Characterization of Recombinant *Saccharomyces cerevisiae* Manganese-Containing Superoxide Dismutase and Its H30A and K170R Mutants Expressed in *Escherichia coli*†

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ABSTRACT: All known Mn-containing superoxide dismutases (MnSODs) have a highly conserved histidine (His-30 in *Escherichia coli* FeSOD) in the active-site channel, and nearly all have an active-site arginine (Arg-170) that has been proposed to play a combined structural and functional role [Chan et al., *Arch. Biochem. Biophys. 279*, 195−201 (1990)]. In *Saccharomyces cerevisiae* MnSOD, the active-site arginine is replaced by a lysine. The *S. cerevisiae* MnSOD gene has been cloned and expressed in *E. coli*, and H30A and K170R site-specific mutants have been prepared. The purified recombinant native (RN) and mutant enzymes were compared to one another and to the native enzyme purified from *S. cerevisiae* (SC) in terms of activity, temperature stability, and sensitivity to 2,4,6-trinitrobenzenesulfonate (TNBS) and phenylglyoxal (PG). All enzymes had high specific activities (SC = 5000, RN = 5600, H30A = 4500, K170R = 4600) (U/mg, using the pyrogallol assay). SC, RN, and H30A were very stable at 75 °C (pH 8.0), with half-lives of 4.7, 2.8, and 2.7 h, respectively, while K170R had a much greater temperature lability, with a half-life of 0.36 h under these conditions. TNBS (0.5 mM, pH 9.0, 25 °C) rapidly inactivated SC, RN, and H30A, with half-lives of 3.5, 5.1, and 5.5 min, respectively, but only slowly inactivated K170R, with a half-life of 101 min. PG (20 mM, pH 9.0, 25 °C) caused very slow inactivation of SC, RN, and H30A by biphasic kinetics, and each enzyme retained ≥25% activity after 3 h of modification. K170R, on the other hand, was completely inactivated by PG under these conditions by first-order kinetics, with a half-life of 7.0 min. The data suggest that His-30, a residue highly conserved in the active-site channel of MnSODs and FeSODs, does not play a crucial role in catalysis or stability. In addition, Lys-170, a residue that is almost always arginine in the numerous other MnSODs and FeSODs sequenced to date, can be replaced by arginine with no loss of catalytic activity, but K170R is less stable and Arg-170 in this mutant is more exposed than the corresponding arginine in other SODs. RN and SC showed some surprising differences. Thus, while the specific activities of RN and SC are very similar, SC is more stable to inactivation at 75 °C, and less susceptible to inactivation by phenylglyoxal, than RN. These data suggest that there may be slight differences in the tertiary structures of SC, the native enzyme expressed in *S. cerevisiae*, and RN, the recombinant native enzyme expressed in *E. coli*.

Superoxide dismutases (SODs) (EC 1.15.1.1) provide a major line of defense against oxygen toxicity in living cells. There are three types of SOD, classified according to the metal ion present at the enzyme active site: Cu,ZnSOD, MnSOD, and FeSOD. Cu,ZnSOD, most often found in the cytosol of eukaryotic cells (*1*), was the first SOD discovered (*2*) and many studies have been carried out on members of this class of enzyme (for reviews, see refs *1, 3, 4*). The characterization of MnSODs and FeSODs has been more recent and the catalytic mechanisms are still not fully understood.

MnSODs and FeSODs are highly homologous to each other (*1, 5*) but appear to be unrelated to the Cu,ZnSODs at all levels of structure (*6*−*8*). The availability of three-dimensional structures and primary amino acid sequences of MnSODs and FeSODs has increased tremendously in the past decade. High-resolution crystal structures determined

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[1] Abbreviations: SOD, superoxide dismutase; Cu,ZnSOD, copper- and zinc-containing SOD; MnSOD, manganese-containing SOD; FeSOD, iron-containing SOD; TNBS, 2,4,6-trinitrobenzenesulfonate; PG, phenylglyoxal; SC, *S. cerevisiae* MnSOD purified from yeast by the method of Bjerrum (*26*); RN, recombinant-native *S. cerevisiae*

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by X-ray diffraction have been reported for FeSODs from *Escherichia coli* (*6, 9*), *Pseudomonas ovalis* (*7, 10*), and *Mycobacterium tuberculosis* (*11*), MnSODs from *Thermus thermophilus* (*12, 13*) and *Bacillus stearothermophilus* (*14*), as well as the human mitochondrial MnSOD (*15, 16*). The residues acting as ligands to the active-site metal have been identified as three histidines and an aspartate in all six enzymes. In addition, a fifth ligand to the metal is contributed by solvent and is likely to be a hydroxyl group at neutral pH (*9*). These five ligands form a distorted trigonal bipyramide in all structures of the resting enzyme examined (*9*). Azide, a presumed superoxide mimic, binds to both *E. coli* FeSOD and oxidized *T. thermophilus* MnSOD to produce a six-coordinate metal center (*9, 17*). A stepwise mechanism for the reaction catalyzed by FeSOD, in which superoxide anion coordinates directly to the metal during both the oxidative and reductive half-reactions and in which the metal geometry alternates between 5-coordinate and 6-coordinate during catalysis, has been proposed (*9*).

MnSODs and FeSODs are homodimers, or occasionally homotetramers (dimers of dimers) (*1*), with two metal-dependent active sites per dimer. Each metal center is surrounded by an array of aromatic residues contributed by both subunits. In *E. coli* FeSOD, including the metal ligands, there are five histidines, four tyrosines, three tryptophans, and one phenylalanine within 10 Å of the active-site metal (*9*) (Figure 1). An array of similar residues has also been reported for the active sites of all other FeSODs and MnSODs examined. The catalytically essential metal ion lies at the bottom of a conical channel (Figure 1), and the path to the metal ion is hindered by the side chains of Tyr-34 and His-30, which presumably move aside during catalysis. Other important residues include Glu-159, a glutamine [Gln-69 for *E. coli* FeSOD or Gln-141 for MnSOD (*9*)], and Arg-170 in most SODs (*5*). This arginine is replaced by a lysine in *S. cerevisiae* MnSOD (*18*, *19*).

