Expression of the Maize *MnSod* (*Sod3*) Gene in MnSOD-Deficient Yeast Rescues the Mutant Yeast Under Oxidative Stress

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ABSTRACT

Superoxide dismutases (SOD) are ubiquitous in aerobic organisms and are believed to play a significant role in protecting cells against the toxic, often lethal, effect of oxygen free radicals. However, direct evidence that SOD does in fact participate in such a protective role is scant. The MnSOD-deficient yeast strain (*Sod2d*) offered an opportunity to test the functional role of one of several SOD isozymes from the higher plant maize in hopes of establishing a functional bioassay for other SODs. Herein, we present evidence that MnSOD functions to protect cells from oxidative stress and that this function is conserved between species. The maize *Sod3* gene was introduced into the yeast strain *Sod2d* where it was properly expressed and its product processed into the yeast mitochondrial matrix and assembled into the functional homotetramer. Most significantly, expression of the maize *Sod3* transgene in yeast rendered the transformed yeast cells resistant to paraquat-induced oxidative stress by complementing the MnSOD deficiency. Furthermore, analyses with various deletion mutants of the maize SOD-3 transit peptide in the MnSOD-deficient yeast strain indicate that the initial portion (about 8 amino acids) of the maize transit peptide is required to direct the protein into the yeast mitochondrial matrix *in vivo* to function properly. These findings indicate that the functional role of maize MnSOD is conserved and dependent on its proper subcellular location in the mitochondria of a heterologous system.

SUPEROXIDE and hydrogen peroxide are produced in all living organisms (FRIDOVICH 1986). The superoxide radical and hydrogen peroxide participate in a metal-catalyzed Haber-Weiss reaction, generating the highly reactive hydroxyl radical which is implicated as the major factor in oxidative stress. Organisms have evolved diverse ways of protecting cells from the toxic and potentially lethal effects of active oxygen species. Superoxide dismutase (SOD; $\cdot\text{O}_2^-:\cdot\text{O}_2^-$ oxidoreductase; EC 1.15.1.1), one of the antioxidant defense enzymes, catalyzes the dismutation of two superoxide radicals into molecular oxygen and hydrogen peroxide. The essential role of superoxide dismutase is to remove the superoxide produced in living cells to prevent cellular damage (BRAWN and FRIDOVICH 1981; FUCCI *et al.* 1983; and MEAD 1976). It has been reported that SOD is induced by superoxide-generating redox compounds, such as the herbicide paraquat, in *Escherichia coli* (HASSAN and FRIDOVICH 1977) and in maize leaves (MATTERS and SCANDALIOS 1986).

In maize, the six superoxide dismutase isozymes are coded by six nonallelic nuclear genes: *Sod1*, *Sod2*, *Sod3*, *Sod4*, *Sod4A* and *Sod5* (BAUM and SCANDALIOS 1982; CANNON and SCANDALIOS 1989). The cytosolic isozymes, SOD-2, SOD-4, SOD-4A, SOD-5 and the chloroplast-associated SOD-1 are copper- and zinc-

containing homodimeric enzymes. SOD-3, the mitochondrial-associated isozyme, is a manganese-containing homotetrameric enzyme (BAUM and SCANDALIOS 1979; BAUM, CHANDLEE and SCANDALIOS 1983). Previous studies have shown that the maize SOD-3 is synthesized on cytosolic polyribosomes as a precursor with a cleavable presequence of 31 amino acid residues at the amino terminus (WHITE and SCANDALIOS 1987). *In vitro* import studies using internal deletion mutations in the transit peptide of maize SOD-3 indicated that each deletion mutant of preSOD-3 is imported and processed in isolated maize mitochondria (WHITE and SCANDALIOS 1989).

