric protein. Despite their spatial proximity, the modulation of these activities is independent, since factors such as Ca^{2+}, ionic strength and limited proteolysis modulate the activity of the reductase but not of the dehydrogenase. Moreover, the maize mutant *o2*[1], known to have a lysine-rich endosperm, shows a severe impairment in reductase activity (~18 fold lower compared to the WT levels) which is not paralleled by a similar effect on dehydrogenase activity (~2 fold reduction). We show here that these differences in the behavior of these two activities are probably the result of distinct phosphorylation and dimerization patterns exhibited by the native enzyme. The dephosphorylated enzyme, which lacks reductase activity, recovered its activity upon treatment with casein kinases I and II, but only in the presence of lysine. Ca^{2+} plays an important role in the dimerization of this bifunctional enzyme. The reductase activity is present only in dimers, whereas the dehydrogenase activity is present in dimers and monomers. A model of the dimeric lysine-oxoglutarate reductase/saccharopine dehydrogenase is described and the possible roles of a leucine-zipper and a low-affinity EF-hand in the reductase domain are discussed.

INTRODUCTION

Lysine is degraded in plants and mammals via the saccharopine pathway (1-13), the first two reactions of which are catalyzed by lysine-oxoglutarate reductase (LOR[1]; E.C. 1.5.1.8) and saccharopine dehydrogenase (SDH; E.C. 1.5.1.9). The reductase activity condenses lysine and α-oxoglutarate to form saccharopine (ε-*N*-(L-glutaryl-2)-L-lysine), which is then oxidized by the dehydrogenase activity to produce α-aminoadipic-δ-semialdehyde (AASA) and glutamate. In maize, these activities are observed only in the endosperm (1,14), and reside on a single, bifunctional polypeptide, the native form of which is a dimer composed of identical subunits of M_r = 117,000 (4). cDNA and genomic sequencing have confirmed the bifunctional nature of the LOR/SDH enzyme in maize[2,3] and *Arabidopsis* (15,16). A monofunctional SDH, probably expressed by the same gene that encodes the bifunctional enzyme, has also been isolated from *Arabidopsis* (16). In baboon and bovine liver, the LOR and SDH activities reside in a single polypeptide, the native form of which is a homotetramer composed of M_r = 115,000 subunits (6, 9-12, 17-20). In rat liver, however, non-linked LOR and SDH polypeptides have been isolated (21). In yeast, where the saccharopine pathway is used to synthesize lysine, LOR and SDH activities are located in separate polypeptides of M_r = 49,000 and 73,000, encoded by *Lys1* and *Lys9* genes, respectively (22-25).

Lysine degradation in plants and mammals is subject to complex regulation. In tobacco plants and mice, the exogenous administration of lysine induces LOR activity by a mechanism that operates, at least in mice, at the transcriptional level[4] (3). Feeding lysine to rats or injecting lysine into mice[4] or developing tobacco pods stimulates LOR activity (3, 26). Increased LOR activity is also observed in lysine-overproducing transgenic tobacco and canola plants expressing

genes coding enzymes for increased lysine production (3,27). Together, these results suggest that, *in vivo*, lysine regulates its own degradation.

Recently, it has been shown that the stimulation of LOR activity in tobacco seeds, is dependent on an intracellular cascade involving Ca^{2+} and protein phosphorylation (28). The LOR, but not SDH domain of the maize LOR/SDH enzyme was shown to be activated by Ca^{2+} and high salt concentrations (29). Separation of the LOR and SDH domains by limited proteolysis revealed that although proteolysis did not affect SDH activity, LOR activity showed a time- and protease concentration-dependent inactivation followed by reactivation (29). The SDH domain was shown to inhibit LOR activity. When separated from the SDH domain by limited proteolysis, the LOR domain retained its ability to be activated by Ca^{2+}, but was no longer activated by high salt concentrations. These results suggest that the LOR activity of the native enzyme is normally inhibited such that after modulation, the enzyme undergoes a conformational alteration to expose the catalytic domain for substrate binding (29).

In this study, we show that Ca^{2+} may play a role in enzyme dimerization and that this effect may account for the observed Ca^{2+} stimulation of LOR activity. We also show that LOR activity is selectively activated by phosphorylation in a lysine-dependent manner. Finally, we provide evidence that the phosphorylation status is altered in the LOR/SDH enzyme isolatedfrom *o2* endosperm, resulting in restricted impairment of LOR activity.

EXPERIMENTAL PROCEDURES

Chemicals and chromatographic materials – $\gamma[^{32}P]$-ATP was obtained from Amershan and CSPD from Tropix. Protein assay dye was purchased from Bio-Rad. The Superdex 200 HR HPLC column was purchased from Pharmacia and the Protein-Pak Q 8 HR column from Waters. All other chemicals were purchased from Sigma.

Enzymes – Rat recombinant casein kinase I (1000 U/µl), Human recombinant casein kinase II (200 µU/µl), the catalytic subunit of protein kinase C from rat brain (10 mU/µl) and rat brain Ca^{2+}/calmodulin kinase II (19 µU/µl) were purchased from Calbiochem. The catalytic subunit of bovine hearth protein kinase A (3 U/µl) was purchased from Sigma. Alkaline phosphatase (1 U/µl) was purchased from Boehringer.

Plant material - The commercial maize (*Zea mays* L) hybrid F-352 from Agroceres SA (Brazil) was crossed with the Oh43*o2* mutant line. The resulting F1 were self-pollinated and seeds from ears segregating WT and *o2* were planted. The plants were self-pollinated and again WT seeds from ears segregating WT and *o2* were planted. Six successive rounds of self-pollination were performed. After the 6^{th} generation, seeds from homozygous WT and *o2* ears were planted and the resulting ears were harvested at 17 days after pollination (DAP) or at distinct developmental stages and stored frozen at -70°C.

Enzyme purification - LOR/SDH was purified from 17 DAP WT endosperms as previously described (29). The following steps were performed at 4°C. Tissues were homogenized with buffer A (50 mM Tris-HCl pH 7.5, containing 1 mM DTT and 1 mM EDTA plus 5 mg leupeptin/l). The homogenate was centrifuged at 20,000g for 30 min and the supernatant was adjusted to 35% saturation ammonium sulfate. The solution was kept on ice for 30 min and the precipitate removed by centrifugation at 20,000g for 10 min. The supernatant was brought to

70% saturation with ammonium sulfate, and after centrifugation at 20,000g for 10 min, the pellet was resuspended in a minimal volume of buffer B (50 mM Tris-HCl, pH 8.5, 1 mM DTT, 1 mM EDTA). The extract was dialyzed overnight against 2 L of buffer B and applied to a DEAE-Sepharose column (2.5 cm x 40 cm) previously equilibrated with buffer B. The enzyme was eluted from the column with a linear gradient of 0 - 0.5 M NaCl in buffer B. The fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20,000g for 10 min. The pellet was resupended in buffer B, dialyzed against the same buffer, and applied to a Protein-Pak Q 8HR column. The enzyme was eluted from the column with a linear gradient of 0 - 0.5 M NaCl in buffer B. The fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20,000g for 10 min. The pellet was resuspended in buffer B and applied to a Superdex 200 HR column previously equilibrated with buffer C (buffer B containing 0.3 M NaCl). The purified enzyme was eluted from the Superdex column with buffer C and stored at -70°C.

Polyacrylamide gel electrophoresis and immunoblotting - Discontinuous PAGE was performed at pH 8.5 in 7% slab gels. After electrophoresis, the gels were developed for SDH activity as described previously (4) or stained with Coomassie blue. SDS-PAGE was performed in 7% slab gels (30) ant the gels stained with Coomasie blue. For immunoblotting, the gel was soaked in 25 mM Tris-base, 190 mM glycine and 20% methanol for 10 min after electrophoresis. Proteins were electrotransferred to nylon membranes (Hybond N, Amersham) in a semidry blotting apparatus (Pharmacia). Membranes were blocked overnight at 4°C in 20 mM Tris, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20, and 5% (w/v) non-fat dry milk and then incubated with anti-LOR/SDH polyclonal antibodies (29). After incubation with anti-rabbit IgG alkaline phosphatase conjugate, the membranes were incubated for 20 min in the dark in a developing mixture

containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and a 1:1000 solution of CSPD (Tropix). Bands were detected by autoradiography.

Enzyme assays - LOR activity was measured spectrophotometrically in the direction of NADPH to $NADP^+$ at 30°C. The reaction mixture had a final volume of 0.5 ml and contained 20 mM L-lysine, 10 mM 2-oxoglutaric acid (neutralized to pH 7.0 with potassium hydroxide), 0.1 mM NADPH, 150 mM Tris-HCl buffer, pH 7.4, and 0.04 - 0.1 mg protein. SDH activity was also measured spectrophotometrically by following the reduction of NAD^+ to NADH at 30°C in a 0.5 ml reaction mixture containing 2 mM L-saccharopine, 2 mM NAD^+ and 100 mM of Tris-HCl buffer, pH 8.5. Activators and/or inhibitors were added to the assay mixtures as indicated in the figure and table legends. Oxidation of NADPH and reduction of NAD^+ were monitored at 340 nm in a DU-65 Beckman spectrophotometer. One unit of enzyme activity was defined as 1 nmol of NADPH oxidized or NAD^+ reduced per min at 30°C. A control reaction without lysine was performed for each assay measure. The protein concentration in the enzyme extracts was determined using the Bio-Rad protein assay dye reagent (31).

RESULTS

Impairment in LOR activity in o2 endosperm – Assays of LOR/SDH in developing 20 DAP WT and 25 DAP *o2* maize endosperms revealed that the LOR and SDH activities were reduced to ca. 9% and 50%, respectively, in the mutant compared to the WT (Fig. 1*A*). We used 20 DAP WT and 25 DAP *o2* endosperm because LKR and SDH activities reach its maximum at these developmental stages in each endosperm[2]. Since LOR and SDH reside in a bifunctional polypeptide, one would expect equimolar amounts of both activities, such that a reduction in one activity in the mutant endosperm should be accompanained by a similar reduction in the other activity. To test whether this impairment could reflect a differential modulation of LOR activity between WT and mutant endosperms, equivalent amounts of 20 DAP WT and 25 DAP *o2* endosperms were partially purified by 35-60% ammonium sulfate fractionation. The protein extracts were separated by SDS-PAGE in 7% slab gels and the LOR/SDH polypeptide was detected by immunoblotting using anti-LOR/SDH polyclonal antibodies (29). Both the WT and *o2* extracts showed a single band of Mr = 125,000 (Fig. 1*B*). When the samples were separated in 7% non-denaturing polyacrylamide gels and developed for SDH activity, the WT enzyme migrated faster than the mutant enzyme (Fig. 1*B*). We designated these enzymatic forms as fast (F) and slow (S) migrating bands. The existence of two native forms of the LOR/SDH enzyme, in contrast to the single band observed in the denaturing gel, suggested that the maize LOR/SDH may be post-translationally modified. Moreover, the difference in band migration between the two genotypes suggested that post-translational modification could, at least in part, account for both the decrease in LOR and SDH activities and the specific impairment of LOR activity in the *o2* endosperm.

