A Cambialistic Superoxide Dismutase in the Thermophilic Photosynthetic Bacterium Chloroflexus aurantiacus

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Superoxide dismutase from the thermophilic anoxygenic photosynthetic bacterium Chloroflexus aurantiacus was cloned, purified, and characterized. This protein is in the manganese- and iron-containing family of superoxide dismutases and is able to use both manganese and iron catalytically. This appears to be the only soluble superoxide dismutase in C. aurantiacus. Iron and manganese cofactors were identified by using electron paramagnetic resonance spectroscopy and were quantified by atomic absorption spectroscopy. By metal enrichment of growth media and by performing metal fidelity studies, the enzyme was found to be most efficient with manganese incorporated, yet up to 30% of the activity was retained with iron. Assimilation of iron or manganese ions into superoxide dismutase was also found to be affected by the growth conditions. This enzyme was also found to be remarkably thermostable and was resistant to H$_2$O$_2$ at concentrations up to 80 mM. Reactive oxygen defense mechanisms have not been previously characterized in the organisms belonging to the phylum Chloroflexi. These systems are of interest in C. aurantiacus since this bacterium lives in a hyperoxic environment and is subject to high UV radiation fluxes.

Superoxide dismutases (SODs) are an essential part of the first line of cellular defense against reactive oxygen species. Manganese-containing SODs (MnSODs) and iron-containing SODs (FeSODs) catalyze the conversion of superoxide to hydrogen peroxide at nearly the diffusion limit of the substrate in the two-step redox reaction (9, 15):

\[ \text{M}^{4+} + \text{O}_2^- \rightarrow \text{M}^{3+} + \text{O}_2 \]
\[ \text{M}^{2+} + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{M}^{3+} + \text{H}_2\text{O}_2 \]

Excessive levels of superoxide, along with hydrogen peroxide and hydroxyl radicals, can cause extensive oxidative damage to lipids, proteins, and nucleic acids. The sources of these reactive oxygen species include ionizing radiation, normal cellular metabolism, and metal-catalyzed oxidation systems (7).

SODs are metalloenzymes and are found in eukaryotes and aerobic and aerotolerant bacteria (10). The two primary families of these enzymes are the copper- and zinc-containing SODs (CuZnSODs) and the iron- or manganese-containing SODs (Fe/MnSODs). The CuZnSODs have little primary sequence or structural homology to the Fe/MnSODs and are sequence or structural homology to the Fe/MnSODs and are capable of using either manganese or iron catalytically. Some Cambialistic SODs normally contain various amounts of both metals, while others contain either iron or manganese exclusively depending on the availability of metals in the growth medium (2, 12).

Chloroflexus aurantiacus is a thermophilic photosynthetic bacterium that lives in shallow alkaline bacterial mats that flourish at pH 8.0 in hot spring water at 52 to 60°C (3, 20). These mats are directly exposed to near-UV radiation during the day, which is a potent source of oxidative stress (13). C. aurantiacus was previously found to have relatively high resistance to damage caused by UV radiation (19). This bacterium grows rapidly as a photolithotroph by using organic carbon, although it can also grow photoautotrophically by using either sulfide or hydrogen as an electron donor for carbon dioxide fixation (21). Chloroflexus cohabitates with various cyanobacterial Synechococcus species at and below the surface of the bacterial mats. This environment is extremely hyperoxic due to the local release of oxygen from Synechococcus photosynthesis during the day. In the top 1 mm of other cyanobacterium-containing mats, the oxygen levels are 200% of the air saturation levels (22). Oxidative stress in C. aurantiacus is also of interest because this bacterium is deeply rooted in the 16S rRNA tree of organisms and is in the earliest branch of phototrophs (26).