The mitochondrial MnSOD isozyme in yeast is encoded by the nuclear genome, synthesized on cytosolic polyribosomes and imported post-translationally into mitochondria (AUTOR 1982). A MnSOD-deficient yeast strain, *Sod2d*, has been reported (VAN LOON, PESOLD-HURT and SCHATZ 1986) and is characterized by hypersensitivity to oxygen under normal growth in liquid media with the nonfermentable carbon sources ethanol and/or glycerol. Because of a highly conserved machinery for protein import into mitochondria in yeast and plants (BOWLER *et al.* 1989; CHAUMONT, O'RIORDAN and BOUTRY 1990; SCHMITZ and LONSDALE 1989), this yeast mutant strain provides an excellent complementation system to study the functional role of MnSOD *in vivo* by expression of a foreign *MnSod* gene in the mutant yeast cells.

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We attempted to investigate the biological role of maize MnSOD (SOD-3) in protecting yeast cells from oxidative stresses. The maize *Sod3* gene was introduced into the yeast mutant strain Sod2d. The results presented herein demonstrate that the maize SOD-3 can sufficiently complement the MnSOD deficiency and effectively act to rescue the transformed yeast cells oxidatively stressed with paraquat. Due to the intracellular compartmentalization of MnSOD, complementation of the MnSOD deficiency and protection against the toxic effects of paraquat in the MnSOD-deficient yeast cells depend on the effective import of maize preSOD-3 into yeast mitochondria. Import studies using deletion mutants in the maize SOD-3 transit peptide show that the initial amino acids of the preSOD-3 transit peptide are required for targeting the maize preSOD-3 into yeast mitochondria *in vivo*.

MATERIALS AND METHODS

Yeast strains and culture media: The *Saccharomyces cerevisiae* strain DL-1wt (*MAT α* , *leu2-3*, *2-112 his3-11*, *3-15*, *ura3-251*, *3-372*, *3-328*, *SOD2*) and MnSOD-deficient strain Sod2d (*MAT α* , *his3*, *ura3*, *Sod2::LEU2*) were obtained from D. J. KOSMAN at State University of New York at Buffalo. Culture media used were: MM (0.5% yeast nitrogen base/3% glucose with 20 μ g of histidine and leucine per ml), YPD (1% yeast extract/2% Bacto-peptone/3% glucose), and YPE (1% yeast extract/2% Bacto-peptone/3% ethanol). In all cases, cells were grown at 30° with constant shaking.

N-terminal deletion mutants of maize SOD-3: The maize SOD-3 cDNA clone used in this study was previously described (WHITE and SCANDALIOS 1988). The *EcoRI* cDNA fragment was isolated from plasmid pSod3.1c. and subcloned into the *EcoRI* site of pBluescript II KS vector (Stratagene). The resulting plasmid was designated pBSod3B. DNA from pBSod3B was first digested by *SalI* to create the 5' overhang which was subsequently filled with dideoxythioderivatives (Thio-dNTPs) using Klenow polymerase. Unidirectional deletion series at the 5' end of the *Sod3* cDNA were produced by the *ExoIII*/mung bean nuclease deletion system (Stratagene) following the *EcoRV* second digestion. The *NdeI* linker (CCATATGG) was added to create an in-frame start codon and the junction sequences of each plasmid construct were confirmed by double-stranded DNA sequencing using the dideoxy chain-termination method (SANGER, NICKLEN and MANIATIS 1977).

Expression of the maize *Sod3* gene and its deletion mutants in yeast: Yeast expression plasmids pYZ0, pYZ6, pYZ13, pYZ18, pYZ30, and pYZ37: The yeast pDB20 (BECKER, FIKES and GUARENTE 1991) vector DNA obtained from K. TATCHELL at NCSU contains the yeast ADC1 (*ADH1*) promoter and terminator. The *NdeI*/*EcoRI* double digested fragments from the plasmids containing the N-terminal deleted *Sod3* genes and the *EcoRI* fragment from the undeleted *Sod3* gene plasmid were subcloned into the *HindIII* linearized pDB20 vector DNA by blunt-ended ligation. The plasmid which expressed the undeleted maize *Sod3* gene was designated pYZ0. The plasmids expressing the N-terminal deleted maize *Sod3* genes were designated pYZ6, pYZ13, pYZ18, pYZ30 and pYZ37 and regarded as N-terminal deletion mutants.

