Escherichia coli iron superoxide dismutase targeted to the mitochondria of yeast cells protects the cells against oxidative stress

(recombinant plasmid/precursor protein/oxygen toxicity/evolution)

RENA BALZAN*†‡, WILLIAM H. BANNISTER†, GARY J. HUNTER*†, AND JOSEPH V. BANNISTER†

* Cranfield University, Cranfield, Bedford MK43 0AL, England; and † Department of Physiology and Biochemistry, University of Malta, Msida MSD 06, Malta

Communicated by Irwin Fridovich, Duke University Medical Center, Durham, NC, January 23, 1995 (received for review July 20, 1994)

ABSTRACT A gene encoding a fusion protein consisting of Escherichia coli iron superoxide dismutase (FeSOD) with the mitochondrial targeting presequence of yeast manganese superoxide dismutase (MnSOD) was cloned and expressed in E. coli and in Saccharomyces cerevisiae DL1Mn− yeast cells deficient in MnSOD. In the yeast cells the fusion protein was imported into the mitochondrial matrix. However, the presequence was not cleaved. In a control set of experiments, the E. coli FeSOD gene without the yeast MnSOD leader sequence was also cloned and expressed in S. cerevisiae DL1Mn− cells. In this case the FeSOD was located in the cytosol and was not imported into the mitochondrial matrix. E. coli FeSOD, with and without the yeast MnSOD presequence, proved to be active in yeast, but, whereas the FeSOD targeted to the mitochondria of yeast cells deficient in MnSOD protected the cells from the toxic effects of oxidative stress, FeSOD without the yeast MnSOD presequence did not protect the yeast cells deficient in MnSOD against oxidative stress.

Apart from its presence in plant chloroplasts (1-4) and in some protozoa (5, 6), iron superoxide dismutase (FeSOD) is not known to occur in eukaryotic cells (7). The SODs of eukaryotic cells are copper/zinc SOD (Cu/ZnSOD) and manganese SOD (MnSOD). The latter is found in the matrix of mitochondria (8). The gene was probably acquired when eukaryotic cells obtained mitochondria as an endosymbiotic event (9). FeSOD is present in primitive protozoa lacking mitochondria, such as Entamoeba histolytica (10). It appears to have been lost in higher protists after the advent of mitochondria. There is no clear evolutionary reason for this. The present work was undertaken to investigate whether yeast mitochondria depleted of MnSOD were able to support the import and assembly of functionally active FeSOD in vivo.

A yeast mutant lacking MnSOD was found to be hypersensitive to oxygen, and increasing concentrations of oxygen led to a progressive inhibition of growth (11). In this report we show that Escherichia coli FeSOD with a yeast MnSOD presequence, on being imported into the mitochondria of yeast cells deficient in MnSOD, is able to replace the function of the eukaryotic manganese protein in protecting the cells against physiological and induced oxidative stress. The FeSOD protein without the yeast MnSOD presequence was located in its active form in the cytosol and was not able to replace MnSOD in its activity against oxidative stress.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions. The E. coli host strain for the cloning procedures was JM105 (supE endA sbcB15 hsdR4 rpsL thi Δ(lac-proAB) F' [traD36 proAB* lacF16 lacZΔM15]) obtained from Pharmacia. The media used were (i) M9 minimal medium (glucose, 0.4%; Na2HPO4·7H2O, 1.28%; KH2PO4, 0.3%; NaCl, 0.05%; NH4Cl, 0.1%) containing thiamine hydrochloride (5 μg/ml) and (ii) 2TY medium (Bacto-tryptone, 1.6%; Bacto yeast extract, 1%; NaCl, 0.5%). For plates 1.5% agar was used. Cells were grown aerobically at 37°C on a rotary shaker (New Brunswick Scientific) at 200 rpm.