Here, we describe cloning, expression, and characterization of the SOD from C. aurantiacus. The SOD was expressed in Escherichia coli as an easily purified fusion construct with maltose binding protein (MBP). We show that this enzyme is cambialistic and is highly resistant to inhibition by hydrogen peroxide. Metal fidelity studies and growth medium metal enrichment experiments demonstrated that this enzyme is most efficient when manganese is incorporated, yet up to 30% of the activity is maintained with iron. Amino acid sequence analysis and phylogenetic analysis revealed that this SOD has an array of residues that are characteristic of both FeSODs and MnSODs.
Cloning and purification of SOD from *C. aurantiacus*. SOD was purified from cell extracts of *C. aurantiacus* strain J-10FL (= ATCC 29366) by ammonium sulfate precipitation, gel filtration chromatography, ion-exchange chromatography, and isoelectric focusing (IEF) for preliminary characterization (23). The SOD activity in partially purified samples was monitored by using the xanthine-xanthine oxidase-based SOD assay (see below). The large quantities of pure SOD that were needed for experiments were obtained by cloning and expression of the SOD gene in *E. coli*. To facilitate this, purified wild-type SOD (wtSOD) from *C. aurantiacus* was chemically N terminally sequenced (Porton 2090E protein sequence; Applied Biosystems, Foster City, Calif.). The N-terminal methionine was retained in the mature protein, indicating that the SOD was cytosolic and not exported to the periplasm. The resulting 52 amino acids were used for a BLAST analysis of the partially sequenced *C. aurantiacus* genome in the National Center for Biotechnology Information databank (taxonomy identification no. 1108). The BLAST search resulted in the first 397 bp of the SOD gene sequence in genome contiguous sequence (contig) 107. This 397-bp sequence expanded the protein sequence to 132 amino acids. The downstream gene region was located at the 5’ end of another segment of the *C. aurantiacus* genome. Sequencing of this contig indicated that the SOD sequence was derived by using incomplete Freund adjuvant. Antibodies were purified by acid elution of pure SOD in the same manner by using incomplete Freund adjuvant. Antibodies were purified by acid elution of pure SOD, which resulted in catalytically inactive SOD apoprotein. Neutral pH was used because this protein readily precipitated when it was exposed to acidic pH. The SOD sample was then divided and placed into five different dialysis bags (12.4 kDa). FeSOD from *C. aurantiacus* was chemically N terminally sequenced (Porton 2090E protein sequence; Applied Biosystems, Foster City, Calif.). A pH gradient was established inside the IEF cell by using Bio-Lyte 3/5 ampholytes (Bio-Rad). Pure SOD was isolated from pH 3.7 to 3.9 and was then thoroughly dialyzed into 20 mM Tris (pH 8.0).

**Western blotting.** Antiserum was prepared by injecting polyclonal antibody gel slices containing approximately 100 μg of wtSOD into rabbits. Gel slices were homogenized in 1 ml of phosphate-buffered saline and 1 ml of complete Freund’s adjuvant. Three boosts of 100 μg of wtSOD were administered in the same manner by using incomplete Freund’s adjuvant. Antibody was produced by Rockland, Inc., Gilbertsville, Pa. Antibodies were purified by the rabbit serum by first desalting it with an Econo-Pac DG-10 column, and then pure immunoglobulin G was isolated by using an Econo-Pac serum immunoglobulin G purification chromatography column (Bio-Rad). Western blotting was performed by electrophoresing 10 μg of *C. aurantiacus* whole-cell extract and 5 μg of pure recombinant SOD on a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gel, which was then transferred to an Immobilon-P transfer membrane (Millipore, Bedford, Mass.) for 8 h at 100 mA. The protein concentrations of SOD samples were quantified by using a Coomassie Plus protein assay reagent kit (Pierce, Rockford, Ill.). The membrane was blocked in TBST (0.1 M Tris HCl [pH 7.5], 0.1% Tween 20) for 2 h containing 5.0% milk (3 h). The membrane was then transferred to a 1:3,000 solution of SOD primary antibody in TBBS for 30 min, and this was followed by three washes with TBSS. The membrane was then soaked in a 1:10,000 dilution of alkaline phosphatase-labeled secondary anti-rabbit antibody (Novagen, Madison, Wis.) in AP buffer (10 mM Tris HCl [pH 9.5], 100 mM NaCl, 5 mM MgCl2) for 30 min. The membrane was then washed three times in TBSS and once in AP buffer. The blot was developed by mixing 66 μl of an immunopure 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) (Pierce) solution (50 mg/ml in 100 mM dimethylformamide [Fisher Scientific, Pittsburgh, Pa.]) and 34 μl of a p-nitroblue tetrazolium chloride (U.S. Biochemicals, Cleveland, Ohio) stock solution (50 mg/ml in 70% dimethylformamide) with 10 ml of AP buffer. When bands appeared, the blot was removed from the solution and dried on filter paper.