Yeast expression plasmids pYB10, pYC38, pYA8, pYB3 and

pYC37: The plasmids carrying the internally deleted maize *Sod3* genes (B10, C38, A8, B3 and C37) were described (WHITE and SCANDALIOS 1989). For expression of these deleted *Sod3* genes in yeast, the *EcoRI* fragments from the above plasmids were repaired by Klenow polymerase and subsequently inserted into the *HindIII* site (made blunt with Klenow polymerase) of the yeast pDB20 vector DNA. The resulting plasmids were named pYB10, pYC38, pYA8, pYB3 and pYC37 and referred as internal deletion mutants.

Transformation of intact yeast cells: The DNAs from the yeast expression plasmid (shown in Figure 1) were introduced into the MnSOD-deficient yeast strain Sod2d by the lithium acetate transformation method (SCHIELT and GIETZ 1989). The URA3 encoded by the plasmid gene in pDB20 vector was used as a selection marker and the transformants were selected for growth in uracil-free MM medium. The wild type yeast strain DL-1wt and the mutant strain Sod2d transformed with the pDB20 vector alone were used as positive and negative controls, respectively.

Complementation assay: Complementation was carried out by growing the transformants in YPE liquid culture as described (VAN LOON, PESOLD-HURT and SCHATZ 1986). Cell densities were monitored at 600 nm for 68 h.

Total protein extraction and isolation of yeast mitochondria: Total protein extracts from maize scutella were prepared as described (BAUM and SCANDALIOS 1982). Total protein extracts were obtained by disruption of the yeast cells with glass beads. Cells were grown in MM medium overnight at 30°. The cells were washed once with ice-cold water and resuspended in 50 ml YPE with a supplement of 0.5% glucose to an OD₆₀₀ about 1.0–1.5. The cells were collected and resuspended in 500 μ l ice-cold buffer (20 mM HEPES, pH 7.1, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 μ g antipain/ml). Following addition of an equal volume of glass beads, the cells were broken by high speed vortexing for 5 times at 1 min each time. The supernatant was collected by centrifugation for 45 min 6000 rpm at 4°. The yeast mitochondria were isolated by Zymolyase digestion as described (DAUM, BOHNI and SCHATZ 1982). Protein concentration was determined by the Lowry protein assay (LOWRY *et al.* 1951).

Proteinase K protection assay: Thirty micrograms of isolated yeast mitochondria were incubated with 1.8 μ g proteinase K on ice for 30 min in the presence of 1% Triton X-100. The incubation was terminated by adding PMSF to 1–2 mM and the reaction was kept on ice for other 15 min. The mitochondria treated with proteinase K were subjected to Western blot analysis.

Western blot analysis and SOD activity assay: The proteins were separated in 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electroblotted onto nitrocellulose filters. The filters were incubated overnight with monospecific maize SOD-3 antibodies at room temperature followed by a 1-hr incubation with horseradish peroxidase conjugated to goat anti-rabbit IgG-specific antibody. The maize SOD-3 proteins were detected by the ECL Western blotting detection system (Amersham). For SOD enzymatic activity, protein samples were separated on nondenaturing 7.5% polyacrylamide gels at 4°. The SOD activity was photochemically determined as described elsewhere (RAVINDRANATH and FRIDOVICH 1975).