A number of studies have used either chemical modification or site-directed mutagenesis to examine the role of specific residues in MnSODs or FeSODs. Chemical modification studies have not been common, in part because the catalytic activities of MnSODs and FeSODs is relatively insensitive to most available group-specific reagents (*20*). However, Beyer and Fridovich (*21*) have shown that inactivation of *E. coli* FeSOD by hydrogen peroxide is accompanied by the loss of tryptophan residues, and Chan et al. (*5*) have used chemical modification with group-specific reagents to show that a positively-charged residue at position 170 in MnSODs and FeSODs is critical for activity. Very recently, Hunter et al. (*22*), Sorkin et al. (*23*), and Whittaker and Whittaker (*24*) have used site-directed mutagenesis to examine the role of Tyr-34 in either *E. coli* FeSOD or *E. coli* MnSOD. In the work reported herein, we have used site-directed mutagenesis, combined with chemical modification, to examine the role of His-30 and Lys-170 in *S. cerevisiae* MnSOD. More specifically, we have prepared and characterized the H30A and K170R mutants of this enzyme, and we now report the results of this work. A preliminary report has appeared (*25*).

MnSOD expressed in and purified from *E. coli*; H30A, RN in which His-30 (*E. coli* FeSOD numbering) has been replaced by alanine; K170R, RN in which Lys-170 (*E. coli* FeSOD numbering) has been replaced by arginine.

[2] Specific residues are numbered using the *E. coli* FeSOD sequence, unless otherwise stated. His-30 and Lys-170, the residues which are a focus of this study, are actually His-30 and Lys-182 in the primary sequence of *S. cerevisiae* MnSOD.

EXPERIMENTAL PROCEDURES

Materials. Native *S. cerevisiae* MnSOD (SC) was prepared by published methods (*26*). Phenylglyoxal and TNBS were purchased from Aldrich Chemical Company. All restriction enzymes, other DNA-handling enzymes, and DNA molecular mass markers were from Pharmacia or BRL. Low-melting point agarose, Sephadex resins, lysozyme, and ribonuclease A were from Sigma, while IPTG was a product of NBL. Microbiological growth media were purchased from Oxoid, while all other chemicals were from either BDH, M&B, Sigma, Fluka AG, or Beecham. Radioactive materials were products of Amersham, DE-52 resin was from Whatman, and DEAE-Sepharose CL-6B and Sephacryl S-300 were from Pharmacia.

Vectors and Bacterial Strains. Protein expression was obtained using the pKK233-2 vector containing the *trp-lac* promoter (*27, 28*). The pKK233-2 vector containing the mature *S. cerevisiae* MnSOD gene, designated pKMnII, was prepared by I. Schrank at UMIST. This vector has the signal peptide sequence deleted, a requirement for the functional expression of the eukaryotic mitochondrial MnSOD in *E. coli* (*29*). The M13 replicative form DNA containing the *S. cerevisiae* MnSOD gene with a synthetic *Dra*I site that allows for the excision of the leader sequence, designated M13amMn, was prepared from M13mp18amIV by Schrank et al. (*29*). M13amMn was used for oligonucleotide-directed mutagenesis, as described below. The TG1 strain of *E. coli* was obtained from Amersham. The QC871 strain (Sod A^-, Sod B^-) of *E. coli*, which lacks functional MnSOD and FeSOD genes and which was used for the production of *S. cerevisiae* native and mutant MnSODs, was a gift from Dr. Danielle Touati.

Mutagenesis of the S. cerevisiae MnSOD Gene Cloned into M13. Site-specific mutagenesis of M13amMn was carried out using the method of Taylor et al. (*30*), using the Amersham oligonucleotide-directed mutagenesis system, version 2. This technique involves a strand selection technique which eliminates the unwanted non-mutant sequence in vitro, generating a pure homoduplex mutant DNA sequence. For the preparation of the M13amMn containing a mutation which results in the conversion of Lys-170 to an arginine (M13MnK170R), the mutagenic oligonucleotide was a 21-mer which was designed to introduce a single base-pair change in the relevant codon, from AAA in the native gene to AGA in the mutant gene. After the final ligation to form the mutant homoduplex, the M13 replicative-form DNA was transformed into *E. coli* TG1 cells and plated on LB/ampicillin plates. Mutant colonies were identified by differential temperature dot-blot hybridization of the ^{32}P-labeled mutant oligonucleotide to the mutant replicative-form DNA at 57 °C, using the procedure recommended by Amersham for their kit. Once isolated, the mutation was confirmed by single-stranded sequencing of the entire gene using the Sanger dideoxy procedure (*31*).

M13amMn containing a mutation which caused the histidine at position 30 to be converted to an alanine

(M13MnH30A) was prepared by the procedure described above by M. Gent at UMIST. This mutation was also confirmed by single-stranded sequencing.

Subcloning the Mutant MnSOD Gene from M13MnK170R and M13MnH30A into pKK233-2. The replicative-form DNA prepared from the mutant (M13MnK170R or M13MnH30A) was digested with *Dra*I/*Hin*dIII. This DNA contained the unique *Dra*I site derived from M13amMn such that scission by the cognate restriction endonuclease yields a DNA fragment that begins with the lysine codon that specifies the N-terminal amino acid of the mature form of *S. cerevisiae* MnSOD. The *Dra*I/*Hin*dIII fragment was purified and cloned into the pKK233-2 vector. Eukaryotic gene fragments lacking a prokaryotic ribosome binding site and an ATG initiation codon can be inserted into the correct reading frame of pKK233-2 by blunt-end ligation to the filled-in *Nco*I site (*28*). The cleavage of the resultant blunt-ended molecule with *Hin*dIII produced a plasmid with asymmetric ends, compatible with the *Dra*I/*Hin*dIII fragment from M13MnK170R or M13MnH30A. The mixture of the treated pKK233-2 and the mutant fragment was ligated to produce either pKMnK170R or pKMnH30A. Each purified plasmid was used to transform *E. coli* QC871 (Sod A^-,Sod B^-) using ampicillin selection.

Expression and Purification of Recombinant Native S. cerevisiae MnSOD and its K170R and H30A derivatives. Large-scale preparation of the enzymes was possible by using the 20 L and 200 L fermenters available at UMIST. A 5 mL starter culture in LB/ampicillin, grown for 8 h at 37 °C from a single colony, was used to inoculate a larger culture. Induction of SOD by IPTG was carried out as previously described (*29*). When the large fermenters were used, a 10% inoculum was employed and the medium kept at 37 °C for 5 h before harvesting the cells by centrifugation. Cell lysis was obtained by suspending the cells in 50 mM phosphate, 1 mM PMSF, pH 7.4, and passing the cell suspension three times through a Gaulin APV homogenizer at 12 000 psi. The insoluble cell debris was removed by centrifugation to give a cleared cell lysate.

