Manganese Activation of Superoxide Dismutase 2 in the Mitochondria of Saccharomyces cerevisiae*

Edward Luksi, Mei Yang, Laran T. Jensen, Yves Bourbonnais, and Valeria Cizewski Culotta

From the Departments of Environmental Health Sciences and Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland 21205 and Département de Biochimie et Microbiologie and Centre de Recherche sur la Fonction, la Structure et l’Ingénierie des Protéines (CREFSIP), Université Laval, Québec G1K 7P4, Canada

Manganese-dependent superoxide dismutase 2 (SOD2) in the mitochondria plays a key role in protection against oxidative stress. Here we probed the pathway by which SOD2 acquires its manganese catalytic cofactor. We found that a mitochondrial localization is essential. A cytosolic version of Saccharomyces cerevisiae Sod2p is largely apo for manganese and is only efficiently activated when cells accumulate toxic levels of manganese. Furthermore, Candida albicans naturally produces a cytosolic manganese SOD (Ca SOD3), yet when expressed in the cytosol of S. cerevisiae, a large fraction of Ca SOD3 also remained manganese-deficient. The cytosol of S. cerevisiae cannot readily support activation of Mn-SOD molecules. By monitoring the kinetics for metalation of S. cerevisiae Sod2p in vivo, we found that prefolded Sod2p in the mitochondria cannot be activated by manganese. Manganese insertion is only possible with a newly synthesized polypeptide. Furthermore, Sod2p synthesis appears closely coupled to Sod2p import. By reversibly blocking mitochondrial import in vivo, we noted that newly synthesized Sod2p can enter mitochondria but not a Sod2p polypeptide that was allowed to accumulate in the cytosol. We propose a model in which the insertion of manganese into eukaryotic SOD2 molecules is driven by the protein unfolding process associated with mitochondrial import. Superoxide dismutase (SOD) enzymes represent a family of metalloproteins that have evolved to catalytically remove toxic superoxide anions. Most eukaryotes express the Cu-Zn SOD and a Mn-SOD that is expressed in response to oxygen status.

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22715
Manganese Insertion into Mitochondrial SOD2

Experimental Procedures

Yeast Strains and Growth Conditions—The yeast strains used in this study, including the wild-type BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and the isogenic smf2Δ::kanMX4 (1878) and sod2Δ::kanMX4 (6605) mutant variants, were purchased from Research Genetics (Huntsville, AL). Yeast cultures were maintained in enriched yeast-peptoned medium supplemented with 2% glucose (YPD) or 2% raffinose (YPR) or in minimal synthetic medium supplemented with 2% glucose (24).

Plasmids—The pEL111 vector was constructed by subcloning the BamHI-Sall fragment of pEL101 (18) cloning the N terminus of \( S.\ cerevisiae \) SOD2 into the pBS415 vector (25) digested with the same enzymes. To construct the vector pEL1G1 bearing a GAL1-SOD2 fusion, a SacI site (GAGCTC) was first introduced in the SOD2-expressing vector pEL101 (18) replacing the sequence TAAAAG 15 bp upstream of the SOD2 start codon by site-directed mutagenesis. This plasmid was subsequently digested with SacI and XhoI, and the resulting 910-bp fragment containing the SOD2 open reading frame and its transcriptional terminator was subcloned downstream of a GAL1 promoter in the pYES2/CT vector (Invitrogen) using the same restriction sites. Sequence integrity was confirmed by DNA sequencing analysis (Core Facility, Johns Hopkins Medical Institutions). The multicopy expression vector for cytosolic Sod2p (amino acids 27 to the stop codon) pEL124 was created by subcloning the BamHI-Sall fragment of pEL104 (18) into the vector pYES2/CT that contains the same restriction enzyme sites. The resulting plasmid pEL124 containing the SOD2 gene promoter (−558 to −1) and terminator (+703 to +889) but lacks the mitochondrial presequence of the S. cerevisiae SOD3 expression vector pVTSOD3 was described previously (20).

Biochemical Assays—For preparation of cell lysates, \( S.\ cerevisiae \) strains were inoculated in 50 ml of YPD at a starting \( A_{600} \) of 0.05 and allowed to grow without shaking at 30 °C for ~15 h. In general, whole cell lysates were prepared by glass-bead agitation as described previously (18). Where needed, antibodies directed against Mas2p (gift from Dr. Rob Jensen, Johns Hopkins University) and cytosolic Pgk1p (Molecular Probes, Eugene, OR) were used as described (18).