Since LOR activity from soybean is modulated by phosphorylation (32), an experiment was done to investigate whether there were differences in the kinase activities of WT and *o2* endosperms, which could explain a possible divergent level of phosphorylation between the two genotypes. Crude extracts from equivalent amounts of WT and *o2* were assayed for 15 different kinases using a membrane containing their specific substrates (Phosphospots^{TM}, Jerini, Berlim, Germany). This assay revealed a decreased activity of casein kinase II in *o2* compared to WT endosperm (data not shown). To investigate the pattern of CK, the crude extracts were separated in 7% SDS-PAGE gels containing casein and the gels then developed for casein kinase II (CKII) activity. Four CK activity bands were detected in the WT endosperm. Surprisingly, one of the four bands was completely absent in the *o2* endosperm. In a control gel without casein, only one band (corresponding to CK autophosphorylation) was detected in both endosperms (Fig. 1C). These results suggest that the missing CK activity in the *o2* endosperm may account, directly or indirectly, for the decrease in LOR/SDH enzyme and the specific impairment of LOR activity in the mutant endosperm.

The bifunctional LOR/SDH enzyme can be phosphorylated in vitro – To test whether phosphorylation is involved in the post-translational modification of maize LOR/SDH, the enzyme was purified to homogeneity from 17 DAP WT endosperm, and phosphorylated *in vitro* using five protein kinases. Incubation with casein kinase I resulted in strong phosphorylation of the LOR/SDH polypeptide (Fig. 2). Prolonged exposures revealed that casein kinase II and protein kinase A also phosphorylated the enzyme, but to a much lesser extent, whereas Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C did not phosphorylate LOR/SDH (Fig. 2).

LOR activity is modulated by phosphorylation – To test whether the phosphorylation status changes LOR and/or SDH activities, a partially purified extract obtained from 17 DAP WT

endosperm by 35-60% ammonium sulfate fractionation was dephosphorylated with AP. The LOR activity decreased to 50% of the control treatment with the addition of 1 unit of AP to the assay mixture and to 2% when the amount of AP was raised from 2.5 to 5 units (Fig. 3*A*). There was no effect on SDH activity. To confirm that the decrease in LOR activity was caused by protein dephosphorylation, enzyme assays were conducted in the presence of 5 units of AP plus increasing amounts of the competitive inhibitor of AP, β-glycerol phosphate. The inhibitory effect of AP decreased with increasing concentrations of β-glycerol phosphate, whereas β-glycerol phosphate alone did not affect LOR activity (Fig. 3*B*).

LOR activity can be recovered after dephosphorylation by treatment with casein kinase in the presence of lysine – The LOR activity of soybean is inhibited by dephosphorylation in a lysine-dependent manner (32). To test whether maize LOR behaves like soybean LOR, the maize enzyme was treated with 1 unit of AP in the presence of increasing concentrations of lysine. Figure 4*A* shows that lysine did not increase the inhibitory effect of dephosphorylation on LOR activity. We also investigated whether LOR activity of the AP-treated enzyme could be recovered by treatment with protein kinase. The enzyme was treated with AP and then chromatographed on a Superdex 200 gel filtration column, to dissociate the LOR/SDH polypeptide from AP. Since LOR and SDH reside in a bifunctional polypeptide and the LOR activity was inactivated by dephosphorylation, the fractions from the gel filtration column were monitored for SDH activity. Fractions containing SDH activity were pooled and treated with a 1:1 mixture of CKI and CKII in the presence and absence of 11 mM lysine. Phosphorylation with CK restored LOR activity, but only in the presence of lysine (Fig. 4*B*). These results indicate that LOR/SDH is a phosphoprotein and that protein phosphorylation/dephosphorylation may be an important mechanism of post-translational regulation of the enzyme's activity.

Dephosphorylation does not affect the LOR/SDH dimerization pattern – To investigate whether dephosphorylation affects LOR/SDH dimerization, the enzyme was treated with AP and chromatographed on a Superdex 200 gel filtration column. If dephosphorylation disrupted the dimer, the enzyme should show a different elution profile from the non-dephosphorylated enzyme. Again, SDH activity was used to monitor the elution profile of the dephosphorylated enzyme. The dephosphorylated and non-dephosphorylated enzymes had identical elution profiles, with a peak corresponding to a *Mr* = 260,000 (Fig. 5). The most active fractions of each enzyme peak were pooled, concentrated and then separated by SDS-PAGE in 7% slab gel. After electrophoresis, the LOR/SDH polypeptide was detected by immunoblotting with anti-LOR/SDH polyclonal antibodies (29). The dephosphorylated and non-dephosphorylated enzymes showed the same migration rate in SDS-PAGE (data not shown). This result indicates that phosphorylation is not involved in dimerization, and that treatment with AP probably removes phosphate groups from sites that are important for LOR activity in the LOR/SDH enzyme.

Ca^{2+} is involved in enzyme dimerization – Recently, we demonstrated that LOR activity is modulated by Ca^{2+} and high ionic strength (29). To investigate the effect of Ca^{2+} on enzyme dimerization, the kinetics of LOR were determined in the presence of different concentrations of lysine, α-oxoglutarate and Ca^{2+}. The kinetic parameters for lysine and α-oxoglutarate in the presence of varying concentrations of Ca^{2+} indicated that LOR behaved as an allosteric enzyme (Fig. 6*A* and *B*). The $K_{0.5}$ and Hill coefficient (η_H) for each curve calculated with a fixed V_{max} for all assays at different concentration of Ca^{2+}, showed that η_H increased with increasing Ca^{2+} concentration: The η_H value of ~2 at maximum Ca^{2+} concentration indicated that the enzyme could dimerize in the presence of Ca^{2+} (Table I). To confirm the involvement of Ca^{2+} in LOR/SDH dimerization, partially purified enzyme preparations, obtained by 35 - 60%

ammonium sulfate fractionation were dialyzed and then chromatographed on a Superdex 200 gel filtration column equilibrated with buffer B containing different concentrations of free Ca^{2+}. The fractions were monitored for SDH and LOR activities. In the absence of Ca^{2+}, only SDH activity was recovered, and the enzyme behaved as a monomer with a *M*r = 115,300 (Table II). In the presence of 99 μM of free Ca^{2+}, two peaks were observed – one with a *M*r = 274,300, corresponding to dimer with both LOR and SDH activities, and another with a *M*r = 115,300, corresponding to the monomer with only SDH activity (Table II). In the presence of 1.3 mM of free Ca^{2+} only the dimer peak with both LOR and SDH activities was observed (Table II). These results indicated that Ca^{2+} has a major role in enzyme dimerization.

The o2 endosperm contains a dimer of LOR/SDH enzyme – The pattern of LOR/SDH oligomerization in WT and *o2* was investigated by fractionating partially purified extracts from 20 DAP WT and 25 DAP *o2* endosperms on a Superdex 200 gel filtration column. Elution of the enzyme was followed by monitoring SDH activity. The WT and *o2* endosperm extracts showed only the peak corresponding to the dimeric enzyme (Fig. 7).

DISCUSSION

We have shown previously that the LOR activity of the LOR/SDH bifunctional maize enzyme is modulated by Ca^{2+}, high salt concentration, organic solvents and Mg^{2+} (29). Digestion with elastase separated the bifunctional 125-kDa polypeptide into two polypeptides of 65 kDa and 57 kDa, containing the functional domains of LOR and SDH, respectively. The LOR activity showed a time- and protease-concentration-dependent inactivation followed by reactivation, while SDH activity was unaffected by limited proteolysis. We further demonstrated that fractions containing the isolated SDH domain could inhibit LOR activity (29). In the present work, we have provided evidence that Ca^{2+} plays a major role in enzyme dimerization, while phosphorylation is involved in lysine-mediated enzyme activation. In addition, we have shown that the phosphorylation status may be altered in the *o2* maize endosperm mutant compared to WT.

Lysine catabolism by the saccharopine pathway is an important mechanism for the control of free lysine levels in maize endosperm cells (33,34). Analysis of the developmental pattern of LOR activity in WT and *o2* endosperms showed that enzyme activity is decreased in the mutant, suggesting that the reduced lysine degradation in *o2* endosperm may reflect the lower content of LOR enzyme (2). We recently cloned a cDNA encoding the maize bifunctional LOR/SDH enzyme[2]. This cytosolic enzyme is encoded by a single gene which is highly expressed in the subaleurone cell layers of the distal part of the developing endosperm. In the *o2* mutant, LOR/SDH mRNA and protein levels are severely reduced and the expression pattern during kernel development is markedly modified, suggesting that the gene encoding LOR/SDH may be transcriptionally regulated by the Opaque2 transcriptional factor[2]. In addition, the LOR activity of *o2* endosperm is reduced to a much greater extent relative to SDH activity[2]. Since both

activities reside in separate domains of the same bifunctional polypeptide, and should therefore be synthesized in equimolar amounts, the impairment of LOR activity may reflect differences in post-translational modification caused by the *o2* mutation.

As with soybean LOR/SDH (32), maize LOR/SDH can also be phosphorylated *in vitro* by casein kinases, and dephosphorylation of the native enzyme by AP drastically inhibits enzyme activity (Figs. 2 and 3). The reductase activity of soybean LOR/SDH is also downregulated by AP *in vitro* in a lysine-dependent manner (32). In contrast, the inhibition of the maize enzyme by dephosphorylation is not dependent on lysine (Fig 4*A*). Since LOR activity would prevent the accumulation of excess lysine in plant tissues (4,27,28), it is somewhat contraditory that the dephosphorylation of soybean LOR is enhanced by lysine (32) since this would prevent lysine-degradation *in vivo*. It would be more reasonable to expect that phosphorylation is lysine-dependent because the accumulation of lysine would activate the enzyme and, therefore, its own degradation. Our results indicate that this is the case for maize LOR/SDH. The dephosphorylated maize LOR recovered enzymatic activity when treated with casein kinases in the presence of lysine. This is in accordance with a physiological role for the enzyme and is in keeping with the stimulation of enzyme activity in transgenic plants overproducing lysine (27,28) or plant tissues treated with exogenous lysine (3,28).

The maize LOR/SDH from WT and *o2* yielded two different bands upon electrophoresis in non-denaturing polyacrylamide gels, and a single band following SDS-PAGE (Fig. 1*B*). The difference in band migration observed in non-denaturing gels could represent enzymes in different states of phosphorylation. The fast-migrating F band may represent a hyperphosphorylated, more acidic form of LKR/SDH found in WT endosperm, while the slow-migrating S band, may represent a hypophosphorylated, less acidic form of LKR/SDH in *o2* endosperm. These results suggest that in the WT, the proportion of phosphorylated LOR protein

is higher while in the mutant the proportion of non-phosphorylated LOR/SDH protein is higher. Since phosphorylation appears to be essential for high rates of LOR activity, this could explain the observation that LOR activity in the mutant endosperm was less than 10% of that observed in the WT (Fig. 1*A*).

We have shown previously that Ca^{2+} and high salt concentration modulate LOR activity but have no influence on SDH activity (29). As shown here, SDH is also not affected by phosphorylation since treatment with AP did not change SDH activity (Fig. 3*A*). These results suggest that the two domains of the bifunctional LOR/SDH undergo different post-translational modification and that modulation of one domain does not affect the enzymatic activity of the other domain.