The recombinant native enzyme and the K170R and H30A mutant forms were purified by slight variations of the procedure described by Ditlow and Johansen (*32*) and modified by Bjerrum (*26*). The MnSOD is quite heat stable (*32*), and an initial step in the purification scheme is the heating of the cell-free lysate to 70 °C for 2 min to denature and/or precipitate unwanted proteins, followed by successive ion-exchange chromatography steps on DE-52 cellulose, and DEAE-Sepharose CL-6B (*32*), followed by a size exclusion chromatography step (*26*). The recombinant native and H30A enzymes were both heat treated, and the former subjected to DE-52 cellulose and Sephacryl S300 HR chromatography to yield a protein that was >97% pure by SDS-polyacrylamide gel electrophoresis (data not shown), while the H30A was purifed to the same extent by DE-52 cellulose chromatography, followed by chromatography on DEAE-Sepharose CL-6B. The K170R mutant proved to be much more susceptible to heat denaturation than the other two, so the heat-treatment step was replaced by a novel crossflow filtration procedure in BioFlo hollow fiber filters (*33*). The lysate was passed successively through 1000, 100, and 54 kDa molecular mass cut-off filters, and the MnSOD was retained in the 54 kDa concentrate. The protein was then subjected successively to DE-52 cellulose, DEAE-Sepharose CL-6b, and Sephacryl S300 HR steps to give a product that was >97% pure, as estimated by SDS-polyacrylamide gel electrophoresis.

Determination of S. cerevisiae MnSOD Concentration. The concentration of the native, recombinant native, and mutant MnSODs was determined from the absorbance at 280 nm using a value of $\epsilon = 1.92 \times 10^5$ M^{-1} cm^{-1} (*32*) and a molecular mass for the tetrameric enzyme of 90 800 Da (*18*).

SOD Assay. SOD activity was determined at pH 8.2 and 25 °C by the ability of the protein to inhibit pyrogallol autooxidation (*34*), as described elsewhere (*5, 35*). One unit of SOD activity is defined as the amount that reduces the rate of autooxidation of 0.2 mM pyrogallol (50 mM Tris, 50 mM cacodylic acid, 0.1 mM diethylenetriaminepentaacetate, pH 8.20, 25 °C) by 50% in 1.00 mL of assay solution.

Specific Activities of the Various S. cerevisiae MnSODs. Aliquots of enzyme samples of known concentration were added to the SOD assay medium, and the percent inhibition of the pyrogallol autooxidation was determined. For each sample, assays were carried out with six or seven different amounts of enzyme, the resulting data were plotted as % inhibition vs. ng MnSOD, and the amount of enzyme necessary to give 50% inhibition was determined from the plot.

Heat Labilities of the Various S. cerevisiae MnSODs. Each enzyme (0.04 mg/mL) in 30 mM Tris-HCl, pH 8.0, was incubated at 75 °C and at the indicated times aliquots were withdrawn, diluted 5-fold with the same buffer, and assayed immediately for SOD activity.

Chemical Modifications. Modifications with phenylglyoxal and TNBS were carried out under conditions described in the figure legends. Reactions were initiated by the addition of a stock solution of the enzyme to an aqueous solution of the reagent. At appropriate times after mixing the enzyme and modifying reagent, aliquots were diluted and assayed for SOD activity. Activity is expressed as a percent of a control subjected to the same conditions, but in the absence of high temperature or chemical modification reagent.

Circular Dichroism Measurements. Circular dichroism spectra were obtained on a Jobin-Yvon Dichrograph Mark V at 25 °C. The intensity of the CD signal was calibrated with (+)-camphorsulfonic acid, assuming a $\Delta\epsilon$ of 2.36 M^{-1} cm^{-1} at 290.5 nm. Cylindrical cells with path lengths of 1.00, 0.10, or 0.0050 cm were used. The actual path lengths of the 0.0050 cm cells were determined interferometrically. All samples had absorptions of <1.0.

MnSOD and FeSOD Sequences and Sequence Alignments. MnSOD and FeSOD sequences were obtained from the National Center for Biotechnology Information, Bethesda, MD, at *www.ncbi.nlm.nih.gov*. The GenBank Sequence Database was searched using the Entrez software available on the server. The >150 MnSOD and FeSOD sequences were manually reduced to the 89 non-redundant sequences used for sequence alignments. These alignments were carried out by first opening the ExPASy Molecular Biology Server at *expasy.hcuge.ch*, opening the TOOLS file therein, and using the Multalin (*36*) and Clustal W alignment programs with default parameters. Each program gave almost identical alignments of the highly conserved SOD residues, with small differences occurring in the four highly variable regions (vide infra). Final adjustments to the aligned

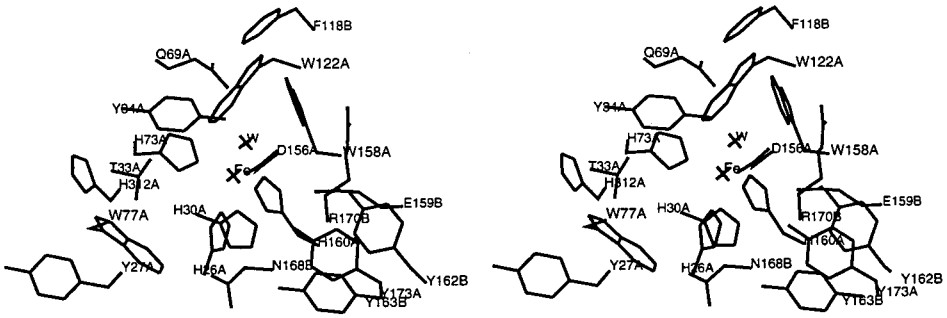

FIGURE 1: Stereoview of the active-site channel in *E. coli* FeSOD. The catalytic Fe from subunit A is in the middle of the view at the bottom of a conical channel, and the approach of substrate to this site is approximately 90° from the plane of the paper. The residues surrounding the metal ion are contributed by both subunit A and subunit B. Including the histidine ligands to Fe(III), there are fourteen aromatic residues shown, and all lie within 10 Å of the metal ion. The two residues which are the focus of this paper are His-30A and Arg-170B.

sequences was made by eye to generate the data used to prepare Figure 5.

Molecular Graphics. The figures showing the active sites of *E. coli* and *M. tuberculosis* FeSODs we generated using Insight II (Biosym Technologies, San Diego, CA). The data files were obtained from the Protein Data Bank at Brookhaven National Laboratory.

RESULTS

Specific Activity of SODs. The specific activity of each of the *S. cerevisiae* MnSODs included was high and, unexpectedly, relatively invariant. SC had a specific activity of 5000 U/mg, while the corresponding values for the other enzymes were RN = 5600, H30A = 4500, and K170R = 4600. The high values for RN and K170R were expected, the latter because the positive charge is conserved and because arginine is almost always found in this position (vide infra). However, results for H30A were unexpected, given the presence of this histidine in the active-site channel and the close proximity to the catalytic metal (Figure 1). The data are however compatible with those of Hunter et al. (*22*), who found that replacement of Tyr-34, a residue even closer to the catalytic metal and implicated in a feasible proposed mechanism for catalysis (*9*), by phenylalanine gives a mutant that still retains 40% of the native catalytic activity.