\( S.\ cerevisiae \) Sod2p (containing the N-terminal mitochondrial targeting sequence) and \( S.\ albicans \) SOD3 were purified as recombinant proteins as described previously (19, 20, 28). Molar concentrations of these SOD molecules were determined by amino acid hydrolysis analysis (Protein Chemistry Laboratory, Texas A & M University).

To follow in vivo mitochondrial import of Sod2p, sod2Δ yeast mutant cells were transformed with the GAL1-SOD2 vector pEL1G1 and cultured for 17 h in YPR medium to an \( A_{600} \) of 1.1–1.5. 2% galactose was then added to induce SOD2 expression. Where needed, 20 \( \mu \)M of the proton uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma) was added to block mitochondrial import (29). Addition of 0.05% (v/v) \( \beta \)-mercaptoethanol (\( \beta \)-ME) served to neutralize CCCP as described previously (29). Cell lysates were prepared by spheroplast homogenization, and mitochondrial and post-mitochondrial supernatant fractions were prepared as described above.

Results

Efficient Metalation of Sod2p Requires Mitochondrial Localization—We tested whether a mitochondrial localization of Sod2p was needed for manganese insertion into the enzyme. A cytosolic version of \( S.\ cerevisiae \) Sod2p was created by removing the \( N \)-terminal mitochondrial presequence (Fig. 1A) (30). The resulting Sod2p molecule (CytSod2p) was expressed in a sod2Δ mutant of \( S.\ cerevisiae \) lacking the endogenous mitochondrial Sod2p. As seen in Fig. 1B, CytSod2p co-localizes with the cytosolic marker Pgk1p and is largely excluded from the mitochondria marked by the mitochondrial matrix protein Mas2p. By comparison, expression of native Sod2p harboring the \( N \)-terminal presequence (MitoSod2p) resulted in a mitochondrial localization of the enzyme as expected (Fig. 1B).

To test for enzymatic activity, lysates from cells expressing CytSod2p or MitoSod2p were applied to a native gel and analyzed for SOD activity by nitroblue tetrazolium staining. As seen in Fig. 1C, the cytosolic Sod2p was largely inactive compared with the mitochondrial enzyme (compare lanes 1 and 6). The activity of CytSod2p was restored by manganese supplementation in vivo, indicating that the lack of CytSod2p activity under physiological conditions results from a manganese deficiency in the enzyme. It is noteworthy that the amount of manganese required to activate CytSod2p is quite high (~100 \( \mu \)M). This is a concentration that is somewhat toxic to the yeast.
FIG. 2. SOD3 from C. albicans is only partially active when expressed in the cytosol of S. cerevisiae (A). Wild-type strain BY4741 and the isogenic smf2Δ and sod2Δ mutants, transformed where indicated (SOD3, +) with pVT-SOD3 (20)-expressing C. albicans (Ca) SOD3, were grown in YPD medium that was supplemented where indicated (Mn²⁺, +) with 100 μM MnSO₄. Total yeast cell lysates were analyzed for SOD activity by the native gel assay as described in the legend to Fig. 1C. The positions of C. albicans SOD3 and the endogenous Sod2p and Sod1p enzymes from S. cerevisiae are indicated. B, the specified amounts of whole cell lysate protein from either the sod2Δ cell-expressing C. albicans SOD3 (left) or from wild-type BY4741 expressing endogenous Sod2p (right) were subjected to immunoblot analysis and compared with known amounts of the corresponding recombinant Mn-SOD molecule, which was purified to homogeneity as described previously (19, 20). The purified recombinant Ca SOD3 and S. cerevisiae (Sc) Sod2p contain an N-terminal His₆ tag (20) and mitochondrial targeting sequence (18), respectively, that account for the slightly higher molecular weights on the immunoblot.

as indicated by slowed growth (19) and (not shown). The cytosolic form of Sod2p is only active when cells hyperaccumulate manganese. Under physiological conditions, Sod2p needs to be inside the mitochondria to be efficiently activated.

Cytosolic SOD3 from C. albicans Is Largely Active When Expressed in S. cerevisiae—The pathogenic fungi C. albicans expresses a manganese-containing SOD in the cytosol (Ca SOD3) that is reported to be active when expressed in the cytosol of S. cerevisiae (20). We therefore addressed whether Ca SOD3 has a unique ability to acquire manganese in the cytosol.