Yeast Strain and Culture Conditions. The parental strain used in this study was Saccharomyces cerevisiae DL1Mn− (α, leu2-3,112 his3-11,15 ura3-251,372,328 sod2::LEU2) (11). Both DL1Mn− and DL1 (α, leu2-3,112 his3-11,15 ura3-251,372,328) were supplied by S. Oliver of Manchester University. The culture media used were (i) YEPD (Bacto yeast extract, 1%; Bacto-yeastpeptone, 2%; glucose, 2%); (ii) minimal medium (Bacto yeast nitrogen base without amino acids, 0.5%; glucose, 2%; L-lactate, 2%; with histidine, tryptophan, methionine, adenine, lysine, uracil, and leucine, each at 20 μg/ml, as required; pH was adjusted to 5.5 with KOH), and (iii) YEPD medium (Bacto yeast extract, 1%; Bacto-yeastpeptone, 2%; ethanol, 3%). S. cerevisiae stocks were maintained at 25°C on minimal medium plates (minimal medium in 2% agar). Aerobic growth in liquid cultures was maintained at 25°C, with constant shaking (250 rpm) in an orbital incubator (Gallenkamp International, England).

Plasmid Vectors. The E. coli FeSOD gene (12) was isolated from the plasmid pH51-8 (a gift from D. Touati, Institut Jacques Monod, Paris) as described in Fig. 1A. The cloning of the gene in yeast, as explained below, was done by means of the plasmid vector YepPGK, which was supplied by S. Oliver, Manchester University. All ligation experiments were carried out as specified by Sambrook et al. (13).

Synthesis of the Yeast MnSOD Gene Leader Sequence. The yeast MnSOD gene (SOD2) leader sequence (14) was constructed from oligonucleotides synthesized by means of an Applied Biosystems 391 DNA synthesizer (PCR-Mate). The same structure as for the S. cerevisiae MnSOD mitochondrial targeting signal peptide (14) was maintained except for glycine in position 15, which was replaced by alanine (Fig. 1B). This produced a small increase in the calculated hydrophobic moment for residues 7-24, the segment of maximum hydrophobicity in the yeast MnSOD presequence.

Subcloning of the FeSOD Gene with the Yeast SOD2 Leader Sequence in E. coli and Yeast. The FeSOD gene with and without the yeast SOD2 leader sequence, in the recombinant plasmids pKL-SOD and pKK-SOD, respectively (Fig. 1A), was subcloned in E. coli JM105 according to Sambrook et al. (13). The FeSOD gene with the yeast SOD2 leader sequence was then isolated from pKL-SOD by digestion of the recombinant plasmid with restriction enzymes.

Abbreviation: SOD, superoxide dismutase.
‡ To whom reprint requests should be addressed at: Department of Physiology and Biochemistry, University of Malta, Msida MSD 06, Malta.
Transformation of *S. cerevisiae* DL1Mn− cells (carrying an inactivated MnSOD gene) (11) by the recombinant plasmids YEp/PGK-S (to give rise to the strain DL1Mn−Fe3+-P) and YEp/PGK-F (to give rise to the strain DL1Mn−Fe4+), was carried out by the lithium acetate method (15). The protocols used for the isolation of the membrane-free cytosolic fraction, mitochondria, intermembrane space fraction, and mitochondrial matrix were as described (16).

**Determination of Protein Expression and Activity.** Protein expression studies were carried out by SDS/polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue (17) and silver staining with the Bio-Rad silver stain kit. SOD activity was determined according to McCord and Fridovich (18) or Marklund and Marklund (19). SOD activity staining was performed according to Beauchamp and Fridovich (20).

**Response to Oxidative Stress.** In the study of the response of the four *S. cerevisiae* strains (DL1, DL1Mn−, DL1Mn−Fe3+-P, and DL1Mn−Fe4+) to induced oxidative stress, a range of parafur (Sigma) concentrations (0.02–8 mM) in both YEPD and minimal medium was used. The cell growth was followed by measuring the optical density (OD) at 600 nm in a Perkin–Elmer Lambda 17 spectrophotometer after appropriate dilution of the cell cultures. Cell counts were carried out on an “improved” Neubauer hemacytometer (Hawksley, London) after dilution of the cells with 5 mM galactose to produce a uniformly dispersed suspension.

**Miscellaneous.** DNA sequencing was carried out using the dideoxy method (21) using Sequenase enzyme (United States Biochemical). Published procedures were used in determining protein concentration (22, 23). Electrophoresis of proteins from SDS/polyacrylamide gels onto nitrocellulose membranes (Hybond-C extra; Amersham) was carried out with a Pharmacia LKB 2117-250 Novablot electrophoresis transfer kit. For subsequent immunoscreening of the proteins the Amersham SuperScreen system was used.