**Mass spectrometry.** Mass spectrometry data were collected with a 10 μM sample of recombinant SOD cleaved from MBP in 20 mM Tris (pH 8.0). The instrument used was a DE STR mass spectrometer from Applied Biosystems. Mass spectra were acquired in the linear, positive-ion mode by using delayed extraction. Protein samples were mixed with an equal volume of a saturated solution of sinapinic acid dissolved in a mixture of 1% trifluoroacetic acid in water and acetonitrile (2:1), and 1 μl of the mixture was dried on a stainless steel sample plate. The spectrum was the average of 100 laser shots.

**Subunit determination.** The molecular weight of native SOD was determined by gel filtration on a Sepharose 100 high-resolution column (1.5 by 100 cm). The column was equilibrated with 20 mM Tris–150 mM NaCl (pH 8.0). The column was calibrated with a molecular mass marker kit (12 to 200 kDa; Sigma) containing blue dextran (2,000 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa). FeSOD from *C. aurantiacus* (37 kDa; Sigma) was also used as a column standard.

**Phylogenetic analysis.** SOD sequences of various bacteria were obtained from the National Center for Biotechnology Information website. Only sequences of SODs whose metal contents have been experimentally characterized were used. Protein sequences were aligned with the program Clustal W. A phylogenetic tree was inferred by using Mega (version 2) and the neighbor-joining tree method with 500 bootstrap replicates (16).

**Metal analysis.** Metal analysis and quantification were performed by using a Varian SpectrAA-400 Zeeman graphite furnace atomic absorption spectrometer. Iron and manganese standard solutions were obtained from VHG Labs, Manchester, N.H. A manganese standard curve was obtained by using 0.1% manganese in 18-MΩ water and Mn2+ concentrations of 0.5, 5.0, 10, 12.5, and 15 μg/liter. Similarly, an iron standard curve was obtained by using 0.1% nitric acid and Fe3+ concentrations of 1, 5, 10, 20, and 25 μl/g liter. The absorbance of manganese and the absorbance of iron were measured at 297.5 and 248.3 nm, respectively. SOD samples were diluted in nanopure water with 0.1% nitric acid until the absorbance fell in the range on the standard curves. Protein concentrations of SOD samples were determined by using a Coomassie Plus protein assay reagent kit (Pierce).

Purified catalytically active MBP-SOD fusion protein was used in metal analysis and electron paramagnetic resonance (EPR) assays. The fusion protein was in 20 mM Tris (pH 8.0). The fusion protein was also concentrated with a Centricon-3 concentrator (Amicon, Beverly, Mass.), and its flowthrough was used as a control for both the atomic absorption and EPR analyses. MBP was cleaved from SOD and purified during the IEF step was also used as a control in the atomic absorption measurements.

**EPR spectroscopy.** EPR measurements were obtained at about 8 K by using a Bruker EPR ES80 ELEXYSX spectrometer with an Oxford Instruments ESR900 liquid helium flow cryostat (Bruker, Billerica, Mass.). The instrument conditions were as follows: microwave frequency, 9.42 GHz; magnetic field modulation frequency, 100 kHz; modulation amplitude, 15 G; sweep range, 2.250 ± 2.000 G; sweep time, 336 s; time constant, 0.082 s; microwave power, 5.0 mW; and horizontal resolution, 2.048 points. Each spectrum was the average of four scans.

**Spectrophotometry-based activity assays.** The specific activities of SOD samples were determined by using the xanthine-xanthine oxidase-based method as previously described (11). All reactions were purchased from Sigma. Samples were then thoroughly dialyzed in 20 mM Tris (pH 8.0) prior to the assay.

**Native polyclonal gel-based SOD activity assays.** Electrophoresis was performed by using a Bio-Rad Mini-Protean 3 cell with 25 mM Tris–0.192 M glycine–0.1% SDS (pH 8.3) as the electrode buffer. Analytical gels with 5% acrylamide stacking gels and 15% acrylamide resolving gels were used. The protein reference was the Benchmark prestained protein ladder (Invitrogen, Carlsbad, Calif.). Denatured samples were boiled for 15 min after addition of 0.1% SDS, 2-mercaptoethanol, 0.5 M Tris (pH 6.8), and 0.5% bromophenol blue. Native samples were prepared in sample buffer without 2-mercaptoethanol and were not boiled. Native gel-based activity assays were performed by the p-nitroblue tetrazolium chloride-based photochemical method (5).