Heat Lability of SODs. It has been noted that *S. cerevisiae* MnSOD is quite stable at elevated temperatures (*26*, *32*). In addition, it was observed early in this study that the K170R mutant was not stable to the heat-treatment step that is a part of the reported purification procedure for native *S. cerevisiae* MnSOD (*26*, *32*). Figure 2 shows the rate at which catalytic activity is lost when the various enzymes examined in this study were incubated at 75 °C and pH 8.0. SC, RN, and H30A were found to be quite stable under these conditions, with the loss of activity in each case occurring by a first-order process with half-lives of 4.7, 2.8, or 2.7 h, respectively. In contrast, the lability of K170R was much greater than that of the others, with a half-life of 0.36 h under the same conditions. It should be noted at this point that the RN form of the enzyme behaved somewhat differently than SC and more like H30A, an observation that is repeated regularly in the data reported in this paper.

Treatment of SODs with TNBS. It has been reported (*5*) that *S. cerevisiae* MnSOD is inactivated much more rapidly by TNBS, a lysine-selective reagent (*37*), and to a much greater extent at the limit of inactivation, than the *E. coli*, *B. stearothermophilus*, and human MnSODs and the *E. coli* FeSOD. The differences were attributed to the fact that the latter four enzymes each have an arginine at position 170, while the former has a lysine at this position (*5*). SC, RN, H30A, and K170R were inactivated by 0.5 mM TNBS, at pH 9.0 and 25 °C, in a pseudo-first-order process, but at widely varying rates that depended on the identity of residue 170 (data not shown). SC, RN, and H30A were rapidly inactivated, with half-lives of 3.5, 5.1, and 5.5 min, respectively. In contrast, K170R was inactivated at an approximately 20-fold lower rate, with a half-life of 101 min.

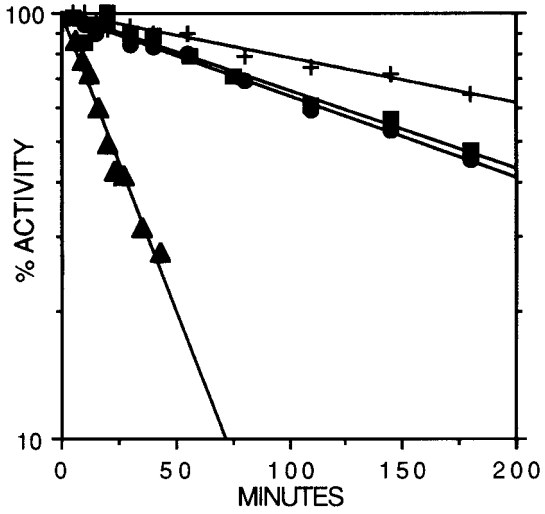

FIGURE 2: Temperature stability of *S. cerevisiae* MnSODs. The enzymes (approximately 0.04 mg/mL) were incubated at 75 °C in 30 mM Tris-HCl, pH 8.0, and at the indicated times, aliquots were withdrawn, diluted, and assayed immediately for SOD activity. (+) SC, (■) RN, (●) H30A, (▲) K170R.

Treatment of SODs with Phenylglyoxal. The sensitivities of MnSODs and FeSODs to phenylglyoxal, an arginine-specific reagent (*38*), has also been shown to be a function of the identity of residue 170; i.e., SODs containing arginine at this position are inactivated more rapidly and more extensively than *S. cerevisiae* MnSOD (*5*). However, it has also been noted (*5*) that the stringent conditions necessary for the inactivation of Arg-170 SODs, i.e., 20 mM phenylglyoxal, pH 9.0, and the extended modification times needed for significant inactivation, suggest that this arginine is much more shielded from the modifying reagent than active-site arginines in other phenylglyoxal-sensitive enzymes. This reduced reactivity of Arg-170 may be attributed in great part

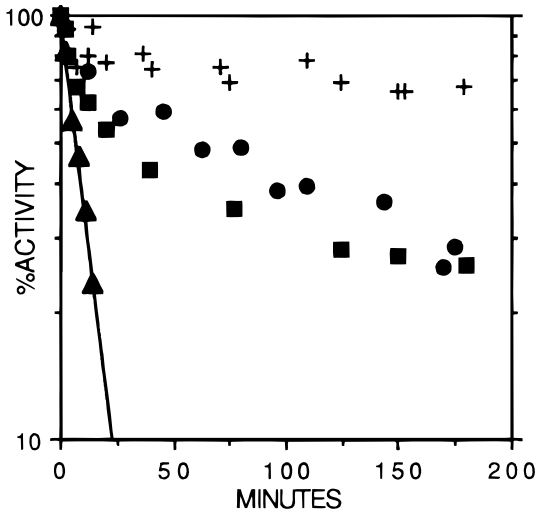

FIGURE 3: Inactivation of *S. cerevisiae* MnSODs by phenylglyoxal. The enzymes (0.05−0.07 mg/mL) were treated at 25 °C with 20 mM phenylglyoxal in 50 mM sodium pyrophosphate, 50 mM $NaHCO_3$, 0.1 mM EDTA, pH 9.0, and at the indicated times aliquots were diluted and assayed for enzymatic activity as described in the text. (+) SC, (■) RN, (●) H30A, (▲) K170R.

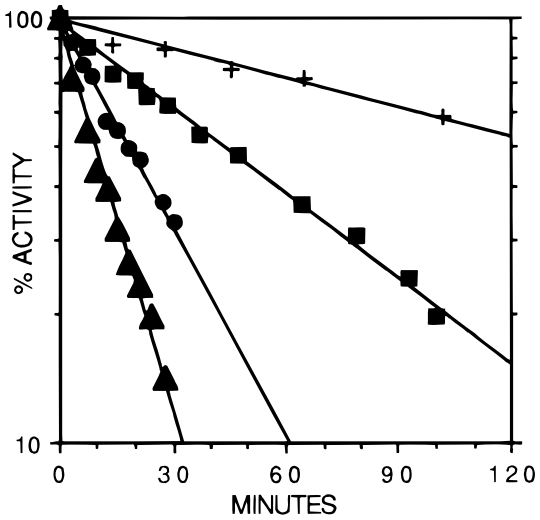

FIGURE 4: Inactivation of *S. cerevisiae* K170R MnSOD as a function of the concentration of phenylglyoxal. The enzyme (approximately 0.07 mg/mL) was treated at 25 °C with (+) 2, (■) 4, (●) 8, or (▲) 16 mM phenylglyoxal in 50 mM sodium pyrophosphate, 50 mM $NaHCO_3$, 0.1 mM EDTA, pH 9.0, and at the indicated times aliquots were diluted and assayed for enzymatic activity as described in the text.

to its extensive hydrogen bonding to backbone carbonyl oxygen atoms (*39*).