**RESULTS**

**Expression of Cloned FeSOD in *E. coli.* There was efficient expression of the cloned FeSOD gene with and without the yeast SOD2 leader sequence in *E. coli* JM105 cells, after induction with isopropyl β-D-thioglactopyranoside. However, whereas the specific activity of SOD (18) in *E. coli* cells carrying pKK-SOD was 160 ± 12 units/mg of protein (estimate ± SE)—i.e., about 5 times that of the SOD in *E. coli* cells carrying the plasmid vector pKK223-3 (31 ± 5 units/mg of protein)—the specific activity of SOD in *E. coli* cells carrying pKL-SOD (where the FeSOD gene has the SOD2 leader sequence) was 56 ± 4 units/mg of protein.

**E. coli FeSOD with Yeast MnSOD Presequence Is Targeted to Yeast Mitochondria in Vivo.** The prokaryotic FeSOD gene (with the SOD2 leader sequence in *S. cerevisiae* DL1Mn−Fe4+ cells) and without the leader sequence in DL1Mn−Fe3+ cells) was expressed in both strains (Fig. 2) and the fusion protein was efficiently targeted to yeast mitochondria in vivo (Fig. 3). The presence of the MnSOD presequence at the N terminus of FeSOD was found to be essential for import of the protein into mitochondria. Protein expression studies, together with electrophoretic and subsequent immunoscreening by means of *E. coli* FeSOD antibody, revealed the presence of FeSOD with the MnSOD presequence in the isolated mitochondrial matrix of *S. cerevisiae* cells (Fig. 3, lane 4). Without the presequence only a negligible amount of authentic *E. coli* FeSOD was observed in the matrix (Fig. 3, lane 6). Also, from a series of immunoscreening experiments with yeast antibodies for hexokinase, cytochrome *b*₂, cytochrome *c* oxidase subunit IV, and 29-kDa porin (all yeast antibodies were supplied by G. Schatz of the University of Basel), the mitochondrial matrix of the *S. cerevisiae* cells...
Biochemistry: Balzan et al.

FIG. 2. *E. coli* FeSOD gene with and without the yeast SOD2 leader sequence is expressed in *S. cerevisiae* cells deficient in MnSOD. Samples (63 μg) of total protein from each of the cytosolic fractions of *S. cerevisiae* DL1Mn−Fe+−P cells (containing *E. coli* FeSOD with yeast MnSOD presenence) (lane 4), and DL1Mn−Fe+−P cells (containing *E. coli* FeSOD) (lane 5) were loaded on an SDS/polyacrylamide [5% (wt/vol) stacking, 12% (resolving)] gel. After electrophoresis, the gel was stained with Coomassie brilliant blue. Lane 1, protein size calibration markers; lane 2, 10 μg of *E. coli* FeSOD (Sigma). The difference in the mobility of the two FeSOD bands in lanes 4 and 5 is due to the presence of the MnSOD presenence at the N terminus of FeSOD in *S. cerevisiae* DL1Mn−Fe+−P cells (lane 4).

(Confirmed as matrix by immunoscreening with anti-Hsp60) was found not to be contaminated by the cytosol, intermembrane space fraction, or mitochondrial membranes (results not shown). The import of the prokaryotic protein into the mitochondria of yeast cells was apparently not accompanied by the proteolytic removal of the eukaryotic presenence as judged by electrophoretic behavior (Figs. 3–5). However, the presence of the presenence at the N terminus of FeSOD did not inhibit the enzymatic activity of the protein (Figs. 4 and 5). SOD activity measurements (19) showed comparable activity in cytosol from DL1Mn−Fe+−P and DL1Mn−Fe+−P cells [8.9 ± 0.3 units/mg of protein (estimate ± SE), respectively]. DL1Mn−Fe+−P cells contained SOD activity in mitochondrial matrix, whereas DL1Mn−Fe+−P cells contained appreciable activity (25 ± 0.9 units/mg of protein) attributable to imported FeSOD.