**Reconstitution of MBP-SOD in the presence of MnSO4 and Fe3Sod.** MBP-SOD was denatured by dialysis into 8 M urea–10 mM EDTA–50 mM Tris (pH 8.0) for 24 h at 25°C. The sample was then dialyzed into 50 mM Tris (pH 8.0) for 24 h at 4°C, which resulted in catalytically inactive SOD apoprotein. Neutral pH was used because this protein readily precipitated when it was exposed to acidic pH. The SOD sample was then divided and placed into five different dialysis bags with molecular mass cutoffs of 6 to 8 kDa (Spectrum, Gardena, Calif.), and it was dialyzed into 8 M urea–50 mM Tris (pH 8.0) with the following metals added for 4 h at 4°C: (i) 10 mM FeSod, (ii) 10 mM FeSO4 and 1 mM MnSO4, (iii) 10 mM FeSO4 and 10 mM MnSO4, (iv) 1 mM FeSO4, and 10 mM MnSO4, and (v) 10 mM MnSO4. Using the same concentrations of metals and Tris buffer, the urea concentration was stepped down from 8 M to 6, 4, and 2 M for 4 h each at 4°C. The samples were then collected in 50 mM Tris (pH 8.0) with 0.5 mM EDTA, and each metal concentration was reduced 10-fold. Finally, each sample was placed in 50 mM Tris (pH 8.0) with 0.5 mM EDTA for 24 h at 4°C. The
specific activity of each sample was measured by the xanthine-xanthine oxidase SOD assay as described above.

Nucleotide sequence accession number. The GenBank accession number for the complete nucleotide sequence of the SOD gene of *C. aurantiacus* is AY289213.

**RESULTS**

The SOD from *C. aurantiacus* was isolated and partially sequenced chemically (23). The gene coding for SOD was cloned, and the protein was expressed as a fusion protein with MBP in *E. coli*. The 65-kDa fusion protein was purified by using an amylose column (Fig. 1A). Pure recombinant SOD was obtained by IEF after the MBP-SOD fusion protein was cleaved with the factor Xa protease (Fig. 1B). Western blotting with antibodies raised against SOD purified from *C. aurantiacus* was performed to verify the integrity of the recombinant SOD (Fig. 1C). The monomeric mass of recombinant SOD was found to be 23.15 kDa by mass spectrometry (Fig. 2). A second peak at 22.96 kDa was found, and this peak probably represented cleavage of a single amino acid from the four residues that were added to the N terminus of SOD during cloning. The molecular mass of pure native recombinant SOD was determined by gel filtration chromatography to be 49.3 kDa, indicating that the molecule is a dimer (standard curve not shown).

The gene sequence of *C. aurantiacus* SOD is 612 bp long, and the translated protein sequence is 204 amino acids long (Fig. 3) (GenBank accession number AY289213). A neighboring tree was constructed with the SOD protein sequence from *C. aurantiacus* by using bacterial and archael SOD protein sequences that have been experimentally determined to contain iron or manganese or to be cambialistic (Fig. 4). This tree shows that *C. aurantiacus* SOD groups with MnSODs and cambialistic SODs, which is consistent with the protein sequence analysis and experimental data.

Atomic absorption analysis of MBP-SOD samples demonstrated that 1.345 ± 0.045 atoms of iron and 0.014 ± 0.002 atom of manganese were present in each monomer of SOD, when it was expressed in the normal *E. coli* system without metal enrichment. The levels of copper, zinc, and nickel were below the detection limits. Buffer extracted from each sample with a Centricron concentrator, as well as MBP cleaved from SOD and purified by IEF, did not contain measurable amounts of Mn, Fe, Cu, Zn, or Ni.