Ca^{2+} affects the oligomerization status of maize LOR/SDH. The kinetics of lysine degradation in the presence of varying concentrations of Ca^{2+} showed a sigmoidal curve, indicating ultrasensitivity of LOR activity to lysine (35) (Fig. 6). This effect may reflect differences in enzyme folding induced by Ca^{2+} and/or the influence of cooperative lysine binding after dimerization promoted by Ca^{2+}. Both alternatives are plausible to occur. Although a Hill coefficient does not directly imply dimerization, the increase in the η_H value as free Ca^{2+} increased suggests a Ca^{2+}-dependent dimerization process (Table I). Interestingly, the kinetics of α-oxoglutarate consuption also showed a rise in the η_H value as free Ca^{2+} rise, but the increase was not as marked as for the lysine curves. Since lysine binds to the active site before α-oxoglutarate, and since the lysine concentration was close to saturation in the α-oxoglutarate kinetics, the rise in the η_H value in the α-oxoglutarate curves is likely to be the consequence of previous lysine cooperative binding. The η_H value clearly indicates that Ca^{2+} confers ultrasensitivity to LOR activity (Fig. 6 and Table I). Indded, this is a common mechanism by

which allosteric enzymes change their activities rapidly over a small range of substrate concentrations (35). Thus, in addition to activation by phosphorylation, LOR may also be activated by Ca^{2+} through dimerization. Ca^{2+} induced LOR/SDH dimerization was also demonstrated by running the enzyme in a gel filtration column equilibrated with buffer containing different free Ca^{2+} concentrations (Table II). Since dimerization did not affect SDH activity, it is likely that only the LOR domain is involved in dimerization.

The maize and *Arabidopsis* LOR/SDH protein sequences show a number of features that strengthen the results of the present work. A putative EF-hand-like motif was found in the carboxy end of the LOR domain (Fig. 8A). EF-hand motifs are Ca^{2+}-binding sites and, at least in one case, are also involved in protein self-assembly (36). Since Ca^{2+} promotes the dimerization of calmodulin (37), this ion may have a role in protein dimerization, like with LKR/SDH.

The LOR/SDH amino acid sequence also contains a putative leucine-zipper motif which is commonly involved in the dimerization of transcription factors and some kinases (38-40). This motif involves amino acids 158 to 179 of the deduced LOR/SDH amino acid sequence (Fig. 8*B*). However, a leucine-zipper would be functional only if the region possesses a defined amphipathic character and can form a coiled-coil structure (41). Careful analysis of the LOR/SDH leucine-zipper region using an MTIDK matrix (42) showed that it contains an amphipathic helices (Fig. 8*B*). Although the probability of this leucine-zipper forming a coiled-coil is low (0.14), the leucine-zipper of the osmosensor histidine kinase Env-Z was found to be functional *in vitro* and *in vivo*, despite its low probability (0.21) of forming a coiled-coil (41,42).

The findings presented here suggest the model illustrated in Fig. 9*A*. In this model, the LOR domain is separated from the SDH domain by an interdomain region. In the active state, two LOR domains interact to form a dimer. Since SDH activity does not change with the dimerization status of the LOR/SDH polypeptide, we postulate that the SDH domains remain

separated from each other in the dimeric molecule (Fig. 9*A*). This structural organization probably allows the enzyme either to rapidly convert saccharopine, the product of the reductase reaction, into α-aminoadipic-δ-semyaldeyde by the SDH reaction or to provide a tight regulation of LOR activity. The latter alternative is more likely since the SDH domain inhibits LOR activity (Fig. 9*B*). On the other hand, phosphorylation may disrupt the inhibitory activity of the SDH domain on the LOR domain. The existence of an LOR inhibitory domain in the SDH polypeptide has been reported (29).

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FOOTNOTES

[1] Abbreviations used in this article: LOR, lysine-oxoglutarate reductase; SDH, saccharopine dehydrogenase; DTT, dithiothreitol; CSPD, disodium 3-{4-methoxyspiro[1,2-dioxethane-3,2'-(5'-chloro)tricyclo(3.3.1.1)decan]-4-yl}phenyl phosphate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; M_r, molecular weight; bZIP, basic domain leucine zipper; DAP, days after pollination; AP, alkaline phosphatase; WT, wild type; *o2*, *opaque2* mutant.

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FIGURE LEGENDS

FIG. 1. **LOR, SDH and CK activities in WT and *o2* endosperm.** A, Activities of LOR and SDH in 20 DAP WT and 25 DAP *o2* endosperms. Aliquots corresponding to 190 μg protein and 380 μg protein of enzymes partially purified by ammonium sulfate fractionation, were used to determine the LOR and SDH activities, respectively. The activities are expressed in terms of tissue weight. The columns represent the mean plus the standard deviation. B, Aliquots of crude extracts containing 150 μg protein of 20 DAP WT and 175 μg protein of 25 DAP *o2* endosperms were separated by SDS-PAGE (upper panel) and non-denaturing PAGE (lower panel) in 7% gels. The SDS-PAGE gels were blotted onto nylon membranes and probed with anti-LKR/SDH polyclonal antibodies. The PAGE gels were developed for SDH activity. S and F are to slow and rapid migrating bands, respectively. The results in panel B are for one gel out of three experiments which produced similar results. C, Casein kinase II activity of crude extracts of 20 DAP WT and 25 DAP *o2* endosperms. Samples from each genotype containing 160 μg protein were separated by SDS-PAGE in 10% gels with (CK) and without (control) casein. The gels were then developed for CKII activity as described by Carter (43).

FIG. 2. **Phosphorylation of LOR/SDH *in vitro*.** Aliquots (1,7 μg protein) of purified LOR/SDH were phosphorylated *in vitro* with CKI, CKII, PKA, CaCamK and PKC in reaction mixtures containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 μl of each kinase, 20 μM ATP and 10 μCi of [γ-^{32}P]ATP in a final volume of 20 μl. After a 30 min incubation at 30°C, the samples were separated by SDS-PAGE on 7% gels. The gels were stained with Coomasie brilliant blue (B) and autoradiographed (A).

FIG. 3. **Effect of dephosphorylation on LOR and SDH activities.** A, LOR and SDH activities were assayed in their reaction mixture, containing 0-5 units of AP and 1 mM $MgCl_2$. B, LOR

activity was assayed in complete reaction mixtures containing 5 units of AP, 1 mM $MgCl_2$ and 0-40 mM of β-glycerol phosphate. Control reactions without alkaline phosphatase (C), contained 40 mM of β-glycerol phosphate. Each point is the average of triplicate assays in panel A and duplicate assays in panel B. The bars indicate the standard deviation. A control reaction without lysine were performed for each assay. Each assay contained 250 μg of protein.

FIG. 4. **Recovery of LOR activity after dephosphorylation with AP and rephosphorylation with CK.** A, Aliquots containing 250 μg protein of WT endosperm crude extract were assayed for LOR activity in complete reaction mixtures containing varying concentrations of lysine and 1 mM $MgCl_2$ in the presence (●) and absence (○) of 1 unit of AP. This experiment was repeated twice with similar results. B, An aliquot (5 mg protein) of enzyme partially purified by 35 - 60% ammonium sulfate fractionation was dephosphorylated with 40 U of AP for 30 min at 30°C. The dephosphorylated enzyme was chromatographed on a Superdex 200 gel filtration column equilibrated with 50 mM Tris-HCl, pH 8.5, 1 mM DTT, 1 mM EDTA and 300 mM KCl. Three runs were done and the peaks containing SDH activity were pooled. Aliquots of the pooled fraction were phosphorylated with a 1:1 (v/v) mixture of CKI and CKII in the absence and presence of 11 mM lysine. After phosphorylation, LOR activity was assayed to test whether casein kinases could restore the LOR activity. The four treatments used were: (1) *CK+ Lys-* 160 μl enzyme, 55 mM Tris-HCl pH 7.3, 11 mM $MgCl_2$, 110 μM ATP, 1500 U casein kinase I and 400 μU casein kinase II. (2) *CK+ Lys+* As for *CK+ Lys-* with the addition of 11 mM lysine. (3) *CK- Lys-* and (4) *CK- Lys+* were controls for the first two reactions, but lacking casein kinases I and II. This experiment was repeated twice with similar results.

FIG. 5. **Effect of dephosphorylation on LOR/SDH dimerization.** An aliquot (1250 μg protein) of enzyme partially purified by 35 - 60% ammonium sulfate fractionation was dephosphorylated with 7.5 U of AP for 30 min at 30°C. The dephosphorylated enzyme was applied to a gel filtration column equilibrated with 50 mM Tris-HCl, pH 8.5, 1 mM DTT, 1 mM EDTA and 300 mM KCl. Fractions were assayed for SDH activity (●). The same profile was obtained with non-dephosphorylated enzyme (○). This experiment was repeated twice with similar results.

FIG. 6. **Effect of Ca^{2+} on LOR kinetics.** Aliquots (186 μg protein) of enzyme partially purified on DEAE-Sepharose were assayed for LOR activity in reaction mixtures containing varying concentrations of lysine (A) or α-oxoglutarate (B) and free Ca^{2+}. Free Ca^{2+} concentrations were 1.8 μM (□), 99 μM (▲), 359 μM (△), 742 μM (●) and 1.3 mM (○) as estimated from Ca^{2+}-EGTA buffers using Schtwarzenbach constants.

FIG. 7. **Elution profile of WT and *o2* LOR/SDH.** Aliquots corresponding to 1250 μg protein and 1900 μg protein of enzyme partially purified by 35 - 65% ammonium sulfate fractionation from 20 DAP WT (○) and 25 DAP *o2* (●) endosperms were chromatographed on a Superdex 200 gel filtration column equilibrated with 50 mM Tris-HCl pH 8.5, 1 mM DTT, 1 mM EDTA and 300 mM KCl and the eluted fractions assayed for SDH activity. This experiment was repeated three times with similar results.

FIG. 8. **LOR/SDH protein contains EF-hand-like and leucine-zipper domains.** A, Alignment of the amino acid sequences of the EF-hand like motifs present in the LOR domain of LOR/SDH from maize and *Arabidopsis*. Six Ca^{2+} binding residues denoted as x, y, z, -y, -x, -z are indicated. B, The amino acid sequence of the leucine zipper motif was plotted in a α-helical wheel to

emphasize its amphipathic nature. Hydrophobic residues are boxed and the four leucine residues are highlighted by black boxes. The EF-hand-like and leucine-zipper motifs are conserved in LOR/SDH from maize and *Arabidopsis* and were derived from Genbank accession numbers U95759 and AF 003551, respectively.

FIG. 9. **Model of the LOR/SDH structure and the role of lysine-dependent CK phosphorylation and Ca^{2+}-dependent dimerization in LOR activation.** A, The results obtained in this work indicate that only the LOR activity is dependent on dimerization. The line between the LOR and SDH domains represents the interdomain region. Since LOR activity is dependent on dimerization, full LOR activity is achieved when the domains exist as a dimer, while the two SDH domain remain as monomers. The leucine zipper motif and the low affinity EF-hand-like motif located in the LOR domain may be involved in dimerization. B, Steps I to IV and II to III represent the hypothetical mechanism involved in the dimerization and activation of the LOR domain. As the SDH domain was previously shown to inhibit LOR activity, we postulated that the phosphorylation by CK derepresses the LOR domain, which then regains full activity. This is represented by steps I to II and II to IV. Our results indicate that only the configuration IV has LOR activity, whereas all configurations exhibit SDH activity.