The time course for inactivation of SC, RN, H30A, and K170R by 20 mM phenylglyoxal at pH 9.0 and 25 °C is shown in Figure 3. Again, SC was inactivated very slowly under these conditions, with 50% activity loss occurring by a complicated kinetic process (i.e., the plot of log % activity vs time is nonlinear) only after 7−8 h of modification. Interestingly, RN and H30A were inactivated more rapidly, with 50% inactivation times of approximately 0.5 and 1.0 h, respectively. However, the kinetic process for inactivation of RN and H30A was again complex, and each enzyme retained >25% activity after 3 h of modification. The data for K170R are in sharp contrast to the data for the others (Figure 3), for it was rapidly inactivated by a pseudo-first-order process with a half-life of 7.0 min under these conditions.

The inactivation of K170R as a function of phenylglyoxal concentration was examined further. Figure 4 shows that treatment of K170R with 2, 4, 8, and 16 mM phenylglyoxal, at pH 9.0 and 25 °C, gave pseudo-first-order inactivation profiles with half-lives of 131, 45, 18.6, and 9.9 min, respectively. A plot of log k' (where k' is the pseudo-first-order rate constant for inactivation) vs log[phenylglyoxal] gives a straight line with a slope of 1.17 (data not shown). This suggests that the inactivation of K170R is an overall second-order process, first-order in [SOD] and first-order in [phenylglyoxal]. These data for the inactivation of K170R by phenylglyoxal are much more in line with comparable data for modification of exposed arginines at enzyme active sites (*40*).

DISCUSSION

There has been an explosion in the availability of MnSOD and FeSOD primary sequences in the past decade. Parker and Blake (*41*) used the alignments of the nine sequences available at that time to identify residues that are likely determinants of whether a given polypeptide is a MnSOD or a FeSOD. Chan et al. (*5*) determined the sequence homology of the nine MnSODs and four FeSODs available and used the data to show that a positively charged residue at position 170, arginine in all but *S. cerevisiae* MnSOD, lysine in the latter, is essential for activity. Several specialized studies subsequently appeared that led to rapid expansion of the number of available sequences. Smith and Doolittle (*42*) used partial sequences to compare the evolutionary rates of MnSODs/FeSODs and Cu,ZnSODs from widely varying organisms. Joshi and Dennis (*43*) compared the primary sequences of eight SODs from primitive *Archaea*, while Zhu and Scandalios (*44*) showed that maize MnSODs originate from a differentially expressed multigene family, with seven different MnSODs identified. These and other studies allowed an August 1997 search to identify 89 non-redundant MnSOD and FeSOD sequences from all life forms, including 11 *Archaea*, 44 *Eubacteriae*, and 34 eukaryotes. After optimal alignment of all sequences, a consensus sequence was generated using *E. coli* FeSOD as a reference (Figure 5).

An analysis of the consensus sequence shows that 101 residues are >50% conserved in the set examined. There are seven fully conserved residues, including the metal ligands (His-26, His-73, Asp-156, and His-160) and three other residues known to be at the active site (Tyr-34, Glu-159, Tyr-163) (Figures 1 and 6). However, fully 31 residues are at least 90% conserved, including the seven mentioned above. All 31 residues are found in *E. coli* FeSOD, and the location of each was examined in the three-dimensional structure of this protein (data not shown). His-30, Tyr-34, Trp-122, and Trp-158 form part of the active-site channel, as do Glu-159, Tyr-162, Tyr-163, Asn-168, and Arg-170 from the other subunit of the dimer (Figure 1). Glu-159, Tyr-162, Tyr-163, and Arg-170 also make important contacts at the monomer-monomer interface of the dimer, as does Ser-120, which forms a hydrogen bond to Ser-120 in the other subunit. The FeSOD monomer is made up of two domains (*9*), and Leu-4, Leu-7, Ala-13, Leu-14, His-31, Val-35, and Trp-77 make important hydrophobic contacts in the

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                                Asn           O                       O
                 D              Lys          His                      I      Thr
                Gln             Ala          Val             Ser     Phe Val Lys
                Met             Gly          Lys     Ser     Gly Lys Ala Met Cys
                Ile             Asp Trp      Leu     Gly Met Gln Ala Tyr Leu Asn
                Phe Ile     Pro Ser Phe      Phe     Asp Phe Ala Asp Val Phe Asp
  1 xxx-xxx-xxx-Leu-Pro-xxx-Leu-Pro-Tyr-xxx  Tyr-xxx-Ala-Leu-Glu-Pro-His-Ile-Ser-xxx
                 92  85      96  74  85       65      90  97  83  93  53  74  62

                                                                          O
            Ile                                                          Phe
            Asn                     Asn              Ile             Ala Val
            Phe                     Arg              Ser             Ser Trp Glu
            Val             Trp     Gly              Gly     Leu     Lys Tyr Thr
            Leu             Tyr     Thr Asn  Leu     Ala     Ile     Gly Ala Lys
 21 xxx-xxx-Met-xxx-xxx-His-His-xxx-Lys His  His-xxx-Thr-Tyr-Val-xxx-Asn-Leu-Asn xxx
             55         100  80      85  97   98      60 100  93      67  60  96

    Val      O
    Asp     Gln
    Ile     Ala
    Gln     Pro
    Thr     Asp
    Leu     Lys
 41 Ala-xxx-Met-xxx-xxx-xxx-xxx-xxx-xxx xxx  xxx-xxx-xxx-xxx-xxx-xxx-xxx-xxx-xxx xxx
     61      62

                                         O
                            Thr         Tyr
                            Ile Asn     Phe
                        Lys Val Ser     Ala                      Leu             Phe
                    His Leu Leu Cys     Ile                      Trp             Ile
                    Phe His Ala Ala Gln Val      Leu             Tyr Phe         Met
 61 xxx-xxx-xxx-xxx-Asn-Asn-Gly-Gly-Gly-His  xxx Asn-His-xxx-xxx-Phe-Trp-xxx-xxx-Leu
                     53  87  54  63  74  72       88 100          71  96          62

                 O   O   O   O                                O
                Thr Thr Lys Arg Glu     Ser                  Asp
        Lys     Cys Lys  D  Gln Val     Lys                  Glu                 Ile
        Thr     Lys Ser Asn Thr Gly     Glu                  Lys                 Tyr
        Ala     Ala  D  Thr Ala Cys     Ala      Ile         Leu                 Leu
         D       D  Ala Asp Lys Leu     Asp      Val         Arg Leu             Trp
 81 xxx-Pro-xxx-Gly-Gly-Gly-Glu-Pro-xxx-Gly  xxx-Leu-xxx-xxx-Ala-Ile-xxx-xxx-xxx-Phe
         80      65  87  72  54  84      83       88          72  94              87