Paraquat treatment: Paraquat (methyl viologen) was purchased from Sigma Chemical Co. Yeast cells were grown overnight in 2 ml MM liquid cultures. The overnight cultures were diluted to an OD₆₀₀ about 0.025 with 50 ml fresh MM. Paraquat was added to the cultures to 0.2 mM when the OD₆₀₀ was almost 0.1. Growth was measured at 600 nm for 20 hr. To test the sensitivity of all transformants to paraquat on MM plates, the transformants were grown in 5

FIGURE 1.—Plasmid constructions of the vectors for expressing maize *Sod3* cDNA and its mutants in yeast. (a) The undeleted maize *Sod3* cDNA in yeast pDB20 vector, (b) The constructions of the altered maize *Sod3* cDNAs by *ExoIII* (N-terminal deletion in yeast pDB20 vector), and (c) the constructs of the maize *Sod3* internal deletion mutants by *Bal-31* in the yeast pDB20 vector. Expression of the maize *Sod3* cDNA and its mutants in all the constructs in this figure was controlled by the yeast *ADC1* (*ADH1*) promoter, denoted by stippled boxes. The transcriptional direction is indicated by an arrowhead. Transcription was terminated by the yeast *ADC1* terminator shown as hatched boxes. The structures of the maize *Sod3* cDNAs inserted are presented as the sequences encoding the transit peptide (solid boxes) and mature protein (open boxes). The sequences deleted are indicated (Δ), and size of the deletions are listed at right. The abbreviations of the restriction enzymes are: A, *Ava*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sac*I; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I.

ml MM liquid culture for overnight and equal numbers of cells were spotted onto MM plates containing 0.2 mM paraquat and incubated at 30° for 72 hr. The yeast cells grown on the plates were photographed.

RESULTS

Complementation of the yeast MnSOD-deficient mutant by maize SOD-3: The MnSOD-deficient yeast strain *Sod2d* grows very slowly in YPE liquid medium. The complementation assay was based on the growth of the transformed yeast cells in YPE liquid medium. The results showed that the *Sod2d* yeast transformed with vector pDB20 alone failed to grow in YPE liquid medium. By contrast, the mutant yeast transformed with the plasmid pYZ0 carrying the maize *Sod3* gene (Figure 1a) grew at the same rate as the wild-type yeast strain DL-1wt (Figure 2a). A proteinase K protection assay (Figure 2b) indicated that the maize preSOD-3 was synthesized and effectively imported into the yeast mitochondria. In addition, we found that the maize SOD-3 extracted from both the transformed yeast cells and maize scutella migrated identically on 15% SDS-polyacrylamide gels. These data suggest that the maize preSOD-3 was effectively imported and cleaved by the yeast mitochondrial matrix protease.

MnSOD-deficient yeast cells are rescued by maize SOD-3 under oxidative stress induced by the redox-active herbicide paraquat: The functional role of maize SOD-3 in transformed yeast cells was investigated under oxidative stress caused by paraquat. First, we tested the resistance of the wild-type and the mutant yeast strains (DL-1wt, and *Sod2d*) to paraquat. In dose response experiments, the wild-type yeast was

FIGURE 2.—Complementation of a MnSOD-deficient yeast strain by the expression of maize SOD-3. (a) Growth of the transformed yeast cells in YPE. The MnSOD-deficient yeast strain *Sod2d* (■), wild-type yeast strain DL-1wt (□), and the transformant pYZ0 (○) were grown in YPE at 30° with constant shaking. Growth was monitored by measuring the absorbance at 600 nm. (b) Detection of maize SOD-3 protein in yeast cells by immunoblotting. Protein samples from the transformed yeast cells were subjected to SDS-polyacrylamide gel electrophoresis and subsequently transferred onto nitrocellulose filters which were then incubated with maize SOD-3 monospecific antibody. Lane 1, 100 μ g total yeast protein; lane 2, 30 μ g yeast mitochondria; lane 3, 30 μ g yeast mitochondria treated with proteinase K in the presence of 1% Triton X-100; lanes 4 and 5, 30 μ g yeast mitochondria from yeast strains DL-1wt and *Sod2d* transformed by pDB20 vector DNA alone, respectively; lane 6, positive control with 25 μ g crude protein extracts from maize scutella.