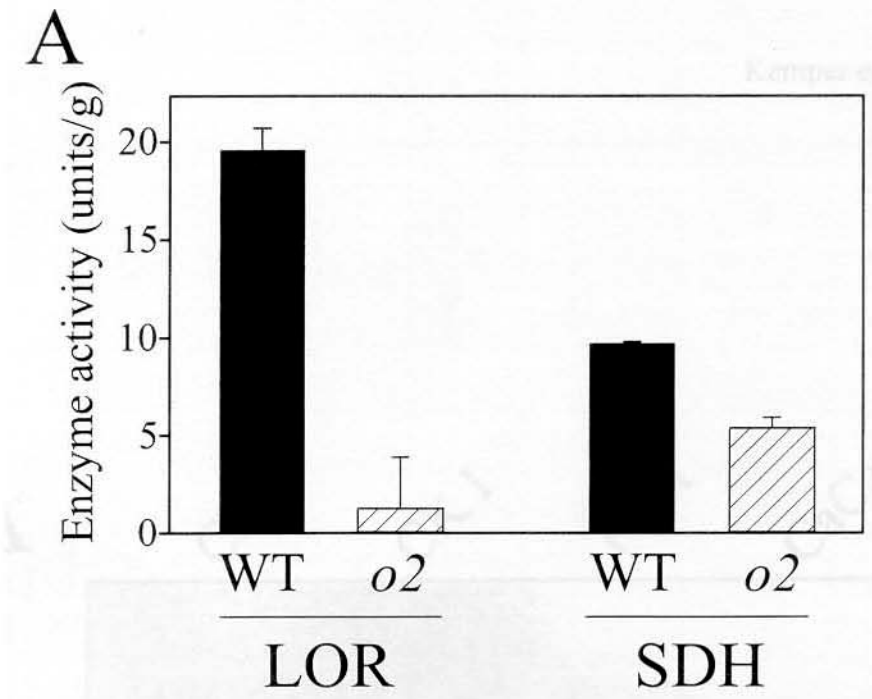
TABLE I *Kinetic constants obtained for lysine and α-oxoglutarate degradation as a function of free Ca^{2+}* concentration. The free Ca^{2+} concentrations were estimated from Ca^{2+}-EGTA buffers using Schtwarzenbach stability constants using the program Mcalc. See figure 6 for the experimental conditions.

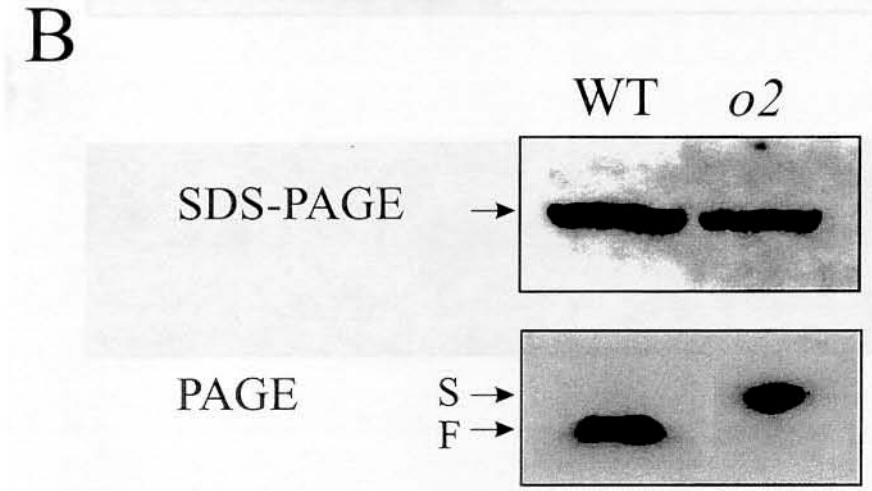
$[Ca^{2+}]$	Lysine			α-Oxoglutarate		
μM	V_{max}	$K_{0.5}$	*n*	V_{max}	$K_{0.5}$	*n*
1.8	67.4 ± 3.8	51.9 ± 13.9	0.95 ± 0.19	56.6 ± 3.1	26.5 ± 8.0	0.83 ± 0.17
99		27.0 ± 3.3	1.47 ± 0.23		12.1 ± 1.9	1.09 ± 0.16
349		16.0 ± 1.6	1.98 ± 0.28		5.8 ± 0.8	1.17 ± 0.14
742		11.8 ± 1.2	2.01 ± 0.29		3.0 ± 0.4	1.27 ± 0.17
1,300		9.0 ± 0.8	2.38 ± 0.39		2.0 ± 0.3	1.42 ± 0.22

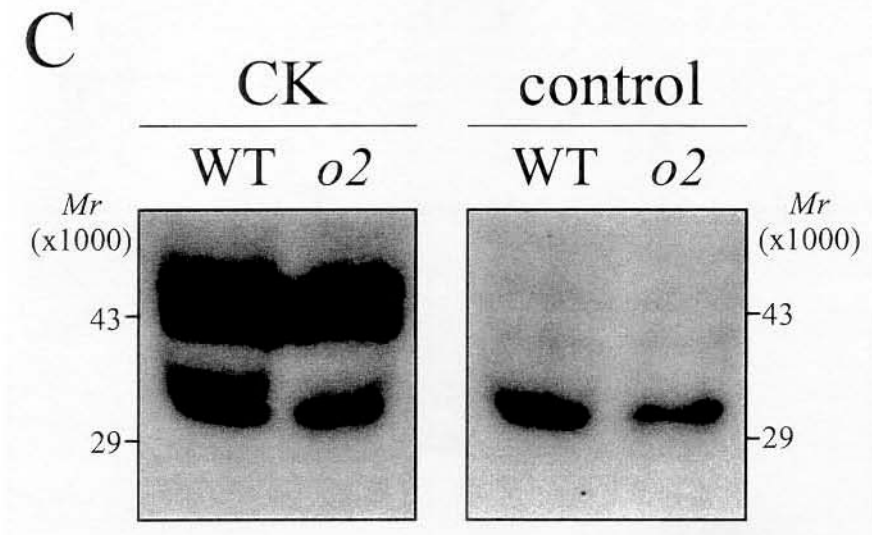
TABLE II *Molecular weight determination of LOR/SDH in different concentrations of free Ca^{2+} after HPLC gel filtration.* Aliquots of partial purified LOR/SDH (1250 µg) obtained by 35 - 60% ammonium sulfate fractionation were applied to a gel filtration column equilibrated with 50 mM Tris-HCl pH 8.5, 1 mM DTT and 25 mM KCl and three different concentrations of free Ca^{2+}. The elution volumes of LOR/SDH were monitored by measuring LOR and SDH activities. For each concentration of free Ca^{2+}, the column was calibrated with a complete set of the following molecular weight standards (Pharmacia Biotech): ovalbumin (M_r = 43,000), bovine serum albumin (M_r = 67,000), catalase (M_r = 232,000), ferritin (M_r = 440,000) and thyroglobulin (M_r = 668,000). The elution volumes of these markers were determined from the elution profiles at 280 nm.

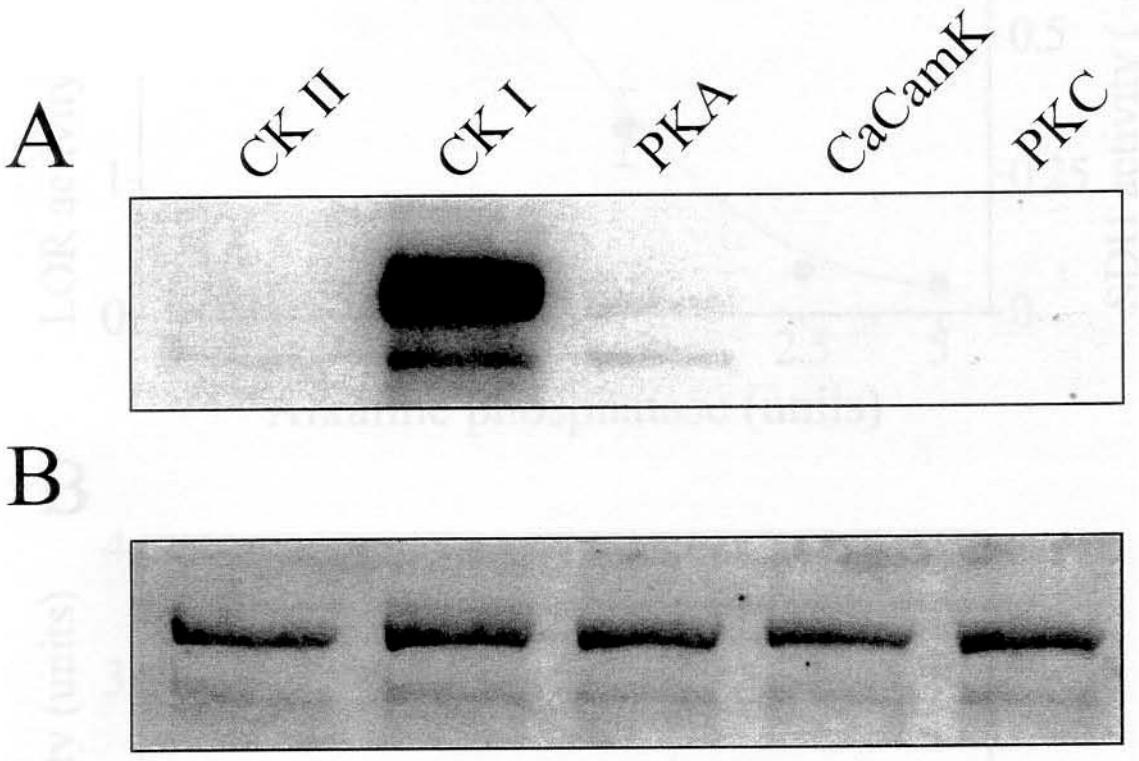
$[Ca^{2+}]$	Molecular weight (x1000)	Activities
0	115.3 ± 2.2	SDH
99 µM	274.3 ± 5.8	LOR and SDH
	115.3 ± 2.6	SDH
1,3 mM	257.5 ± 0.0	LOR and SDH

		Molecular weight (x1000)		
		$[Ca^{2+}]$		
Enzymatic form	Activity	0	99 µM	1,3 mM
Dimer	LOR	absent	274.3 ± 5.8	257.5 ± 0.0
Dimer	SDH	absent	282.0 ± 8.6	241.5 ± 16.2
Monomer	SDH	115.3 ± 2.2	115.3 ± 2.6	absent