                            Tyr                       O
                            Leu                      Ile                 Glu
            His             Gln         Thr          Thr Leu             Leu      D
        Asn Val         Met Ile         Ile          Val Thr             Pro Arg Phe
    Thr Thr Tyr         Trp Val         Met          Ser Ser              D  Ala Gly
    Lys Gln Leu         Leu Arg         Leu          Glu Gly             Gln  D  Ala
101 Gly-Ser-Phe-xxx-xxx-Phe-Lys-xxx-xxx-Phe  xxx-xxx-Ala-Ala-xxx-xxx-xxx-Phe-Gly-Ser
     97  88  55          69  61          71           54  60              52  88  93

             O                               Glu                                  D
             D  Ser                          Arg                         Leu     His
            Phe Ala                          Gln                         Ser     Gly
     D   D  Thr Phe                          Asp                         Tyr     Gln
    Ser Ser Val Val Ala                      Asn     Asn                 Cys     Asp
    Arg Leu Gly Leu Ile                      Lys     Val                 Val     Lys
121 Gly-Trp-Ala-Trp-Leu-xxx-xxx-xxx-xxx-xxx  Gly-xxx-Leu-xxx-xxx-xxx-xxx-Thr-xxx-Asn
     97  96  55  81  98                       52      98                  60      81

         O                                                O
        Glu     Phe                                      Ile         Ala
        Asn     Asn                                  Ile Val         Leu
    Gly Val     Val                          Ile     Val Gln         Glu
    His Ala     Gly                          Val Val Met Ala         Ile
    Ala Gly     Leu                          Ala Ile Phe Thr         Met Phe
141 Gln-Asp-xxx-Pro-xxx-xxx-xxx-xxx-xxx-xxx  Pro-Leu-Leu-Gly-xxx-Asp-Val-Trp-Glu-His
     62  62      54                           94  63  82  52     100  88  98 100 100

                                                 Thr              O           O
                        Val                      Gly             His     Val Ser
                Leu     Leu              X    D  Ala Asp         Lys Val Leu Aso Ala
                Val     Thr             Ser  Gly Lys Val         Glu Leu Met Ala His
                Tyr     His     Thr     Leu  Val Ser His         Thr Trp Tyr Glu Ile
    Ser Phe     Ile     Phe     Pro     Lys  Ala Glu Phe         Asn Ile Phe Lys Leu
161 Ala-Tyr-Tyr-Leu-xxx-Tyr-xxx-Asn-xxx-Arg  Pro-Asp-Tyr-xxx-xxx-Ala-Phe-Trp Asn-Val
     91  93 100  72      80      90      90   69  60  84          57  56  72  51  66