EPR spectroscopy confirmed the presence of coordinated iron and some manganese in the SOD (Fig. 5A and B). Iron was the primary metal present in this SOD. The EPR-active form of iron (as determined by using standard EPR methodology) is Fe(III), and the features near 1,570 G (about *g* = 4.3) in Fig. 5A and B are characteristic of high spin (S = 5/2 ferric iron in a strongly rhombic environment) (24). Adventitiously bound Fe(III) can produce similar spectra. However, the fact that SOD activity was retained in the presence of diethylene-triaminepentaacetic acid (Sigma), an effective chelator of iron, along with the enzyme’s ability to replace Fe(III) with Mn(III) in manganese-enriched growth media (see below), indicates that the absorption at *g* = 4.3 was not due solely to nonspecifically bound iron. Less intense peaks extending below 1,000 G in Fig. 5A were associated with the iron signal at *g* = 4.3.

**FIG. 1.** SDS-polyacrylamide gel electrophoresis and Western blotting of purified recombinant *C. aurantiacus* SOD. (A) The protein fraction was isolated from *E. coli* ER2508 expressing *C. aurantiacus* SOD as the MBP-SOD fusion protein. MBP-SOD was isolated by using an amylose column. Lane 1, protein ladder; lane 2, *E. coli* whole-cell extract with MBP-SOD; lane 3, amylose column flowthrough; lane 4, pure MBP-SOD eluted from the amylose column. (B) Pure SOD was obtained by factor Xa cleavage of MBP-SOD and was isolated by IEF. Lane 1, protein ladder; lane 2, pure recombinant SOD. (C) Western blot with anti-wtSOD antibodies. Lane 1, prestained protein ladder; lane 2, *C. aurantiacus* whole-cell extract; lane 3, pure recombinant SOD.

**FIG. 2.** Mass spectrum of SOD and MBP cleaved by factor Xa. The SOD peak is at 23,153.01 Da, and there is a second peak at 22,963.33 Da. This second peak is most likely a secondary cleavage product produced by factor Xa. MBP is at 42,491.32 Da, with the doubly charged peak at 21,236.06 Da.

**FIG. 3.** Deduced amino acid sequence of *C. aurantiacus* SOD. This sequence is available from GenBank under accession number AY289213. The N-terminal factor Xa cleavage site and putative secondary structure regions are indicated, and metal-coordinating residues are indicated by boldface type.

**FIG. 4.** Joining tree was constructed with the SOD protein sequence from *C. aurantiacus* by using bacterial and archael SOD protein sequences that have been experimentally determined to contain iron or manganese or to be cambialistic. This tree shows that *C. aurantiacus* SOD groups with MnSODs and cambialistic SODs, which is consistent with the protein sequence analysis and experimental data.

**FIG. 5.** In Fig. 5A and B are characteristic of high spin (S = 5/2 ferric iron in a strongly rhombic environment) (24). Adventitiously bound Fe(III) can produce similar spectra. However, the fact that SOD activity was retained in the presence of diethylene-triaminepentaacetic acid (Sigma), an effective chelator of iron, along with the enzyme’s ability to replace Fe(III) with Mn(III) in manganese-enriched growth media (see below), indicates that the absorption at *g* = 4.3 was not due solely to nonspecifically bound iron. Less intense peaks extending below 1,000 G in Fig. 5A were associated with the iron signal at *g* = 4.3.
Since Mn(III) was incorporated into the active site, it was EPR silent and therefore was not found in the spectra of the native samples (Fig. 5A and C). The characteristic hydrated Mn(II) spectrum was obtained only when Mn(III) was released from the protein active site upon acidification of the sample (Fig. 5B and D). Thus, the complex set of features in the range from 3,000 to 4,000 G was due to Mn(II). There were six lines, spaced 90 to 100 G apart, with nearly the same amplitudes. This so-called hyperfine structure arose from the magnetic interaction of the electron spin ($S = 5/2$) on Mn(II) and the
nuclear spin (I = 5/2) of $^{55}$Mn. In the general case of nuclear spin I, 2I + 1 hyperfine lines are observed. The origin of the sharp step near 3,400 G in the spectrum in Fig. 5B is not known, but it was probably an artifact arising from a radical contaminant.

To determine if C. aurantiacus SOD can coordinate manganese instead of iron, E. coli expressing the C. aurantiacus SOD gene was grown in medium enriched with 1 mM MnSO$_4$. SOD was purified as described above, and the metal content was analyzed. Atomic absorption analysis indicated that this SOD contained 0.004 ± 0 atom of iron and 0.199 ± 0.007 atom of manganese per SOD subunit. The EPR spectra indicated that manganese was the sole metal ion in this sample (Fig. 5C and D).