Consistent with earlier studies (19, 20), Ca SOD3 expressed in S. cerevisiae exhibits some activity under physiological conditions (Fig. 2A, lanes 2 and 8). Expression was observed in both a sod2Δ strain (lane 8) and a strain expressing the endogenous mitochondrial Sod2p of S. cerevisiae (lane 2). Activity of Ca SOD3 expressed in S. cerevisiae is limited by manganese bioavailability. Decreasing intracellular manganese through a deletion of the Smf2p manganese transporter (18) abolished Ca SOD3 activity (Fig. 2A, lane 3), and activity was rescued by growing cells in the presence of 100 μM manganese (lane 6). In fact, such manganese supplementation also had a dramatic effect on Ca SOD3 activity in SMP2 wild-type cells (lanes 3 and 9). Hence, there appears to be a large inactive pool of Ca SOD3 that is manganese-deficient and can be activated at high intracellular manganese, reminiscent of the scenario seen with S. cerevisiae cytSod2p (Fig. 1C).

The expression of Ca SOD3 in S. cerevisiae is driven by a high copy vector and the strong constitutive ADH1 promoter (20). To estimate how much Ca SOD3 is being produced relative to endogenous Sc Sod2p, purified Sc Sod2p and Ca SOD3 proteins of known concentrations were used as standards in a semiquantitative immunoblot against lysates from cells expressing Sc Sod2p and Ca SOD3. As seen in Fig. 2B, Ca SOD3 is expressed in S. cerevisiae on a per mole basis at levels that are roughly 10-fold higher than the endogenous Sc Sod2p. This overexpression of Ca SOD3 protein may explain why activity can be detected in S. cerevisiae, despite the fact that a large fraction of the protein lacks manganese. Overall, the findings obtained with Ca SOD3 and with cytosolic Sod2p demonstrated that, in S. cerevisiae, efficient activation of Sod2p requires a mitochondrial localization. There is clearly a component absent from the cytosol that is required for efficient activation of Sod2p.

Insertion of Manganese into Sod2p Requires New Protein Synthesis—How is mitochondrial Sod2p activated with manganese? We know that, in the case of copper-containing Sod1p, a pre-existing apopool of the enzyme is rapidly activated with copper in the absence of new protein synthesis (13, 14). We tested whether the same was true for manganese-containing Sod2p of the mitochondria.

To monitor activation of a pool of Sod2p that is largely apo for manganese, we utilized the manganese-deficient smf2Δ mutant. In these cells, the Sod2p polypeptide still accumulates in the mitochondria, but is largely inactive because of low mitochondrial manganese (18). Sod2p activity is fully restored in this mutant by culturing cells in the presence of 10 μM manganese (Fig. 3, A and C, lanes 3). We monitored the time required to activate Sod2p following the addition of manganese to the growth medium. As shown in Fig. 3, A and C, Sod2p was activated very slowly by manganese and required at least 2–3 h of treatment with the metal. By comparison, activation of cytosolic Sod1p by copper in S. cerevisiae cells is complete in <5 min (13). The slow activation of mitochondrial Sod2p is not a result of slow trafficking of the metal to the mitochondria, as mitochondrial manganese was restored to near wild-type levels after 15 min of treatment with manganese (Fig. 3B). Such a delay in metalation of the enzyme suggests that new protein synthesis may be required.

To address the requirement for protein synthesis, the time course for Sod2p activation was monitored under conditions in which in vivo protein translation was blocked by cycloheximide. Fig. 3C shows that cycloheximide treatment (lanes 5, 7, and 9)
Mitochondrial Import and Synthesis of Sod2p Appear Closely Coupled—Synthesis of the Sod2p polypeptide occurs outside the mitochondria, whereas maturation of Sod2p takes place within mitochondria. Based on these distinct cellular locations, why would polypeptide synthesis be required for manganese insertion? As a likely explanation, the synthesis of Sod2p may be closely coupled with mitochondrial import, and it is the import process that facilitates manganese insertion. In fact, it has been suggested that certain mitochondrial proteins are imported co-translationally (31–34), because folding of the polypeptide in the cytosol would prohibit mitochondrial uptake. We tested whether this was the case for Sod2p.

An experiment was designed in which Sod2p synthesis was controlled via the S. cerevisiae GAL1 promoter. A sod2Δ deletion strain was transformed with an inducible Sod2p expression vector (Gal-Sod2p), and following 15–30 min of treatment with galactose, newly synthesized Sod2p became apparent (Fig. 4A). All of the newly synthesized Sod2p migrated as a single species (Fig. 4A), representing the mitochondrial processed (“P”) form. Unprocessed, precursor Sod2p (“U”) is completely absent in the induced samples, suggesting that the mitochondrial import of Sod2p occurs immediately following, or concomitant with, Sod2p synthesis.