                                     O
                         D           D
    Leu Thr             Arg         Leu
    Ala Lys             Pro         Gln
    Met Ser Asn         Ile         Val
    Ile Asp Phe         Ala         Asn
181 Val-Asn-Trp-xxx-xxx-Val-xxx-xxx-Arg-xxx  xxx-xxx
     54  80  98          52          56
```

FIGURE 5: A consensus sequence for MnSODs and FeSODs. To be included in the sequences used in this analysis, a sequence had to differ from any other sequence in the GenBank database by two or more residues. The numbering is that of the *E. coli* FeSOD. Each amino acid identified in the consensus sequence is present in >50% of the sequences examined, with the frequency percent given immediately below the amino acid. The other residues that also occur in a consensus position are listed above each residue, with the frequency of occurrence decreasing as one reads away from the consensus residue. Abbreviations: D, deletion at this position in one or more sequences; I, insertion at this position in one or more sequences; O, occurrence of at least two residues more than the ones shown. The 89 sequences used (GenBank accession number, type of SOD, source) include the following: (1) 44 *Eubacteriae* sequences: 1070457, Fe, *Escherichia coli*; 1070455, Mn, *Escherichia coli*; 1498060, Fe, *Pseudomonas putida*; 1498062, Mn, *Pseudomonas putida*; 2117673, Mn, *Pseudomonas aeruginosa*; 1711436, Fe, *Pseudomonas aeruginosa*; 586007, Fe, *Bordetella pertussis*; 1711438, Mn, *Bordetella pertussis*; 1711422, Mn, *Plectonema boryanum*; 1711423, Mn, *Plectonema boryanum*; 1711424, Mn, *Plectonema boryanum*; 1711435, Fe, *Plectonema boryanum*; 628856, Fe/Mn, *Haemophilus influenzae*; 1075332, Fe/Mn, *Haemophilus influenzae*; 98822, Fe, *Mycobacterium tuberculosis*; 98698, Mn, *Listeria monocytogenes*; 2117680, Mn, *Nocardia asteroides*; 134650, Mn, *Bacillis stearothermophilus*; 1711456, Mn, *Thermus aquaticus thermophilus*; 134644, Fe/Mn, *Porphyromonas gingivalis*; 134638, Fe, *Coxiella brunetii*; 134649, Mn, *Bacillus caldotenax*; 134666, Mn, *Listeria ivanovii*; 401109, Fe, *Legionella pneumophila*; 1174387, Mn, *Salmonella typhimurium*; 1174379, Fe, *Helicobacter pylori*; 1351085, Mn, *Mycobacterium avium*; 1711440, Mn, *Lactococcus lactis lactis*; 1711432, Fe, *Bacteriodes fragilis*; 1711455, Mn, *Thermus aquaticus*; 1711457, Mn, *Xanthomonas campestris pv. campestris*; 1711458, Mn, *Yersinia enterocolitica*; 1711437, Mn, *Bacillus subtilis*; 464776, Mn, *Propionibacterium freudenreichii shermanii*; 77812, Fe, *Pseudomonas sp.*; 79688, Fe, *Synechococcus sp.*; 80649, Mn, *Mycobacterium leprae*; 1072933, Fe/Mn, *Campylobacter jejuni*; 743362, Fe, *Leishmania donovani chagasi*; 79229, Fe, *Photobacterium leiognathi*; 95281, Fe/Mn, *Methylomonas sp.*; 282314, Mn, *Streptococcus mutans*; 1399534, Mn, *Actinobacillus pleuropneumoniae*; (2) 11 *Archaea* sequences: 148814, Mn, *Halobacterium halobium*; 148820, Mn, *Halobacterium sp.*; 134663, Mn, *Halobacterium cutirubrum*; 134662, Mn, *Halobacterium cutirubrum*; 548947, Mn, *Halobacterium sp. GRB*; 417789, Mn, *Haloferax volcanii*; 417790, Mn, *Haloferax volcanii*; 417791, Mn, *Haloarcula marismortui*; 479542, Fe/Mn, *Sulfolobus acidocaldarius*; 1076135, Fe/Mn, *Methanobacterium thermoautotrophicum*; 80947, Fe/Mn, *Methanobacterium thermoautotrophicum*; and (3) 34 eukaryotic sequences: 401110, Mn, *Caenorhabditis elegans*; 542489, Mn, *Caenorhabditis elegans*; 1078882, Mn, *Caenorhabditis elegans*; 630794, Fe/Mn, *Onchocera volvulus*; 514917, Mn, *Drosophila melanogaster*; 409457, Mn, *Drosophila melanogaster*; 279455, Mn, rat; 134655, Mn, human; 1351084, Mn, mouse; 1174380, Mn, cow; 1174386, Mn, rabbit; 1351083, Mn, guinea pig; 469163, Mn, maize; 469164, Mn, maize; 539066, Mn, maize; 539067, Mn, maize; 1174389, Mn, maize; 469162, Mn, maize; 82728, Mn, maize; 134675, Mn, pea; 945044, Mn, pea; 100284, Fe, tobacco; 82145, Mn, tobacco; 228415, Fe, soybean; 542013, Mn, para rubber tree; 99759, Fe, thale cress; 102388, Fe, *Tetrahymena pyriformis*; 323073m Fe, *Entamoeba histolytica*; 66379, Mn, *Saccharomyces cerevisiae*; 1519036, Mn, *Ganoderma microsporum*; 1078795, Fe, *Plasmodium falciparum*; 1066332, Fe, *Trypanosoma cruzi*; 973176, Mn, *Chaldomonas reinhardtii*; 2117676, Fe, *Chaldomonas reinhardtii*.

core of the N-terminal domain. In a similar fashion, Ile-96, Leu-125, Leu-133, Pro-151, and Trp-183 are key players in the core of the C-terminal domain. Ala-161 lies within 5 Å of the Fe ion and makes van der Waals contact with His-26. Asn-39 makes a hydrogen bond to the backbone carbonyl oxygen of residue Phe-2 at the N-terminus of the polypeptide chain, while Pro-16 lies on the surface of the protein in a tight turn that leads to the first helix of Domain 1. Finally, Gly-121 makes possible the close approach of two polypeptide chains in the core of Domain 2 and Gly-101 lies at the surface of the protein and is likely to play a role in a 180° turn of the polypeptide backbone.

Relatively few studies using chemical modification or site-directed mutagenesis to explore the roles of specific residues in the catalytic mechanisms of MnSODs and FeSODs have appeared. Chan et al. (*5*), using chemical modification with arginine-specific and lysine-specific reagents, have shown that a positively charged residue at position 170 is critical for catalytic activity. This residue was arginine in the large majority of sequences examined at that time, with the only exception being *S. cerevisiae* MnSOD, where it was a lysine. The arginine makes multiple hydrogen bonds to backbone carbonyl oxygens, making it a prime example of a structural arginine, i.e., one that interacts strongly with a polypeptide backbone and stabilizes the tertiary structure of a globular protein (*39*). Arg-170 in MnSODs and FeSODs has been proposed to play a combined structural/functional role, molding the active-site channel as well as providing a diffuse electrostatic attractive force to guide superoxide into the cone that leads to the catalytic metal center (*39*). These functions are apparently taken over by lysine in *S. cerevisiae* MnSOD.

Hunter and co-workers (*22*) have used site-directed mutagenesis to examine the role of Tyr-34 in *E. coli* FeSOD. This residue lies within 5.5 Å of the catalytic metal and has been proposed to play a role in the catalytic mechanism (*9*). Hunter et al. (*22*) concluded that Tyr-34 plays multifunctional roles, including a structural one, where it hydrogen bonds to a glutamine residue in the active-site channel, a stabilizing role at high pH, and a steric role in obstructing access to the catalytic metal site. Sorkin et al. (*23*), using NMR techniques, have shown that Tyr-34 in *E. coli* FeSOD is responsible for the active-site p*K* of 8.5 for reduced FeSOD and is likely to be responsible for the p*K* of 9 that has been identified by kinetic studies (*45*). Whittaker and Whittaker (*24*) have shown that the Y34F mutant of *E. coli* MnSOD has almost full catalytic activity, but the pH sensitivity of the active site metal ion is altered and the ligand binding is perturbed.