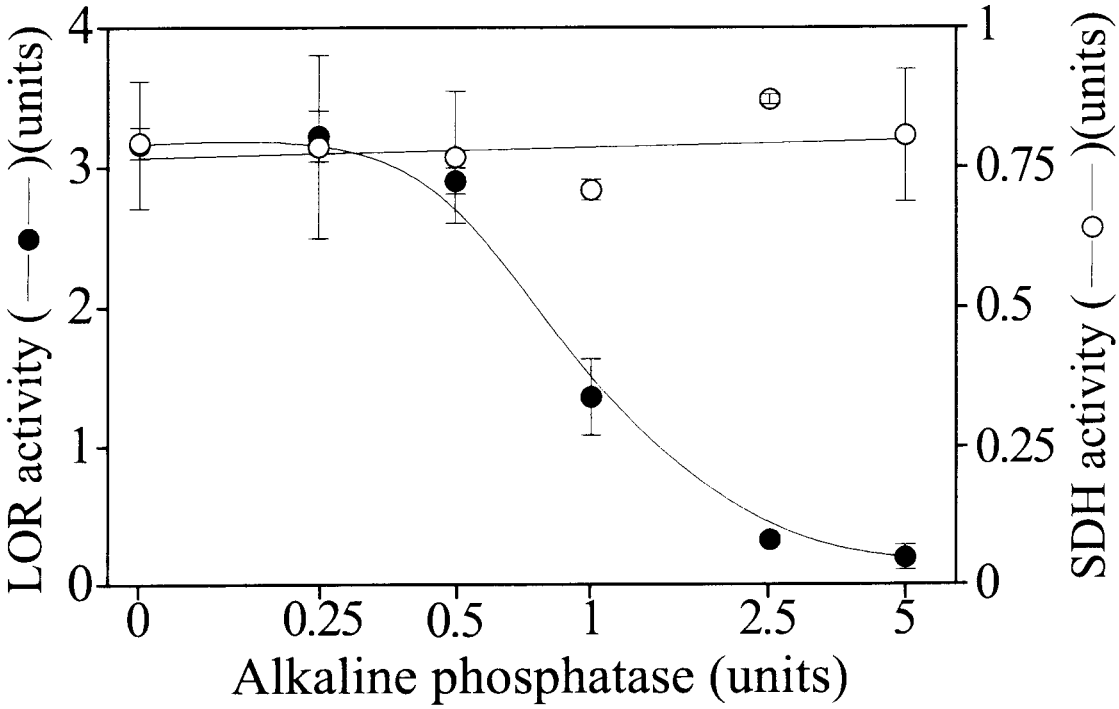

A
Enzyme activity (units/g)
20
15
10
5
0
WT
o2
WT
o2
LOR
SDH


B
WT
o2
SDS-PAGE
PAGE
S
F

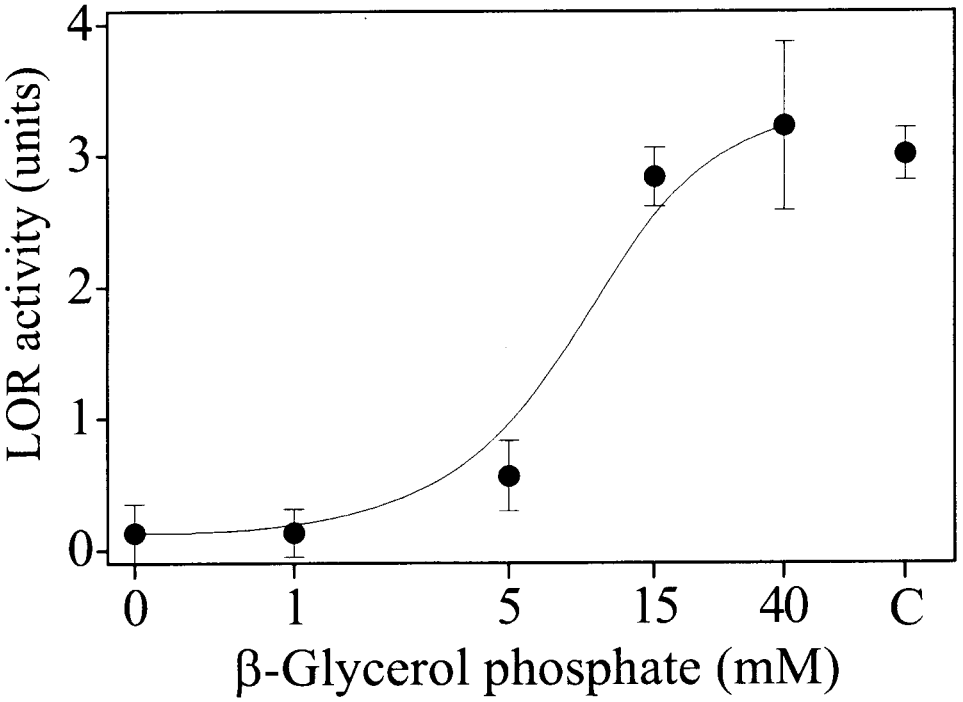

C
CK
control
WT
o2
WT
o2
Mr
(x1000)
43
29
Mr
(x1000)
43
29

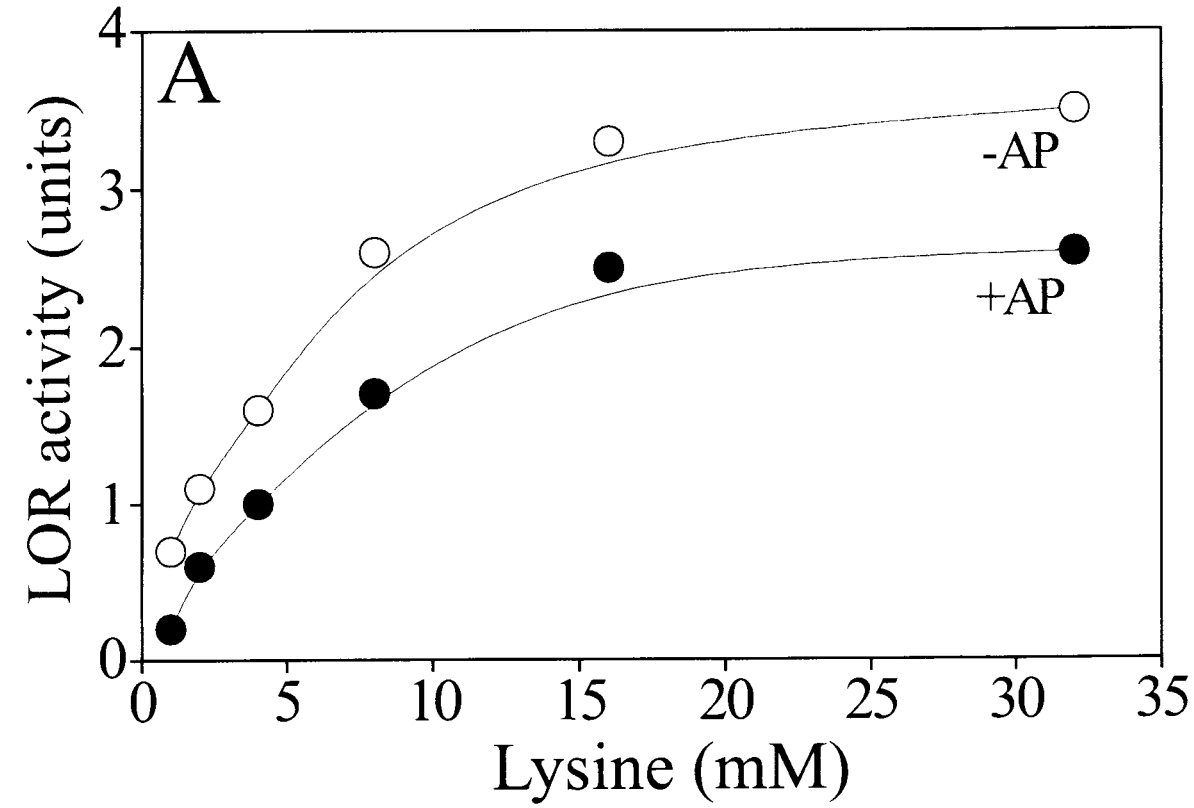

A
CK II
CK I
PKA
CaCamK
PKC
B

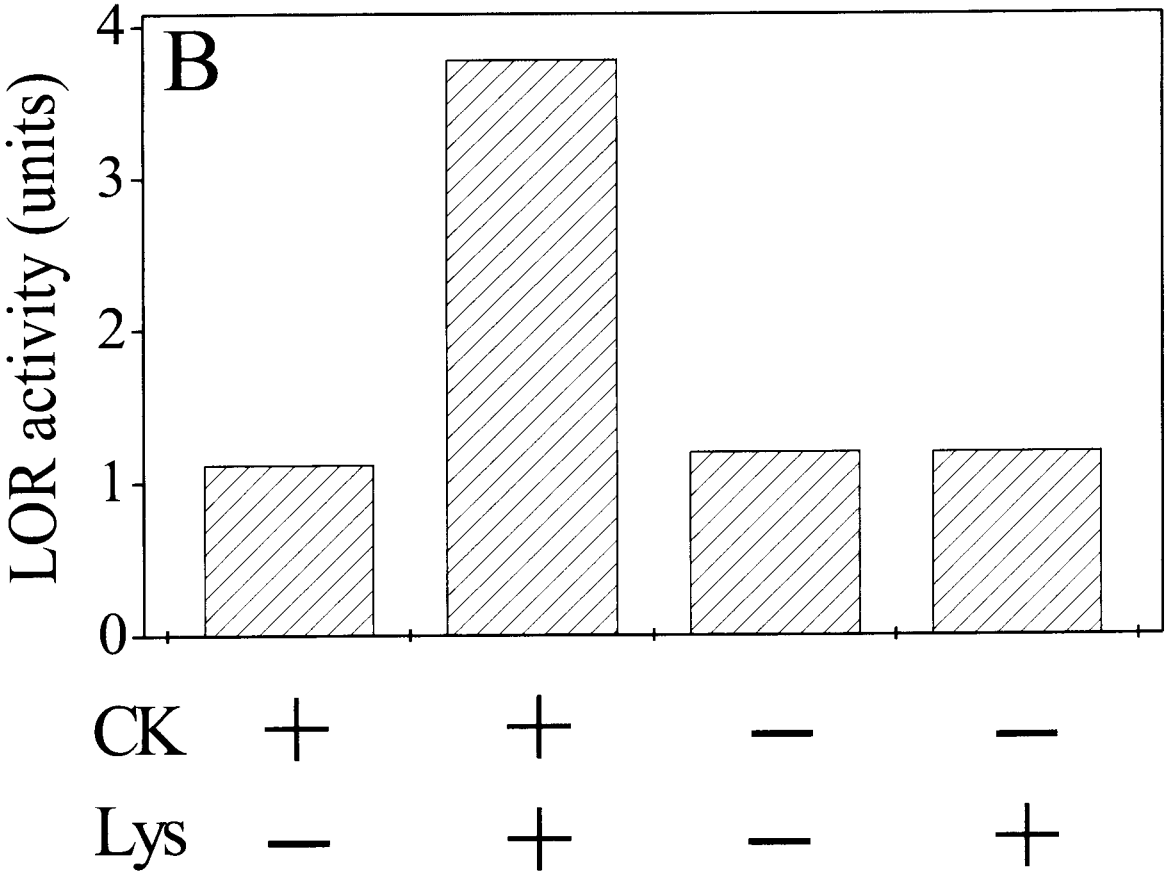
A

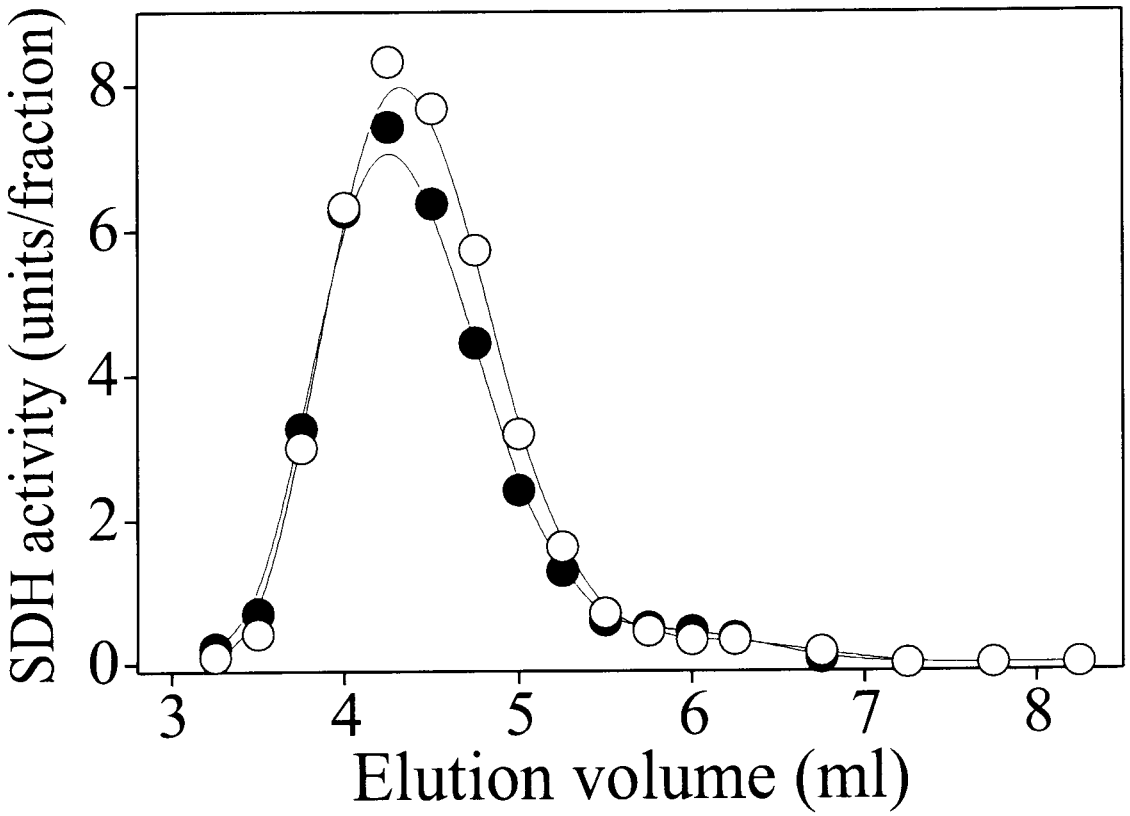

LOR activity (—●—)(units)
SDH activity (—○—)(units)
0
1
2
3
4
0
0.25
0.5
0.75
1
0
0.25
0.5
1
2.5
5
Alkaline phosphatase (units)

