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 incapable 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 phototroph 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 and is 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-10 FL (= 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 cysteolic 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 database (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 (sequence C. aurantiacus contig 862) containing 132 contiguous bases of sequence. To find the remaining downstream region of the SOD gene, the expectation value of the BLAST search was increased to 10 to reveal more distantly related sequences. There was enough overlap between the 132 known residues and the correctly sequenced portion of contig 862 to reveal the remaining 3' portion of the SOD gene. The rest of the 3' SOD gene region was sequenced by reverse primer direct sequencing of SOD on contig 862. The resulting complete gene sequence was used to construct the following primers containing unique EcoRI and PstI restriction sites (underlined): forward primer 5' CGAGAGGATTACGTGCCCCTGATGTTCCG 3' and reverse primer 5' CGACC[CGTGCAG]CCAGTGTGCCATAAGCCAGGGC 3'. The PCR product was cloned into the pMal-c2 vector and transformed into E. coli strain ER2508 (New England Biolabs, Beverly, Mass.). E. coli was grown both in Luria-Bertani (LB) media and in LB media supplemented with 1 mM MnSO4. The MBP-SOD fusion protein was expressed and purified by using methods described in the New England Biolabs manual for the pMal-c2 expression system. Cleavage of the pure fusion protein was performed by incubation with 5% Factor Xa protease at 4°C for 24 h. The 42-kDa MBP was separated from the 23.2-kDa SOD by IEF with a Rotorod preparative IEF cell (Bio-Rad, Hercules, Calif.). A pH gradient was established inside the IEF cell by using Bio-Lyte 3/5. The membrane was then soaked in a 1:10,000 dilution of alkaline phosphatase-reagent kit (Pierce, Rockford, Ill.). The membrane was then blocked in TTBS (0.1 M sodium bicarbonate pH 8.0) for 24 h. The membrane was transferred to a 1:3,000 solution of SOD primary reagent kit (Pierce). The membrane was blocked in TTBS (0.1 M sodium bicarbonate buffer) (16). The membrane was placed in 50 mM Tris (pH 8.0) with 0.5 mM EDTA for 24 h at 4°C. The membrane was then placed in 50 mM Tris (pH 8.0) with 0.5% milk (3%H). The membrane was then transferred to a 1:3,000 solution of SOD primary antibody in TTBS for 30 min, and this was followed by three washes with TTBS. 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 7.5], 0.15% NaCl, 0.01% Tween 20) containing 5% milk (3%H) for 30 min. The membrane was then transferred to a 1:3,000 solution of SOD primary antibody in TTBS for 30 min, and this was followed by three washes with TTBS. 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 TTBS and once in AP buffer. The blot was developed by mixing 66 µl of an immunopure 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) (Pierce) solution (50 mg/ml in 100% 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), cytochrome c (12.4 kDa), FeSOD from E. coli (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 SpectraAA-40 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% nitric acid in 18-MΩ water and Mn2+ concentrations of 0.5, 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 µg/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 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 E850 ELEXYS 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 the xanthine-xanthine oxidase-based method as previously described (11). All reactions were purchased from Sigma. Samples were then dialyzed in 20 mM Tris (pH 8.0) prior to the assay.

Native polyacrylamide 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 photophysical method (5).

Reconstitution of MBP-SOD in the presence of MnSO4 and FeSO4. 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 FeSO4, (ii) 10 mM FeCl3, and 1 mM MnSO4, (iii) 10 mM FeCl2, 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 resulting samples were placed 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 molecular 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* is 612 bp long, and the translated protein sequence is 204 amino acids long (Fig. 3) (GenBank accession number AY289213). A neighbor-joining tree was constructed with the SOD protein sequence from *C. aurantiacus* by using bacterial and archaeal 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 Centricon 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.
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 

![EPR spectra](http://jb.asm.org/)

FIG. 4. Neighbor-joining tree of experimentally characterized SODs from bacteria and archaea. The GenBank accession numbers for the protein sequences used are AAL26890, A38461, P00448, CAA44556, CAAl227, P09738, AAC64207, AAT2217, NP_341862, AAF36989, BAA00489, 1BT8_B, P17670, CAA50266, AAA91964, and NP_743076.

FIG. 5. EPR spectra of MBP-SOD isolated from E. coli grown under two conditions. (A and B) Normal LB growth medium, with production of native MBP-SOD (A) and acidified MBP-SOD (B). (C and D) LB medium supplemented with 1 mM MnSO₄, with production of native MBP-SOD (C) and acidified MBP-SOD (D). Iron and, to a lesser extent, manganese were present in MBP-SOD, and manganese was the only metal detected when E. coli was grown in the presence of 1 mM MnSO₄.
TABLE 1. Specific activities of SOD samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sp act (U/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>MBP-SOD</td>
<td>224.0 ± 14.4</td>
</tr>
<tr>
<td>Pure recombinant SOD</td>
<td>421.0 ± 28.0</td>
</tr>
<tr>
<td>MBP-SOD from E. coli medium enriched</td>
<td>683.5 ± 68.4</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>11.0 ± 0.01</td>
</tr>
<tr>
<td>MBP-SOD apoprotein</td>
<td>572.7 ± 62.9</td>
</tr>
<tr>
<td>Mn reconstruction</td>
<td>447.0 ± 44.7</td>
</tr>
<tr>
<td>Mn-Fe (10:1) reconstitution</td>
<td>288.7 ± 18.8</td>
</tr>
<tr>
<td>Mn-Fe (1:10) reconstitution</td>
<td>68.0 ± 6.2</td>
</tr>
<tr>
<td>Fe reconstitution</td>
<td>63.0 ± 5.7</td>
</tr>
<tr>
<td>Mn reconstitution, boiled for 5 min</td>
<td>44.7 ± 28.4</td>
</tr>
<tr>
<td>Controls (Mn and Fe reconstitution, ac</td>
<td>0</td>
</tr>
</tbody>
</table>

*The specific activity of each sample was determined by the xanthine-xanthine oxidase-based SOD assay, as described in Materials and Methods. One unit was defined as the amount of SOD that reduced the rate of cytochrome c reduction by 50%.

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 MnSO₄ 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 normal 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 specificity of 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 hydrogen peroxide sensitivity of the purified recombinant SOD (Fig. 6). The SOD remained active, and the activity was not significantly changed by exposure to H₂O₂ 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 *Bacteroides 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 reconstructed 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 twofold 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 sequence H-Y-H-H-D-N-H-H of *C. aurantiacus* SOD is more representative of MnSODs than FeSODs, 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 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 archael clade (Methanothermabacter thermotrophicus, 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, Methylomonas sp., and Aeromonas hydrophila) is interrupted by a gram-positive bacterium (*Bacillus caldothermal*) 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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