Cloned Mitochondrial But Not Cytosolic FeSOD in *S. cerevisiae* Protects the Cells from Paraquat Toxicity. Yeast cells deficient in mitochondrial MnSOD activity are sensitive to elevated concentrations of oxygen (11, 24). In this work, increasing concentrations of paraquat in rich YEPD medium and in minimal medium produced different effects on four *S. cerevisiae* strains DL1, DL1Mn−, DL1Mn−Fe+−P, and DL1Mn−Fe−. At a concentration of 4 mM paraquat in YEPD medium (Fig. 6D), the DL1Mn−Fe+−P cells (with cytosolic FeSOD), like the DL1Mn− cells deficient in MnSOD, showed practically no growth, whereas the DL1Mn−Fe−P cells (containing FeSOD in the mitochondria), although showing a moderate lag in the growth curve, eventually grew to the same level as the parent strain DL1. At 1 mM and 2 mM paraquat, the DL1Mn−Fe+−P cells showed a better growth rate than the cells of the parent strain DL1, which has MnSOD (Fig. 6B and C). This increase in the growth rate of the DL1Mn−Fe+−P

Biochemistry: Balzan et al.

FIG. 3. Immunoscreening for *E. coli* FeSOD in *S. cerevisiae* DL1Mn−Fe+−P cells (containing *E. coli* FeSOD fused to the yeast MnSOD presenence) and in DL1Mn−Fe+−P cells (containing *E. coli* FeSOD). Electrophorizing onto a nitrocellulose membrane followed SDS/polyacrylamide (5% stacking, 12% resolving) gel electrophoresis. Staining of the protein markers (lane 1), after transfer from the gel on to the nitrocellulose membrane, was carried out with 0.2% (wt/vol) Ponceau S in 1% acetic acid for 1 min, followed by destaining with deionized water. Lane 2, 7 μg of *E. coli* FeSOD (Sigma); lanes 3 and 5, 13.5 μg of total protein from the membrane-free cytosolic fraction of DL1Mn−Fe+−P cells and DL1Mn−Fe+−P cells, respectively; lanes 4 and 6, 13.5 μg of total protein from the mitochondrial matrix of DL1Mn−Fe+−P cells and DL1Mn−Fe+−P cells, respectively.

Biochemistry: Balzan et al.

FIG. 4. Activity stain of *E. coli* FeSOD (with yeast MnSOD presequence) in the cytosolic fraction of *S. cerevisiae* DL1Mn−Fe+−P cells. Equal amounts (~60 μg) of total protein from each of the membrane-free cytosolic fractions from DL1Mn−P cells (lanes 4 and 9) and DL1Mn−Fe+−P cells (lanes 5 and 10) were loaded in their respective lanes on two nondenaturing 10% polyacrylamide native gels (A and B). Lanes 1 and 6, 10 μg of *E. coli* MnSOD (Sigma); lanes 2 and 7, 3 μg of human Cu/ZnSOD (Sigma); lanes 3 and 8, 10 μg of *E. coli* FeSOD (Sigma). Gel in B was treated with 10 mM KCN to inactivate Cu/ZnSOD prior to the activity staining.

Biochemistry: Balzan et al.

FIG. 5. Uncleaved presenence does not inhibit FeSOD from being assembled into its active form after mitochondrial import. Samples (10 μg) of total protein from each of the membrane-free cytosolic fraction (lanes 3 and 7) and mitochondrial matrix (lanes 4 and 8) of *S. cerevisiae* DL1Mn−Fe+−P cells (containing *E. coli* FeSOD with the yeast MnSOD presenence) were loaded in their respective lanes on two nondenaturing 10% polyacrylamide native gels (A and B). Lanes 1 and 2, 5 μg of human Cu/ZnSOD (Sigma); lanes 1 and 6, 10 μg of *E. coli* FeSOD (Sigma) as markers. Prior to the activity staining, the gel in B was treated with 10 mM KCN to inactivate Cu/ZnSOD, which is present in the cytosolic fraction but not in the mitochondrial matrix of yeast cells.


**DISCUSSION**

In this work, a prokaryotic (E. coli) FeSOD gene (with and without the yeast MnSOD gene leader sequence) was expressed in both E. coli and S. cerevisiae and the fusion protein (FeSOD plus yeast MnSOD presequence) was efficiently targeted to yeast mitochondria in vivo. The prokaryotic FeSOD proved to be active in eukaryotic cytosol. Also, with a mitochondrial targeting signal at its N terminus, FeSOD retains its activity in the mitochondria of yeast cells. In fact, the E. coli FeSOD targeted to the mitochondria of yeast cells was found to protect S. cerevisiae cells deficient in MnSOD against both physiological and induced oxidative stress, whereas the FeSOD without the MnSOD presequence, which was located in the cytosol, although active, did not protect the yeast cells against either form of oxidative stress.