The work reported herein on the K170R mutant of *S. cerevisiae* MnSOD is consistent with the results of Chan et al. (*5*), with important variations. K170R is inactivated much more slowly by TNBS and much more rapidly by phenylglyoxal (Figure 3) than the SC, RN, or H30A forms of *S. cerevisiae* MnSOD, all of which have a lysine at position 170. However, K170R is inactivated much more rapidly than *E. coli* FeSOD, *E. coli* MnSOD, *B. stearothermophilus* MnSOD, or human MnSOD under identical conditions (*5*). The latter four enzymes also have an arginine at position 170 that was introduced during the course of evolution and which is the norm for MnSODs and FeSODs (Figure 5). Arg-170 plays a structural role, making multiple hydrogen bonds to backbone carbonyl oxygen atoms (*39*). A likely explanation for the greater rate of inactivation of K170R by phenylglyoxal is that the arginine in this mutant enzyme is more available for reaction than Arg-170 in other MnSODs and FeSODs because it does not engage in the extensive hydrogen bonding that is normally found for Arg-170.

As shown in Figure 5, Arg-170 is found in more than 90% of all MnSODs and FeSODs examined. When it is replaced by another residue, that residue is usually lysine. Of the six different MnSODs and FeSODs for which high resolution X-ray crystal structures are available, five have arginine at

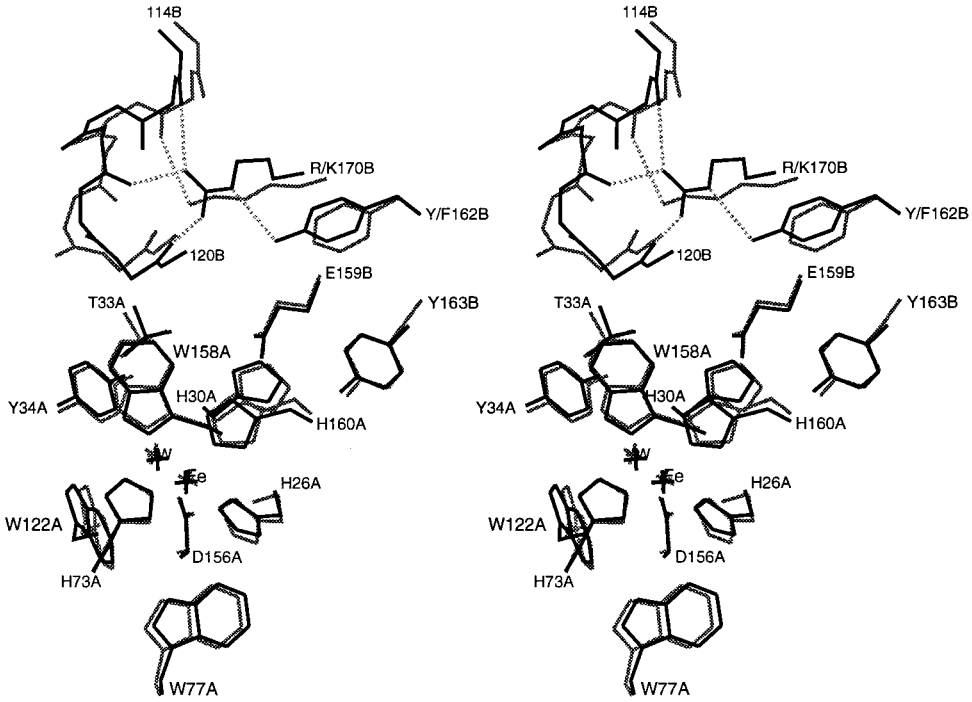

FIGURE 6: Stereoview comparing the active sites of *E. coli* FeSOD (PDB code 1ISB) and *M. tuberculosis* FeSOD (PDB code 1IDS). The two structures were overlaid by taking the root mean square of all residues shown, including backbone atoms, except Y/F162B, R/K170B, and the peptide backbone 114B−120B. The region of interest lies at the top of the figure. *E. coli* FeSOD has an arginine at position 170 and a tyrosine at position 162. It can be seen that R170 makes four hydrogen bonds, three to backbone carbonyl oxygen atoms of residues 114 (top), 117 (middle), and 119 (bottom), and a fourth to the OH group of Tyr-162. *M. tuberculosis* FeSOD has a lysine at position 170 and a phenylalanine at position 162, and it can be seen that both residues move a significant distance relative to the corresponding residues in *E. coli* FeSOD. Although the stretch of peptide backbone from 114B to 120B retains approximately the same orientation, there is a significant twist such that residue 114B of *M. tuberculosis* FeSOD lies to the left of the corresponding residue in *E. coli* FeSOD in the view shown while residue 120B lies to the right of the corresponding *E. coli* FeSOD residue. Lys-170 in *M. tuberculosis* FeSOD makes none of the hydrogen bonds to backbone carbonyls that Arg-170 makes, but instead makes a single hydrogen bond to the carbonyl oxygen of residue 115B.

position 170, while only the FeSOD from *M. tuberculosis* (*11*) has a lysine at this position. Figure 6 shows a stereoview in which the active sites of *E. coli* FeSOD and *M. tuberculosis* FeSOD are compared. It can be seen that most of the differences in the two active sites lie at the top of the figure in the region where Arg-170 or Lys-170 is positioned. In the *E. coli* enzyme, Arg-170 from subunit B makes three hydrogen bonds to the backbone carbonyl oxygen atoms of residues 114, 117, and 119 of the same subunit and a fourth hydrogen bond to the OH group of Tyr-162, consistent with the hydrogen bonding pattern of Arg-170 first reported for the *T. thermophilus* MnSOD (*39*). It should be noted that Tyr-162 is also highly conserved (Figure 5) and that all MnSODs and FeSODs that have an arginine at position 170 have a tyrosine at position 162. Lysine is found at position 170 in six of the 89 sequences examined to generate Figure 5, and in five of the six Tyr-162 is converted to a phenylalanine; such is the case for *M. tuberculosis* FeSOD. Figure 6 shows that Lys-170 in *M. tuberculosis* FeSOD makes no hydrogen bonds to the backbone carbonyl oxygens shown for Arg-170 in *E. coli* FeSOD, but instead makes a single hydrogen bond to the backbone carbonyl oxygen of residue 115. As a consequence, Lys-170 moves approximately 3.3 Å further out into the active-site channel, making it more exposed to solvent. In addition, the ring of Phe-162 moves approximately 1.0 Å into the channel, relative to the position of Tyr-162, and the stretch of polypeptide backbone from residue 114 to residue 120 moves 1-2.5 Å and assumes a slightly different orientation. Our results on the K170R mutant of *S. cerevisiae* MnSOD discussed above suggest that it is more exposed than the Arg-170 found in most MnSODs and FeSODs, and we propose that it does not form the hydrogen bonding network shown for *E. coli* FeSOD (Figure 6). Rather it lies further out into the active-site channel, as does Lys-170 in *M. tuberculosis* FeSOD and, by inference, *S. cerevisiae* MnSOD.

H30A has properties and behavior remarkably similar to those of RN. They have comparable specific activities and indistinguishable heat labilities at 75 °C (Figure 2) and sensitivities to phenylglyoxal (Figure 3) and TNBS. Collectively, these data suggest that His-30 plays no critical role in MnSOD, though this residue is highly conserved (Figure 5) and lies in the active-site channel (*9, 11*).

The differences between RN and SC are much more pronounced than the differences between RN and H30A. The specific activities of RN and SC are very similar, but SC is more stable to inactivation at 75 °C (Figure 2) and less susceptible to inactivation by phenylglyoxal (Figure 3) than RN. These data suggest that there may be slight differences in the tertiary structures of SC and RN. In *S. cerevisiae*, the mitochondrial MnSOD is synthesized in the cytosol from a nuclear gene with a 26-residue N-terminal leader sequence (*19*), then transported to the mitochondria where it is processed to form the mature enzyme (*46*). Molecular chaperones are likely to be involved in the folding of the final product (*47*). An absolute requirement for the expression of active *S. cerevisiae* MnSOD in *E. coli* is the deletion of the 26-residue leader sequence (*29*). The absence of the

leader, along with the unavailability of the normal molecular chaperones, leads to the possibility that RN may be folded in a different manner than SC. The differences in the data noted above could be explained by this. However, if SC and RN have different folding patterns, the difference between the two must be slight, since they have nearly the same specific activity. In addition, the CD spectra of RN and SC in the far-UV region (190−260 nm) were indistinguishable (data not shown), suggesting that the overall folding of RN and SC are the same, and the CD spectra of RN in the near-UV (260−350 nm) and visible (350−800 nm) regions were nearly identical to the published CD spectra of SC (*26*). Nonetheless, investigators working on a eukaryotic mitochondrial MnSOD expressed in *E. coli* should take care to confirm that the recombinant native enzyme behaves in a fashion similar to that of the enzyme isolated from its natural source.

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