The specific activities of various SOD samples were measured by using the xanthine-xanthine oxidase-based SOD activity assay, as described above (Table 1). The iron- and manganese-containing MBP-SOD purified from the E. coli culture enriched with 1 mM MnSO$_4$ was found to have an activity of 683.5 U/mg of protein. This is about threefold higher than the activity of the primarily iron-containing MBP-SOD from E. coli grown in LB broth alone. The iron-containing sample of SOD was enzymatically separated from MBP and purified by IEF and was found to have a specific activity of 421 U/mg of protein. Since there was approximately threefold less SOD present per milligram of protein in the fusion protein sample than in the purified cleaved SOD sample and since the pure cleaved SOD had been subjected to significantly more purification procedures, the presence of MBP does not seem to significantly hinder the activity of SOD.

The effect of metal fidelity on the enzymatic activity of C. aurantiacus SOD was investigated by an in vitro reconstitution experiment with manganese sulfate and ferrous sulfate (Table 1). The SOD sample that was reconstituted exclusively in the presence of manganese had a ninefold-higher specific activity than the SOD reconstituted in the presence of only iron. Some precipitation of protein occurred during the reconstitution of SOD with iron alone and with manganese and iron at a ratio of 1:10. The precipitate was removed by centrifugation, and the activity of the soluble fraction was assayed. In this case, the specific activity of the MBP-SOD from the E. coli culture grown in normal LB broth should have been a more accurate representation of the activity of iron-substituted SOD from C. aurantiacus. The activity of SOD was shown to decrease as manganese was replaced by iron. However, the in vivo iron-substituted SOD still retained up to 30% of its original activity. Apo-SOD retained a minimal amount of activity, which was probably due to the neutral pH required to make the apoprotein. The samples reconstituted in the presence of iron only and in the presence of manganese only were acidified and were found to have no measurable activity. This demonstrated that the free metals in solution are not responsible for the activity. Atomic absorption spectrometry was performed with the reconstituted samples, and the results were inconclusive.

It is interesting that this enzyme also resisted thermal inactivation. The enzymatic activity of SOD reconstituted with manganese was not significantly affected by boiling for 5 min. Furthermore, addition of 2-mercaptoethanol did not affect the activity of a sample, which was probably due to the absence of any cysteine residues in the SOD.

Native gel activity assays were done to visualize the hydroperoxide sensitivity of the purified recombinant SOD (Fig. 6). The SOD remained active, and the activity was not significantly changed by exposure to H$_2$O$_2$ at concentrations up to 80 mM, which is consistent with the properties of MnSODs. E. coli MnSOD and FeSOD were included in the experiment as controls to illustrate the inactivation of FeSODs by peroxide and the natural resistance of MnSODs to peroxide (8).

**DISCUSSION**

The SOD from C. aurantiacus is a remarkably stable enzyme that resists the inactivating and denaturing effects of boiling, SDS, and high levels of hydrogen peroxide. Purified recombinant C. aurantiacus SOD isolated from E. coli was found to contain 100-fold more iron than manganese. This SOD was
also found to be resistant to inactivation by hydrogen peroxide, a characteristic of MnSODs. Indeed, when the E. coli culture was enriched with 1 mM MnSO₄, the SOD was found to primarily contain manganese. The amounts of Fe and Mn were quantified and found to be substoichiometric in this case, although the reason for this is not clear. The two cultures were both grown aerobically. Cambialistic SODs can primarily incorporate oxidized Mn(III) upon aeration of the medium, while Fe(II) dominates during anaerobic growth, as shown for Bac teroides gingivalis (1). However, SOD from C. aurantiacus, like the cambialistic SOD from Propionibacterium shermanii, incorporated Fe(III) in aerated media (12). Mn(III) was incorporated into this SOD only when excess manganese was added to the medium. This SOD has a significant affinity for iron, yet the activity of the manganese-containing enzyme is about threefold higher than the activity of the iron-containing enzyme. This trend was supported by the activities of the reconstituted samples. Clearly, this SOD has the ability to use either metal and, assuming that the secondary structures are the same, acquires the two metals to different degrees depending on their availability in the culture medium.