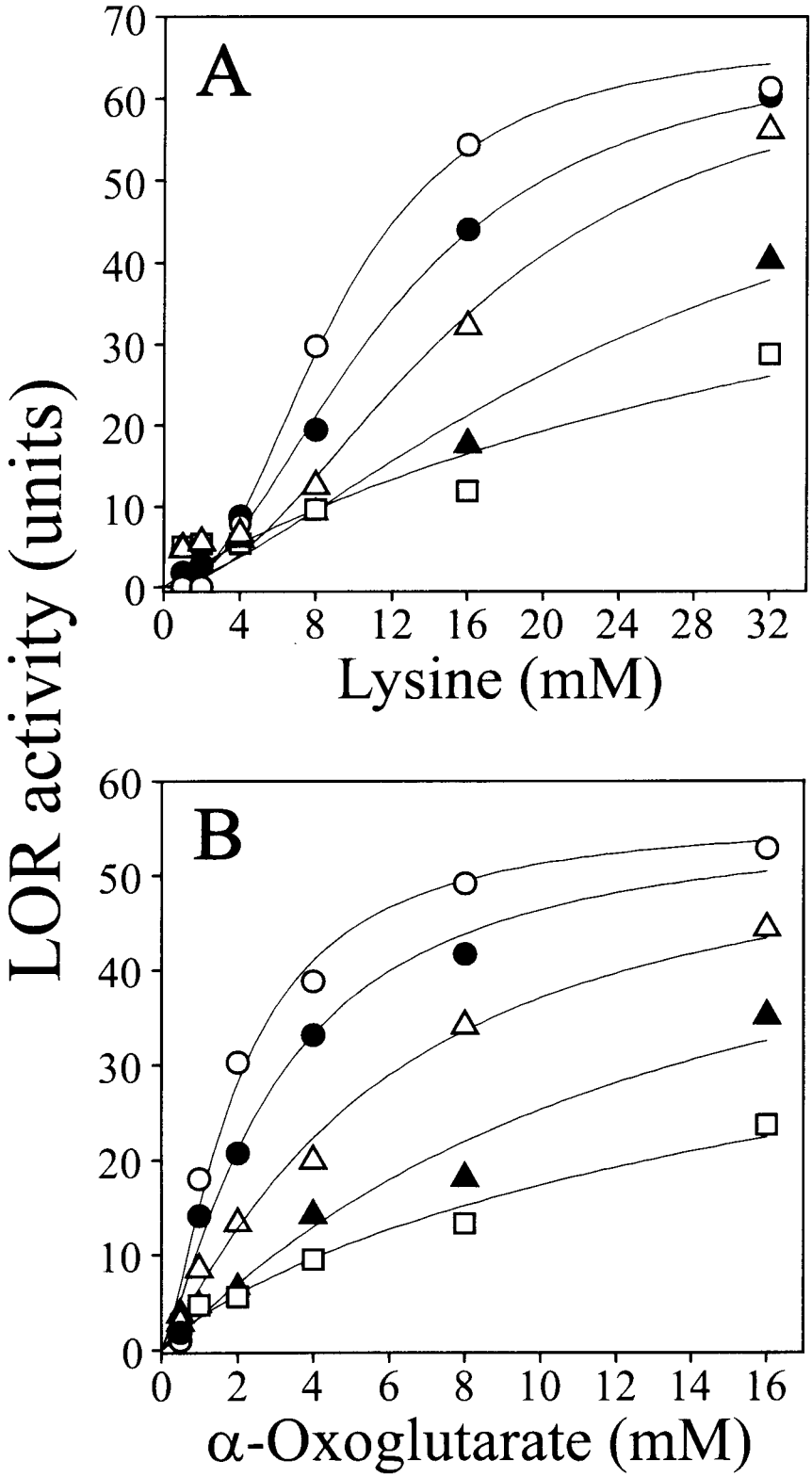
B

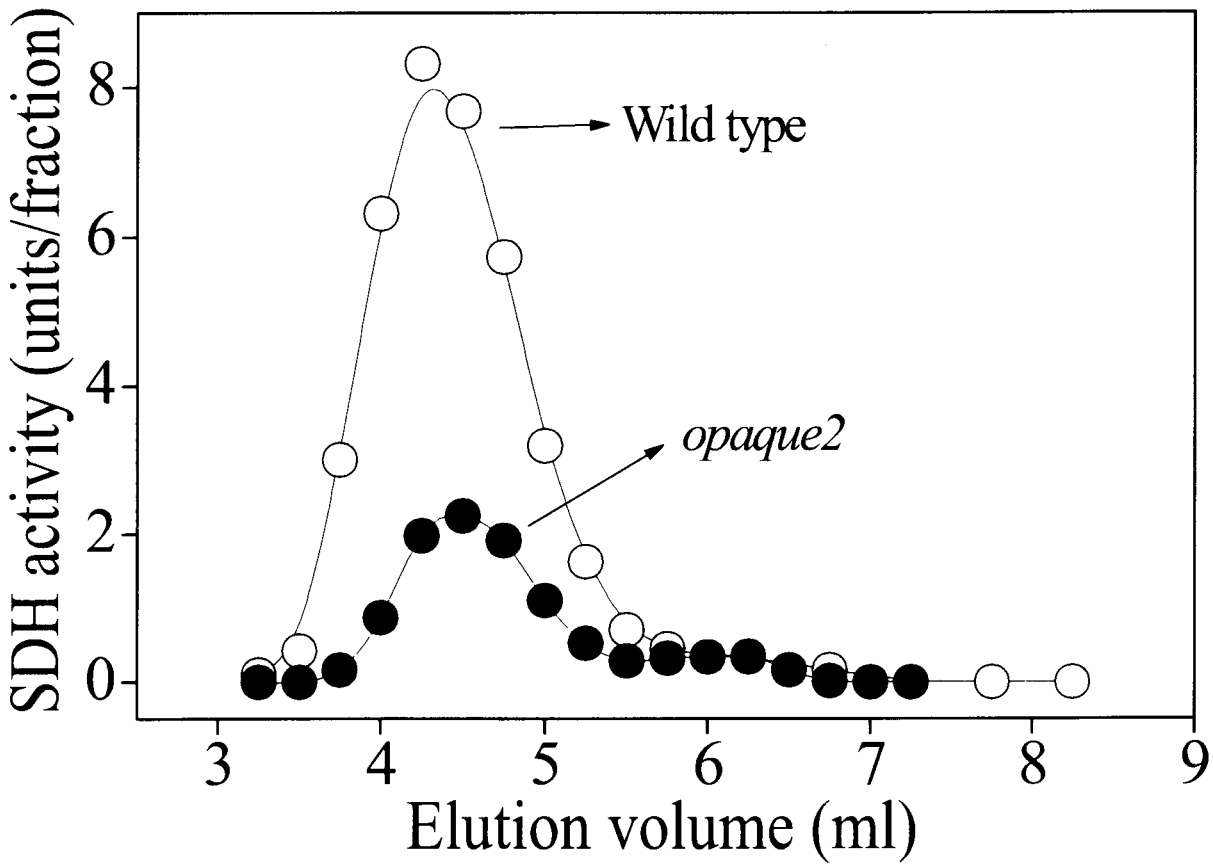

LOR activity (units)
0
1
2
3
4
0
1
5
15
40
C
β-Glycerol phosphate (mM)


A
LOR activity (units)
Lysine (mM)
-AP
+AP
0
1
2
3
4
0
5
10
15
20
25
30
35


B
LOR activity (units)
CK + + − −
Lys − + − +
0
1
2
3
4


SDH activity (units/fraction)
0
2
4
6
8
3
4
5
6
7
8
Elution volume (ml)

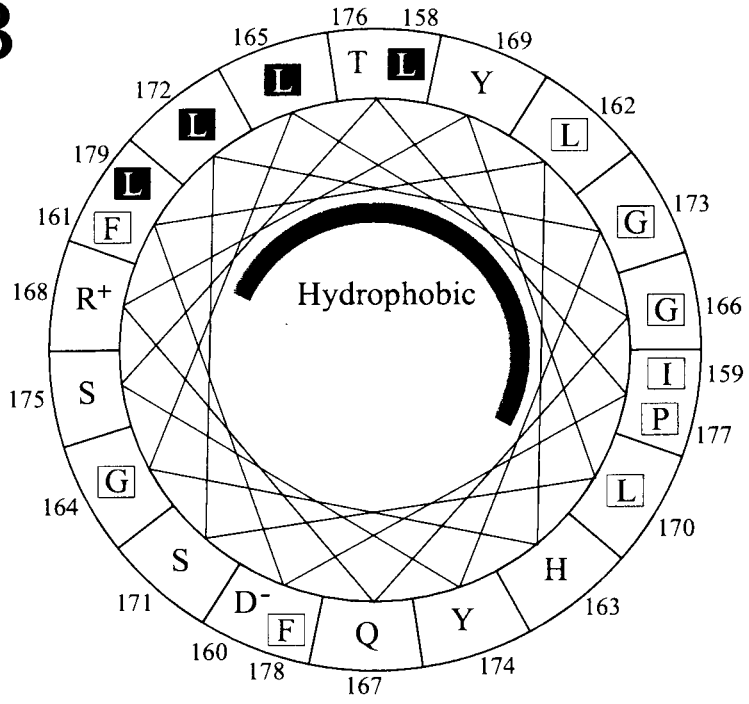

A
B
LOR activity (units)
Lysine (mM)
α-Oxoglutarate (mM)
0
10
20
30
40
50
60
70
0
4
8
12
16
20
24
28
32
0
2
4
6
8
10
12
14
16