resistant to 0.5 mM paraquat, but the mutant yeast strain *Sod2d* grew very slowly in even 0.1 mM paraquat (data not shown). The paraquat resistance was

FIGURE 3.—Growth of the yeast strains treated with 0.2 mM paraquat. Sensitivity of the transformed yeast strains to paraquat was tested by growing the yeast cells in MM medium containing 0.2 mM paraquat as described in MATERIALS AND METHODS. (a) and (b) Growth of the transformed yeast cells with the plasmids bearing the N-terminal deleted maize *Sod3* cDNAs in liquid culture and plates, respectively. In panel (a), (■), the MnSOD-deficient yeast strain Sod2d, and the transformants of pYZ6, pYZ13, pYZ18, pYZ30 and pYZ37; (○), the transformant of pYZ0; (□), the wild type yeast strain DL-1wt. In panel (b), 1, the wild-type yeast strain DL-1wt; 2, the mutant yeast strain Sod2d; 3–8, the transformants of pYZ0, pYZ6, pYZ13, pYZ18, pYZ30 and pYZ37. (c) and (d) Growth of the transformed yeast cells with plasmids carrying the internal deleted maize *Sod3* cDNAs. In panel (c), the MnSOD-deficient yeast strain Sod2d (■); the wild type yeast strain DL-1wt (□); the transformants of pYB10, pYC38 and pYA8 (○); the transformant of pYB3 (Δ); and the transformant of pYC37 (▲). In panel (d), 1, the yeast strain DL-1wt; 2, the mutant yeast strain Sod2d; 3–7, the transformants of pYB10, pYC38, pYA8, pYB3, and pYC37. In b and d, opaque (white) colony indicates growth; clear (gray) colony indicates no growth.

analyzed by growing the mutant yeast cells transformed with the plasmid pYZ0 bearing maize *Sod3* cDNA (Figure 1a) in MM medium containing 0.2 mM paraquat. The transformants grew as well as wild type yeast strain DL-1wt under the paraquat treatment (Figure 3, a and b). The results indicated that the maize SOD-3 can functionally protect the yeast cells from the toxic effects of the superoxide-generating herbicide paraquat. The paraquat resistant assays were consistent with the results of complementation, which clearly show that the maize SOD-3 can functionally substitute for the yeast MnSOD and protect the yeast cells from oxidative stress.

Complementation of the MnSOD deficiency and

FIGURE 4.—Complementation of the MnSOD-deficient yeast strain by maize SOD-3 deletion mutants. Growth of the strain Sod2d transformed by plasmids containing the N-terminal and internal deleted maize *Sod3* cDNAs. Assays were performed as indicated in panel (a) of Figure 2. (■), the MnSOD-deficient strain Sod2d and the transformants of pYZ6, pYZ13, pYZ18, pYZ30 and pYZ37; (□), the wild type yeast strain DL-1wt; (○), the transformants of pYB10, pYC38 and pYA8; (▲), the transformant of pYB3; and (Δ), the transformant of pYC37.

protection against the lethal effects of paraquat depend on effective import of the maize preSOD-3 into yeast mitochondria:

We have obtained a number of deletion mutants in the transit peptide and amino terminus of the maize mature SOD-3 protein (Figure 1b). These mutant *Sod3* genes were subcloned in the yeast pDB20 vector and transformed into Sod2d yeast (see MATERIALS AND METHODS). Results of complementation and paraquat resistance assays demonstrate that none of the transformants containing N-terminal deleted maize *Sod3* genes were able to restore wild-type growth in MM medium containing 0.2 mM paraquat (Figure 3, a and b) and YPE liquid media (Figure 4). Surprisingly, removal of only 5 amino acids of the transit peptide (pYZ6) was sufficient to abolish complementation and protection. These results suggest that the targeting information for maize preSOD-3 import into the yeast mitochondria is located in the initial portion of the preSOD-3 transit peptide. If this were the case, the internally deleted maize *Sod3* genes encoding SOD-3 proteins containing the first 8-amino acid residues of the transit peptide should be able to complement the MnSOD deficiency in the Sod2d yeast. To test this possibility, mutant yeast were transformed with the plasmids containing the internally deleted *Sod3* genes (Figure 1c). The results of paraquat treatments (Figure 3, c and d) and complementation (Figure 4) show that all of the clones containing the first 8 amino acids of the transit peptide could restore wild-type growth in YPE liquid media and protect the yeast cells from the toxic effects of paraquat. In addition, the transformants bearing pYB10, pYC38, and pYA8 grew as well as the transformant of plasmid pYZ0 which contains the undeleted *Sod3* gene. In contrast, the transformants of plasmid pYB3 and pYC37 had lower complementation and protection efficiencies (up to 75% for pYB3 but only 20% for pYC37). Immunoblot analyses of yeast protein

FIGURE 5.—Immunoblot detection of the mutant maize SOD-3 proteins in the yeast cells. Samples of 100 μ g yeast total protein extracts (a) and 30 μ g yeast mitochondrial protein (b) prepared from strains DL-1wt, Sod2d and the transformed strains were separated on 15% SDS-polyacrylamide gel electrophoresis, and were transferred onto nitrocellulose and immunoblotted with the maize SOD-3 monospecific antibody. Lane 1, plant protein extracts; lane 2, wild-type yeast strain DL-1wt; lane 3, mutant yeast strain Sod2d; lanes 4 to 14, yeasts transformed by plasmids pYB10, pYC38, pYA8, pYB3, pYC37, pYZ0, pYZ6, pYZ13, pYZ18, pYZ30 and pYZ37. Plant protein extract was obtained from maize scutellum.

extracts and isolated yeast mitochondria confirmed that failure of complementation and resistance to paraquat by the N-terminal deletion mutants was due to absence of protein import and not degradation of the proteins. The results demonstrated that all mutant SOD-3 proteins were made in the yeast cells and detected in the total protein extracts (Figure 5a), and only the SOD-3 proteins containing the initial 8 amino acids of the transit peptide (pYB10, pYC38, pYA8, pYB3 and pYC37) were found in the yeast mitochondrial fraction (Figure 5b). The data showed that the unimported maize SOD-3 which was retained in the yeast cytosol did not function to complement the yeast MnSOD-deficiency nor did it render resistance to paraquat. Furthermore, we found that the mutant SOD-3 recovered from the transformed yeast mitochondria migrated to the same position on 15% SDS-polyacrylamide gels as SOD-3 from maize scutella. Such comigration suggests that the mutant maize preSOD-3 proteins were also processed by the yeast mitochondrial matrix protease. The complementation results together with the observations of paraquat resistance are consistent with the role of the N-terminal 8 amino acids for maize preSOD-3 to be imported into precise intracellular location of yeast mitochondria to carry out the biological function.

For the transformant pYC37, the lower efficiency of complementation and paraquat resistance may be due to the unprocessed preSOD-3 inside the yeast mitochondria. The 32 amino acids deleted in this mutant SOD-3 protein included codons 16–31 at the carboxy terminus of the transit peptide and codons 32–47 at the amino terminus of the mature protein; therefore, this mutant SOD-3 did not contain the proteolytically cleavable site. It was expected that if this mutant SOD-3 had been processed, it should have had a smaller molecular weight and greater mobility on 15% SDS-polyacrylamide gels because of the dele-