The observation that FeSOD retains its activity even though the MnSOD presequence is not cleaved in the mitochondrial matrix conforms with the previous observations of Endo and Schatz (26) that an artificial mitochondrial precursor protein folds as tightly as, and refolds with similar kinetics to, the corresponding presequence-free protein. In the targeting of E. coli FeSOD to the mitochondria of yeast cells, the efficiency of the presequence to effect import of the corresponding fusion protein to the mitochondrial matrix did not seem to have been adversely influenced by the passenger protein. However, the prokaryotic passenger protein may have lacked the protease recognition sequence, or it could have exerted an influence on the structural integrity of the presequence, thereby inhibiting proteolytic maturation.

From this study, it has been observed that eukaryotic (yeast) mitochondria, depleted of MnSOD, can support the import and assembly of enzymatically active prokaryotic FeSOD in vivo. In a previous study, MnSOD from Bacillus stearothermophilus was reported to be functional in yeast cytosol and to provide protection against concentrations of paraquat and hydrogen peroxide which are normally toxic to S. cerevisiae cells lacking Cu/ZnSOD activity (27). Also Cu/ZnSOD has been found to function in E. coli almost as efficiently as the endogenous MnSOD and FeSOD normally present (28). Thus, despite the structural differences, the functional capacities of the three enzymes are equivalent. The homologies in the mitochondrial MnSOD and prokaryotic MnSOD (and FeSOD) sequences have been taken as evidence for the endo-

---

**FIG. 6.** E. coli FeSOD targeted to the mitochondria of yeast cells deficient in MnSOD protects the cells against paraquat toxicity. The S. cerevisiae DL1 (wild type, A), DL1Mn− (MnSOD-deficient mutant, ○), DL1Mn−Fe+−P (with cloned FeSOD fused to the yeast MnSOD presequence, △), and DL1Mn−Fe+ (containing FeSOD without a mitochondrial targeting signal, ○) were grown in liquid minimal medium. Logarithmically growing cells were then inoculated into fresh aliquots of YEPD medium containing 0 mM (A), 1 mM (B), 2 mM (C), or 4 mM (D) paraquat and grown at 25°C in air.

---

**FIG. 7.** S. cerevisiae strains deficient in MnSOD (DL1Mn−) or in cloned mitochondrial FeSOD (DL1Mn−Fe+−P) are sensitive to air when grown on a nonfermentable carbon source. S. cerevisiae DL1 (wild type, A), DL1Mn− (MnSOD-deficient mutant, ○), DL1Mn−Fe+−P (with cloned FeSOD fused to the yeast MnSOD presequence, △), and DL1Mn−Fe+ (containing FeSOD without a mitochondrial targeting signal, ○) were grown in liquid YPE medium. Logarithmically growing cells were inoculated into fresh aliquots of the same medium and grown at 25°C in air.
symbiotic hypothesis of mitochondrial origin (9). The mitochondrial MnSOD, imported from the cytosol, has a mitochondrial targeting signal that locates it efficiently in the mitochondria. In this work, bacterial FeSOD substituted for the eukaryotic MnSOD in its protective activity only as long as it was targeted to the mitochondria of the yeast cells. Without the signal peptide, FeSOD was not imported into the mitochondria and did not protect the cells from the toxic effects of physiological and induced oxidative stress. If, in the course of evolution, the FeSOD gene had ever been transferred from the mitochondrial genome of prokaryotic origin to the genome of the eukaryotic host, any cytosolic FeSOD would probably not have gained access to the mitochondrial matrix without a mitochondrial targeting signal. The question arises whether the facility of mitochondrial import of MnSOD could have enhanced the selection pressure in favor of MnSOD with the resultant elimination of FeSOD synthesis in eukaryotes. However, more inherent (e.g., physiological) differences between the two enzymes (29) could also have contributed to this selection pressure in favor of the eukaryotic MnSOD.

We thank Professor D. Touati for providing us with the plasmid pH51-8 and the antibody for E. coli FeSOD. We thank Professor S. Oliver for supplying us with the S. cerevisiae DL1 and DL1Mn⁺ strains and the plasmid vector YEp/PKG. We thank Professor G. Schatz for supplying us with antibodies for yeast mitochondrial marker proteins and hexokinase.