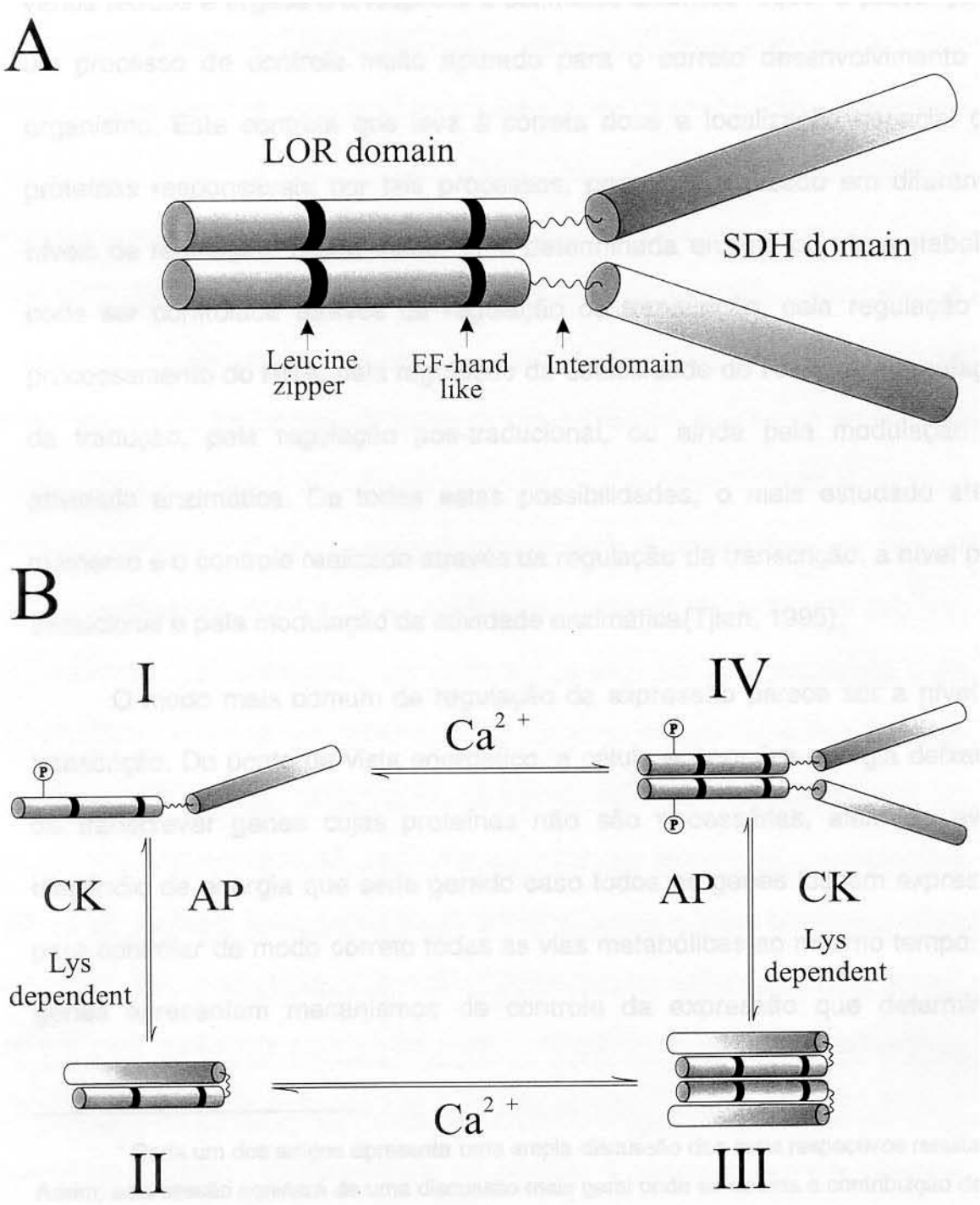

SDH activity (units/fraction)
0
2
4
6
8
Wild type
opaque2
3
4
5
6
7
8
9
Elution volume (ml)

A

```
                X  Y  Z -Y -X    -Z
                *  *  *  *  *  *  *  *  *  *  *  *
Maize (382-396) — DMEGAGVVCLAVDI
Arabidopsis (383-397) — DMDGDGVLCMAVDI
```

B




A
LOR domain
SDH domain
Leucine zipper
EF-hand like
Interdomain
B
I
IV
Ca2 +
CK
AP
Lys dependent
AP
CK
Lys dependent
Ca2 +
II
III

DISCUSSÃO[1]

O crescimento e o desenvolvimento dos organismos, a diferenciação dos vários tecidos e órgãos e a resposta a estímulos externos requer a presença de um processo de controle muito apurado para o correto desenvolvimento do organismo. Este controle que leva à correta dose e localização espacial das proteínas responsáveis por tais processos, pode ser realizado em diferentes níveis de regulação. Deste modo, uma determinada enzima ou via metabólica pode ser controlada através da regulação da transcrição, pela regulação do processamento do RNA, pela regulação da estabilidade do RNA, pela regulação da tradução, pela regulação pós-traducional, ou ainda pela modulação da atividade enzimática. De todas estas possibilidades, o mais estudado até o momento é o controle realizado através da regulação da transcrição, a nível pós-traducional e pela modulação da atividade enzimática(Tjian, 1995).

O modo mais comum de regulação da expressão parece ser a nível de transcrição. Do ponto de vista energético, a célula economiza energia deixando de transcrever genes cujas proteínas não são necessárias, além de evitar dispêndio de energia que seria gerado caso todos os genes fossem expressos para controlar de modo correto todas as vias metabólicas ao mesmo tempo. Os genes apresentam mecanismos de controle da expressão que determinam

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[1] Cada um dos artigos apresenta uma ampla discussão dos seus respectivos resultados. Assim, esta sessão constará de uma discussão mais geral onde se aborda a contribuição destes trabalhos no entendimento da regulação do catabolismo de lisina em plantas e no estudo da regulação coordenada da expressão gênica no endosperma em desenvolvimento.

quando, onde e em que extensão a transcrição deve ser realizada (Tjian, 1995). Embora necessária, muitas vezes tal tipo de regulação não é suficiente para o correto funcionamento da proteína codificada pelo gene transcrito.

Assim, para garantir uma regulação mais precisa dos diversos processos, a célula dispõe de mecanismos adicionais, entre eles o controle da atividade enzimática de diferentes vias metabólicas. Existem quatro mecanismos principais de modulação da atividade enzimática: controle alostérico, proteínas moduladoras, modificação covalente e proteólise (Stryer, 1996). O controle alostérico é o resultado de mudanças conformacionais em decorrência da ligação de pequenas moléculas em motivos diferentes do sítio ativo. Muitas vezes tais moléculas são os produtos finais da via metabólica cuja primeira reação é catalisada pela enzima. As mudanças conformacionais permitem grandes alterações da atividade enzimática em função de pequenas variações na concentração de seus substratos, propriedade chamada de hipersensibilidade. Outras enzimas são reguladas por proteínas estimuladoras ou inibidoras. Exemplos de proteínas reguladoras são a calmodulina ou subunidades reguladoras de proteínas quinases. As propriedades catalíticas de muitas enzimas são acentuadamente modificadas pela ligação covalente e reversível de diferentes grupamentos químicos, como fosfato, acil, metil, entre outros. Deste tipo de modificação, o mais comum e estudado é sem dúvida a fosforilação. Finalmente, algumas enzimas são ativadas, ou inibidas, através de digestão proteolítica, após a retirada de algum domínio inibitório ou ativador, respectivamente (Stryer, 1996). A atividade das enzimas pode ser modificada

alternando estados ativo e inativo, quando a modulação é realizada pelos três primeiros mecanismos citados acima e na prática, estes mecanismos podem se sobrepor. Já a ativação ou inibição por proteólise transforma irreversivelmente a atividade de uma determinada enzima.

Os estudos apresentados nesta tese indicam que muitos destes importantes pontos de regulação, desde a expressão gênica, regulação da tradução e modulação da atividade são encontrados na via de degradação de lisina na semente de milho. No trabalho 2 são apresentadas evidências de que o ativador Opaco2 regula a transcrição do gene que codifica a enzima LKR/SDH da via de degradação de lisina. As questões referentes a esta regulação transcricional não serão comentadas aqui, uma vez que já foram exploradas em uma tese anterior, desenvolvida em nosso laboratório pelo co-autor do trabalho 2 (Cord-Neto, 1998).

Inicialmente verificou-se que apenas a atividade de redutase (LKR) da enzima bifuncional LKR/SDH é modulada por Ca^{2+}, Mg^{2+}, força iônica e solventes orgânicos. A ativação da LKR por Ca^{2+} foi completamente eliminada por antagonistas de calmodulina. Entretanto não foi possível demonstrar que a calmodulina regula a atividade de LKR. É provável que o Ca^{2+} afete a atividade de LKR diretamente através de algum domínio sensível a antagonistas de calmodulina. Posteriormente, obteve-se evidências de que o Ca^{2+} auxilia no processo de dimerização da enzima LKR/SDH, atuando provavelmente, apenas no domínio LKR.

O Ca^{2+} é utilizado como mensageiro secundário no controle de diversos processos celulares, como contração muscular e excitabilidade neuronal em mamíferos, secreção, metabolismo geral e controle da morte celular programada. O nível citossólico de Ca^{2+} é mantido baixo, entre 10 e 100 nM. Sob certos estímulos verificasse um aumento na concentração de Ca^{2+} para níveis de 500 a 1000 μM, que leva a ativação dos processos celulares citados acima. A célula conta com a duas fontes de Ca^{2+}: do meio externo e de estoques intra-celulares (Berridge, 1993; Clapham, 1995; Simpson et al., 1995). A entrada de Ca^{2+} nos vários compartimentos celulares é controlada pela atuação de bombas de Ca^{2+}. Estes mecanismos de entrada e saída de Ca^{2+} são organizados de modo a produzir breves ondas e picos estimulatórios de Ca^{2+} (Clapham, 1995; Amudson e Clapham, 1995). Deste modo, as células conseguem evitar eventuais efeitos citotóxicos de elevada concentração de Ca^{2+}. Ainda não se sabe se o Ca^{2+} está ou não envolvido em alguma via de transdução de sinal que leva a ativação ou inativação da LKR/SDH, apesar de existir evidências de que o metabolismo de lisina é controlado por uma via de sinalização envolvendo cálcio e fosforilação em tabaco (Karchi et al., 1995). Por outro lado verificamos um efeito estimulatório do Ca^{2+} diretamente na atividade da enzima. Apasar de não ter conseguido demonstrar o binding de Ca^{2+} na enzima no trabalho 1, evidências são apresentadas de que este íon esteja envolvido com o processo de dimerização no trabalho 3. Recentemente demonstrou-se que a calmodulina e a proteína E-cadherin são capazes de formar dímeros e tetrâmeros apenas na presença de Ca^{2+} (Alattia et al., 1997;

Lafitte et al., 1999), indicando que este íon esteja envolvido no processo de oligomerização de proteínas. É importante ressaltar que a LKR/SDH apresenta no domínio LKR, um provável motivo "EF-hand", o mesmo tipo de domínio responsável pela ligação de cálcio na calmodulina. Esta observação, aliado ao resultado de que inibidores de calmodulina previnem o efeito estimulador do Ca^{2+}, sugerem que o íon liga-se de forma direta na enzima LKR/SDH, promovendo a dimerização via domínio LKR. Os resultados obtidos no trabalho 3 indicam que a dimerização é fundamental para a modulação da atividade de LKR, mas não de SDH. Em ratos e em *Arabidopsis* observou-se formas de SDH monofuncionais (Papes et al., 1999; Tang et al., 1997) e pelo menos em camundongos estas proteínas são monoméricas (Papes et al., 1999). Outra evidência da dependência da atividade da LKR da dimerização é a obtenção de dímeros de peptídeos contendo a atividade de LKR após proteólise limitada da LKR/SDH nativa de fígado bovino e posterior separação cromatográfica (Markovitz e Chuang, 1987).