FIGURE 6.—Maize SOD-3 (MnSOD) enzymatic activity assay. Total protein extracts (25 μ g) from each transformed strain were separated on nondenaturing 7.5% polyacrylamide gels at 4° and stained for MnSOD activity as described in MATERIALS AND METHODS. Position of the endogenous yeast MnSOD (◼) in the wild type yeast strain DL-1wt and of the maize SOD-3 (◁) in plant and the transformed yeast cells were marked. Lane 1, plant protein extracts; lane 2, the wild type yeast strain DL-1wt; lane 3, the mutant yeast strain Sod2d; lanes 4 to 9, the transformants of pYZ0, pYB10, pYC38, pYA8, pYB3 and pYC37.

tion of the 15 amino acids at the amino terminus of the mature SOD-3. This protein, however, exhibited the same migration as all the other correctly processed SOD-3 proteins. It is likely that although this protein was imported into the yeast mitochondria, it was not processed in the mitochondrial matrix. The activity assay was carried out to test whether the lower complementation and protection efficiencies in pYB3 and pYC37 may be caused by different enzymatic activity of the mutant maize SOD-3 proteins in the yeast cells. The results (Figure 6) indicate that the unprocessed SOD-3 in the yeast cells showed lower enzymatic activity than other mutant SOD-3 proteins.

DISCUSSION

We have demonstrated that maize MnSOD (SOD-3) can complement a MnSOD deficiency and rescue the transformed yeast cells from oxidative stress induced by paraquat. In addition, the results indicate that the maize preSOD-3 was efficiently targeted and processed by yeast mitochondria. The active mature form of maize SOD-3 was assembled in the yeast mitochondrial matrix.

Oxidative stress enhances or induces the activity of superoxide dismutase in both prokaryotes and eukaryotes (HASSAN and FRIDOVICH 1977; MATTERS and SCANDALIOS 1986). The herbicide paraquat (methyl viologen) is known to cause increased production of cellular superoxide and to produce oxidative stress (HALLIWELL 1984). The yeast MnSOD-deficient mutant strain (Sod2d) is hypersensitive to oxidative stress due to inactivation of its *MnSod* gene (VAN LOON, PESOLD-HURT and SCHATZ 1986). Therefore, by introducing the maize *Sod3* gene into the Sod2d mutant yeast, the biological function of maize SOD-3 under oxidative stress could be investigated. Strong homologies of sequence and structure between maize and yeast MnSOD have been reported (WHITE and SCANDALIOS 1988). The present findings demonstrate that the biological function of MnSOD is also highly conserved in maize and yeast. The maize SOD-3 can functionally replace the yeast MnSOD and protect the

yeast cells from the lethal effects of reactive oxygen species produced by paraquat. The results also imply that maize MnSOD (SOD-3) may play an important role in protecting transgenic plants (and perhaps other eukaryotes) from environmentally imposed oxidative stress.

Expression of various deletion mutants of the maize *Sod3* gene in the yeast MnSOD-deficient mutant strain *Sod2d*, has demonstrated that the first 8-amino acid residues of the transit peptide of maize preSOD-3 are essential for targeting the maize preSOD-3 into the yeast mitochondria *in vivo*. It has been previously shown that most of the internally deleted maize SOD-3 proteins were differentially imported into isolated maize mitochondria *in vitro*, regardless of the deletion size within the preSOD-3 (WHITE and SCANDALIOS 1989). This can now be explained by the present findings that the *in vitro* import of all internally deleted maize preSOD-3 proteins was due to the presence of the first 8 amino acids, which directed the mutant maize preSOD-3 proteins into the isolated maize mitochondria *in vitro*. This was further supported by the evidence that deletion of the initial amino acids of the preSOD-3 transit peptide by the *ExoIII*/mung bean nuclease system prevented the import of the mutant preSOD-3 proteins into yeast mitochondria *in vivo* (Figure 4). These findings not only confirm that maize MnSOD functions to protect yeast cells from oxidative stress produced by paraquat, but also the functional role depends on targeting the maize preSOD into yeast mitochondria. The results further verified that the maize SOD-3 encoded by our cDNA clone is a *bona fide* mitochondrial-associated isozyme.