To address this further, we uncoupled mitochondrial import and protein synthesis. This was achieved through use of the proton ionophore CCCP, which blocks import by disrupting the mitochondrial membrane potential (29). Fig. 4B, lanes 4–6, shows that CCCP completely blocked import of Sod2p into the mitochondria. As a result, the unprocessed Sod2p precursor (U) accumulated in the cytosolic fraction (Fig. 4B, lane 5). The effect of CCCP can be neutralized by β-ME (29), and when β-ME is added shortly following CCCP, there is no inhibition of import, and newly synthesized Sod2p was taken into mitochondria and processed (P) (Fig. 4B, lanes 7–9).

Using this system, we tested whether pre-existing cytosolic Sod2p can be chased into mitochondria. In the experiment of Fig. 4C, Sod2p synthesis was induced for 3 h in the presence of CCCP to allow accumulation of unprocessed cytosolic Sod2p (U) (lane 1). Where indicated, β-ME was then added for an additional 45 min. β-ME clearly reversed the effects of CCCP during this time frame, because the shorter form of Sod2p representing processed mitochondrial Sod2p (P) became apparent (Fig. 4C, lane 3). However, the unprocessed (U) Sod2p that accumulated prior to β-ME treatment was unchanged when mitochondrial import function was restored with β-ME (compare U in Fig. 4C, lanes 1 and 3). Furthermore, the appearance of processed mitochondrial Sod2p required new protein synthesis, as cycloheximide specifically prevented formation of processed (P) Sod2p in β-ME treated cells (lane 4). Together these findings are consistent with the notion that mitochondrial import requires freshly translated Sod2p. Overall, the synthesis, mitochondrial import, and manganese insertion steps for Sod2p are closely coordinated in time.

**DISCUSSION**

The mitochondrial SOD2 enzyme is well known for its role in eukaryote survival and fitness (35–42). Yet despite this widespread importance, virtually nothing is known about the maturation of the SOD2 polypeptide in vivo. How is the inactive protein encoded by the nucleus converted into an active manganese-containing enzyme in the mitochondrial matrix? We have shown here that activation of S. cerevisiae Sod2p through insertion of the manganese cofactor must occur within the mitochondria. When expressed in the cytosol of S. cerevisiae, Mn-SOD molecules are poorly activated. Efficient manganese activation also requires new protein synthesis and mitochondrial import. Our data are consistent with a model in which the translation, mitochondrial import, and manganese activation of Sod2p are closely coupled in time.

Although Sod2p is largely inactive when expressed in the cytosol of S. cerevisiae, activity could be restored by exposing cells to high toxic concentrations of manganese. Under normal physiological conditions, the bioavailability of manganese in the cytoplasm appears too low to activate newly synthesized Sod2p. This may be a universal phenomenon, because most eukaryotes do not express a cytosolic Mn-SOD. However, there are rare exceptions, as in the case of the cytosolic Mn-SOD of C. albicans (20) and of decapped crustaceans (21). Our studies here show that C. albicans SOD3 does not possess an inherent ability to acquire cytosolic manganese, as a large fraction of the protein remained inactive when expressed in the cytoplasm of S. cerevisiae. Instead, C. albicans, as well as the crustaceans, may have evolved novel methods for delivering manganese to Mn-SOD in the cytosol, e.g., mechanisms that involve a manganese chaperone or elevated bioavailability of the metal.

Our studies show that import of S. cerevisiae Sod2p into mitochondria requires a freshly synthesized Sod2p polypeptide. If allowed to accumulate and fold in the cytosol, Sod2p is refractory to mitochondrial uptake. When folded, Sod2p is a notoriously stable molecule. Human SOD2 is stable at 60 °C (43), and the S. cerevisiae enzyme can be purified following treatment at 70 °C with little loss in activity (44, 45). As such, it is not surprising that import of SOD2 into mitochondria must occur before the protein has a chance to fold in the cytosol. In this regard, it is noteworthy that the mRNA for SOD2 in mammalian cells and the mRNA/ribosomes for Sod2p in S. cerevisiae are both found associated with the outer membrane of mitochondria (46, 47). With other mitochondrial proteins, the 3'-
Overall, our studies have provided a more detailed mechanistic picture for the post-translational activation of Sod2 with manganese. As shown in our model of Fig. 5, the ribosomes for S. cerevisiae Sod2p synthesis are juxtaposed to the outer mitochondrial membrane (46, 47). This allows for the coupling of Sod2p synthesis and mitochondrial import. As the polypeptide emerges from the inner membrane, manganese ions are inserted through a process that is facilitated by Mtm1p in the mitochondrial matrix and perhaps other accessory proteins as well. Last of all, the manganese-containing protein is folded in the mitochondrial matrix in a stable quaternary tetramer. With the clear importance of SOD2 in eukaryotic survival and fitness (35–42), these ordered steps must be carefully controlled.

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Manganese Insertion into Mitochondrial SOD2

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