A partially purified sample of wtSOD from C. aurantiacus was also analyzed by atomic absorption spectrometry to determine its metal content. This SOD sample contained two-fold more manganese than iron. This is consistent with the fact that the medium used to grow laboratory cultures of C. aurantiacus contains 3.75-fold more manganese than iron (4).

Proteins of hyperthermophilic bacteria have previously been found to contain a larger portion of charged residues for the purpose of forming stabilizing ion pairs (17, 18). C. aurantiacus SOD actually contains 35% fewer charged residues than the mesophilic MnSOD from E. coli. This may indicate that C. aurantiacus, which grows at temperatures up to 62°C, does not live at high enough temperatures to require the stabilizing properties of ion pairs in its proteins.

The protein sequence of SOD from C. aurantiacus has characteristics of both FeSODs and MnSODs, as well as some unique features. The tertiary structure of each monomer in dimeric Fe/Mn-type SODs consists of two domains. The secondary structure of the first domain is α-helical, and the second domain is α-β fold. The four conserved residues of C. aurantiacus that ligate the metal atom are most likely His27 and His79 in the first domain and Asp165 and His169 in the second domain, based on alignment with SODs whose crystal structures are known, such as the SOD of P. shermanii (data not shown).

An analysis of the primary amino acid sequence revealed features of the C. aurantiacus SOD that are common to both FeSODs and MnSODs of bacteria. An extensive review of the primary sequences and structures of FeSODs, MnSODs, and cambialistic SODs was performed by Jackson and Cooper in 1998 (14), and the results will be compared to our data for C. aurantiacus SOD. There are some unusual features of the C. aurantiacus SOD in the first helix starting at His25 with the sequence H-Y-H-H-D-N-H-H. The first two residues, His25 and Tyr26, are basic and aromatic residues, respectively. Other FeSODs and MnSODs normally have an acidic Glu residue and a neutral Leu residue or similar residues at these positions. Asn30 is similar to the normally conserved Lys at this position, which has been found to be involved in guiding the O₂ sub-strate into the active site (25). Interestingly, His32 is not typically conserved in cambialistic SODs.

The other helix in the first domain starts with Arg73, and its sequence is R-N-N-G-G-H-W-N-H-T-F-F-W; His82 is the second metal ligand. This sequence is more representative of MnSODs; the only exception is the Asn residue at position 74, at which there is usually an aromatic residue, such as Phe, while Asn is found in FeSODs at this position. Also, the cambialistic SODs of Bacteroides fragilis and B. gingivalis contain Phe at position 86, whereas C. aurantiacus cambialistic SOD has the conserved Trp residue at this position.

In the second domain, the first β-strand begins with Gly128, and its sequence is G-S-G-W-A-W-L; the G-W is typical of MnSODs, and the last Leu is conserved in FeSODs. The second β-strand of SODs does not exhibit high homology, but this strand may begin at Leu142 and extend for about seven residues. The sequence between the second and third β-strands is N-Q-D, which is also conservatively changed in MnSODs. The third β-strand contains the other two metal ligands, Asp165 and His169.

A neighbor-joining tree was constructed with the SOD protein sequence from C. aurantiacus by using bacterial and archaeal SOD protein sequences. It is interesting that the archaeal clade (Methanothermibacter thermoautotrophicus, Halobacterium cutirubrum, Sulfolobus solfataricus, and Acidianus ambivalens) grouped with the host-dependent pathogenic mycobacteria (P. shermanii, Mycobacterium tuberculosis, Mycobacterium fortuitum, and Nocardia asteroides). Also, the monophyletic proteobacterial group (Pseudomonas putida, Rhodobacter capsulatus, E. coli, Methylo monas sp., and Aeromonas hydrophila) is interrupted by a gram-positive bacterium (Bacillus caldothermalis) and by C. aurantiacus, which is a photosynthetic green nonsulfur bacterium. This phylogenetic tree is not consistent with 16S rRNA data and may be evidence that there was horizontal gene transfer.

The SOD from the filamentous anoxygenic photosynthetic bacterium C. aurantiacus was found to be cambialistic. This was the only SOD found in the soluble protein fraction and therefore fulfills the vital need in C. aurantiacus for an efficient and flexible defense against the accumulation of the toxic superoxide anion. In future experiments we will focus on the inducibility of the SOD in C. aurantiacus under oxidative stress conditions, such as high UV radiation and high levels of hydrogen peroxide.

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