The initial portion of the transit peptide from several mitochondrial proteins has been found to direct reporter proteins into the mitochondrial matrix (DOUGLAS, GELLER and EMR 1984; EMR *et al.* 1986; HURT, PESOLD-HURT and SCHATZ 1984; HURT *et al.* 1985). Although it is still unclear which sequence or structural motif is required for the targeting function of the mitochondrial transit peptide, computer analysis of 23 mitochondrial transit peptides has shown that most of them exhibit a potential for forming an amphiphilic structure (VON HEIJNE 1986). The functional significance of the amphiphilicity in mitochondrial transit peptides has been demonstrated (ALLISON and SCHATZ 1986). Therefore, we speculate that the deletion of the initial portion of the maize preSOD-3 transit peptide prohibits mitochondrial import by destroying a secondary structure which may be required for the binding of a cytosolic chaperone, such as a presequence binding factor (PBF) (MARAKAM and MASATAKA 1990). The presequence binding factor or other protein factors may keep the preSOD-3 in an unfolded competent form in order to interact with specific receptor proteins at the mitochondrial surface

for proteins to cross mitochondrial membranes. It is likely that the functional role of the initial portion of the maize preSOD-3 transit peptide may be in the formation of a specific secondary and/or tertiary structure rather than in the primary amino acid sequence *per se*.

The mutant preSOD-3 encoded by the plasmid pYC37 had a low enzymatic activity (Figure 6). Interestingly, this mutant protein was effectively imported into the yeast mitochondria with a rate indistinguishable from that of the wild type preSOD-3 and other mutant preSOD-3 proteins, but it was not processed by the yeast mitochondrial matrix protease (Figure 5, a and b). As a result, the capacity of this mutant preSOD-3 to complement the MnSOD deficiency and resistance to paraquat was lower. These results indicate that the lack of processing observed in the mutant preSOD-3 encoded by the plasmid pYC37 was not due to the loss of the targeting information. It seems more likely that the amino acid sequence and structural motif(s) required for mitochondrial binding, import, and processing reside in distinct locations in the maize preSOD-3, and function independently. The lower enzymatic activity may be caused by the unprocessed mutant protein with an amino-terminal extension of its transit peptide. Sequences around the cleavage sites of mitochondrial import proteins do not show sequence similarity, even though they are likely to be processed by the same mitochondrial protease (HURT and SCHATZ 1987). This implies that a specific secondary structure is needed for processing by the mitochondrial protease. We believe that, besides the absence of a proteolytically cleavable site in the mutant protein, the lack of processing may be due to a deletion in the amino-terminal portion of the mature protein. The deletion mutation may cause conformational changes in maize preSOD-3 and prevent the protease from recognizing the specific secondary structure required for cleavage. This is the case for F_1 -ATPase β -subunit, as the processing of its transit peptide requires not only the sequences located within its transit peptide, but also the sequences within its mature protein (VASSAROTTI, STROUD and DOUGLAS 1987). The mature sequences near the proteolytically cleavable site may be involved in the formation of a secondary structure needed for processing the transit peptide by the mitochondrial matrix protease (CONBOY, FENTON and ROSENBERG 1982; MIURA *et al.* 1982; MOONEY, PILGRIM and YOUNG 1990). Because studies of the structure of MnSOD from *Thermus thermophilus* HB8 at 2.4 Å resolution suggest that the helical conformation of the amino-terminal sequence around the first metal ligand site (conserved histidine-rich sequence 28–36) may be crucial for enzyme catalysis (STALLINGS *et al.* 1985), another possible explanation for the low enzymatic activity of this mutant preSOD-3 may be a result of the deletion of the 16

amino acids located in the mature protein. These 16 amino acids may be necessary to form the local protein domain structures involved in the metal binding, which is required for catalytic function of the enzyme.

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