Experimentos de proteólise limitada foram delineados para estudar se os domínios responsáveis pela atividade de LKR e SDH são funcionalmente independentes na enzima nativa. Estes experimentos indicaram que os domínios LKR e SDH são independentes e que o domínio SDH apresenta atividade inibitória sobre o domínio LKR. Muitas outras enzimas demonstram este tipo de relação, onde a atividade enzimática é inibida por algum domínio inibidor. Este domínio inibidor pode ser inativado por algum tipo de modificação pós traducional ou mesmo pelo efeito de algum íon ou proteína regulatória. Um

exemplo deste efeito é aquele verificado na enzima GAD, onde um domínio inibidor é inativado após ligação de Ca^{2+}-calmodulina (Arazi et al., 1995). A expressão de clones truncados de GAD indicaram que esta enzima possui três domínios. Um catalítico, um inibidor e outro regulatório. A partir da ligação da Ca^{2+}-calmodulina no domínio regulatório, há uma mudança conformacional que impede a ligação do domínio inibidor ao catalítico, permitindo que este se mantenha ativo. A expressão de clones truncados de GAD sem o domínio regulador não possuem atividade enzimática mesmo na presença de Ca^{2+}-calmodulina. Já a expressão de clones contendo apenas o domínio catalítico produzem enzimas permanentemente ativas, onde não há ganho de atividade com a adição de Ca^{2+}-calmodulina (Baum et al., 1993; Arazi et al., 1995; Baum et al., 1996).

Este modelo se ajusta bem aos observado para a enzima LKR/SDH, onde o domínio SDH parece ser, ou conter, um domínio inibidor da atividade de LKR. Entretanto, qual é o mecanismo que faz com que a o domínio inibidor deixe a LKR ativa? Aparentemente a fosforilação da LKR/SDH desempenha este papel, retirando um possível motivo inibidor contido no domínio LKR.

No trabalho 2, verificou-se que a atividade de LKR é especificamente reduzida no endosperma *opaco2*. Esta redução indica que a LKR/SDH deve sofrer alguma modificação pós-traducional, uma vez que a regulação transcricional conferida pelo fator Opaco2 não justifica *per se* tal diferença (Cord-Neto, 1998), já que a tradução do mRNA para LKR/SDH gera quantidades equimolares dos domínios de redutase e desidrogenase. De fato, foi

demonstrado *in vitro* que a LKR/SDH é uma fosfoproteína. Para estudar o efeito da fosforilação na LKR/SDH de milho, estudou-se o efeito da defosforilação na atividade de LKR e SDH. Mais uma vez a atividade de SDH mostrou-se insensível ao efeito da defosforilação, enquanto que a atividade de LKR foi especificamente inibida com a defosforilação. Para provar se a atividade de LKR realmente é dependente de fosforilação, a proteína LKR/SDH defosforilada foi tratada com caseína quinase. Como esperado, a atividade de LKR recuperada após a fosforilação, porém somente na presença de lisina. Estes resultados corroboram uma hipótese levantada há certo tempo em nosso laboratório de que a atividade da LKR está sempre ativada no endosperma de milho (Brochetto-Braga et al., 1992), a despeito da modulação da atividade da LKR por Ca^{2+} e fosforilação. Do ponto de vista fisiológico, a enzima deve estar ativa, já que o excesso de lisina deve ser degradado para adequar o pool de amino ácidos, à síntese de proteínas de reserva que ocorre na semente em desenvolvimento.

Um dos pontos mais interessantes deste trabalho é sem dúvida a queda da atividade de LKR no endosperma *opaco2* que não encontra paralelo na atividade de SDH. Ao tentar elucidar qual seria o mecanismo da regulação da atividade de LKR especificamente no endosperma *opaco2*, encontramos a expressão diferencial de uma caseína quinase. A fosforilação de proteínas celulares oferece a célula um interruptor molecular para a regulação de diferentes vias metabólicas. Em adição a efeitos bem estudados da fosforilação no metabolismo, esta modificação possui um importante papel em processos que ocorrem durante o desenvolvimento como divisão celular e morte celular

programada. Embora outras modificações covalentes possam ocorrer, a fosforilação de proteínas é uma importante ferramenta de controle para a célula, geralmente usada na modulação de vias metabólicas e de expressão gênica reguladas por sinais externos às células. A fosforilação reversível de proteínas ocorre via proteínas quinases, que constituem a maior família de proteínas conhecidas (Knighton et al., 1991). Sabe-se que o genoma dos mamíferos contém mais de 2000 genes codificadores de proteínas quinases (Hanks e Hunter, 1995). Esta miríade de quinases fosforilam entre 30 e 50% das proteínas celulares, como indicado por géis de eletroforese em duas dimensões de proteínas marcadas com ^{32}P (Pinna e Ruzzene 1996).

O fato de o endosperma *opaco2* não possuir uma proteína quinase é intrigante. É possível que a falta da atividade desta caseína quinase explique muitos dos efeitos pleiotrópicos observados no endosperma opaco2; já que as cascatas de sinalização intracelular dependem de quinases e originam redes complexas com a habilidade de interconectar sistemas de proteínas que regulam sistemas celulares específicos (Karin e Hunter, 1995). Muitas proteínas tem sido observadas com alteração de expressão diferencial no endosperma *opaco2* (Haben et al., 1993; Damerval e Guilloux, 1998). Esta talvez seja a maior função do ativador transcricional Opaco2 – coordenar a expressão de diferentes genes e proteínas responsáveis pelo metabolismo geral da célula do endosperma, principalmente na camada de subaleurona, que parece ser o local de "preparo" para o depósito de proteínas de reserva.

A dimerização dependente de cálcio e a fosforilação dependente de lisina estão de acordo com uma predição de Jacques Monod. Esta predição diz que enzimas que exibem efeitos cooperativos e inibição por "feedback" ou ativação dependente do substrato são oligômeros compostos de subunidades capazes de realizar transições entre duas ou mais estruturas pelo arranjo diferencial das subunidades e por diferenças nas ligações entre elas (Perutz, 1988). O efeito de recuperação da atividade de LKR após certo tempo de proteólise ou concentração de elastase corrobora esta hipótese na enzima LKR/SDH. A perda inicial da atividade pode ser devido a um corte inicial na enzima pela elastase, que a leva a um rearranjo que inativa a atividade de LKR. Já um segundo corte na enzima pela protease deve favorecer uma nova mudança conformacional que a leva a ter novamente atividade de LKR. Após prolongamento do tempo de incubação ou aumento do número de unidades de elastase, a atividade de LKR é finalmente perdida. Até onde sabemos este foi o primeiro exemplo de reativação da atividade enzimática por proteólise, mesmo que transiente.

O fato de a enzima LKR/SDH estar localizada na região da sub-aleurona é importante, pois como visto, este tecido parece ser o responsável pelo crescimento do endosperma e é justamente onde ocorre expressão mais acentuada do fator de transcrição Opaco2 (Robert Schmidt, comunicação pessoal) além deser o local da expressão de genes regulados por Opaco2, como α-zeínas e cyPPDK (Gallusci et al., 1996). Todas estas expressões colocalizadas podem ser fruto de regulação coordenada dos genes correspondentes. Esta hipótese parece ser plausível, uma vez que os genes

codificadores das α-zeínas e de cyPPDK são regulados a nível transcricional pelo ativador transcricional Opaco2 (Schmidt et al., 1992; Gallusci et al., 1996). Provavelmente a proteína Opaco2 desempenha um papel mais importante do que o verificado até agora. Inicialmente pensava-se que este fator regulasse a síntese de proteínas de reserva, mas o conjunto dos resultados de outros genes que são regulados a nível transcricional e que possuem expressão colocalizada com a proteína Opaco2 indicam que este regulador transcricional pode orquestrar a síntese de proteínas de reserva e o metabolismo de amino-ácidos.

Em leveduras, onde a via da sacaropina funciona para sintetizar lisina (Bhattacharjee, 1985), o transativador GCN4, similar e análogo funcional da proteína Opaco2, controla o metabolismo geral de nitrogênio, inclusive o de lisina (Hinnebusch, 1988). Em cada via de síntese ou degradação de aminoácidos em levedura atua o fator GCN4 e um ou mais fatores específicos de cada via metabólica. No caso da via de síntese de lisina, os genes codificadores das enzimas da via, são controlados também pelo produto de gene LYS14, que curiosamente requer o intermediário AASA para se ligar aos promotores dos genes da via (ver Figura 4)(Feller et al., 1994). Como visto, no endosperma mutante *opaco2*, ocorre, além da redução no nível de mRNA, uma mudança temporal e de intensidade no pico de expressão do gene (Cord Neto, 1998). A expressão do gene da LKR/SDH mesmo no endosperma mutante indica que a proteína Opaco2 não controla de maneira "tudo-ou-nada" a expressão do gene da LKR/SDH e que outros fatores podem estar contribuindo

para tal expressão. Um sistema similar ao LYS14 é um candidato para exercer um controle adicional sobre a via de degradação de lisina na semente de milho.

A LKR/SDH está localizada no citosol. Anteriormente, a enzima tinha sido localizada apenas em mamíferos, onde é encontrada na mitocôndria. Por outro lado, todas as enzimas da via do aspartato, estão localizadas em plastídeos, provavelmente por que muitos passos da síntese de lisina e treonina envolvam enzimas que necessitam de compostos de alta energia (Galili, 1995). Entretanto, as enzimas envolvidas na síntese de metionina estão localizadas no citoplasma (Galili, 1995). Embora o citoplasma seja pobre em NADPH, a LKR/SDH deve estar localizada no citoplasma das plantas, pelo menos no endosperma de milho, para permitir a rápida adequação do pool de amino ácidos àquele requerido para a síntese de proteínas de reserva.

CONCLUSÕES

1. Apenas o domínio LKR da enzima bifuncional LKR/SDH é ativado por Ca^{2+}, Mg^{2+}, força iônica e solventes orgânicos.
2. A ativação dependente de Ca^{2+} do domínio LKR foi prevenida por antagonistas de calmodulina.
3. Os experimentos de proteólise limitada indicam que os domínios LKR e SDH são estruturalmente independentes.
4. O domínio SDH é, ou contém um motivo inibidor da atividade de LKR
5. O domínio LKR isolado mantém a propriedade de ser ativado por cálcio, mas não é mais ativado por força iônica.
6. Os níveis da proteína LKR/SDH, bem como as atividades enzimáticas da LKR e da SDH são diminuídos no endosperma *opaco2*. Ocorre também um atraso de ~5 dias do pico de atividade de LKR e SDH no endosperma mutante em relação ao normal.
7. Aparentemente a expressão do gene *ZLKRSDH* é regulado a nível pós-transcricional por algum mecanismo do tipo de estabilização do mRNA no endosperma *opaco2*.
8. O padrão temporal e o local da enzima LKR/SDH associado ao padrão de expressão dos genes de zeínas de 22-kDa, da enzima cyPPDK e da proteína Opaco2 indicam que este fator transcricional pode coordenar uma série de vias metabólicas do endosperma em desenvolvimento.
9. A atividade da LKR é especificamente reduzida no endosperma *opaco2* em relação ao normal, o que não ocorre com a atividade de SDH, indicativo de que a atividade de LKR é regulada a nível pós-traducional.

10. A atividade de LKR é perdida após tratamento com fosfatase alkalina e recuperada por tratamentos com caseína quinase I e II, indicando que a enzima LKR/SDH é controlada a nível pós-traducional por fosforilação.

11. A recuperação da atividade de LKR por tratamentos com caseína quinase I e II ocorre apenas na presença de lisina, indicando que a concentração do substrato da enzima é fundamental para a ativação da enzima por fosforilação.

12. O processo de fosforilação não altera o padrão de dimerização da enzima.

13. Aparentemente o Ca^{2+} regula a atividade de LKR através do controle do padrão de dimarização da enzima bifuncional via domínio LKR.

14. A dimerização da enzima bifuncional provavelmente ocorre através do domínio LKR.

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