Iron- and Manganese-Containing Superoxide Dismutases from *Methylomonas* J: Identity of the Protein Moiety and Amino Acid Sequence

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Received May 4, 1990; Revised Manuscript Received December 6, 1990

ABSTRACT: Mn-superoxide dismutase (SOD) and Fe-SOD were isolated from *Methylomonas* J, an aerobic methylotrophic bacterium, grown in methylamine media containing either manganese (Mn-rich medium) or iron (Fe-rich medium), respectively. The specific activity of the Mn-SOD was 2250 units mg^{-1} (mol of Mn)$^{-1}$ (mol of dimer)$^{-1}$, and the metal content of the enzyme was 0.98 mol of Mn and 0.12 mol of Fe per mole of dimer, while those of Fe-SOD were 88.5 units mg^{-1} (mol of Fe)$^{-1}$ (mol of dimer)$^{-1}$ and 1.04 mol of Fe and 0.02 mol of Mn. The electrophoretic mobilities in the presence of sodium dodecyl sulfate, with or without urea, and the chromatographic behavior on an HPLC column using an octadodecyl silicated column and a gel permeation column were identical. Amino acid compositions were practically indistinguishable in both SODs. The enzyme activity was restored by dialysis of an apoprotein obtained from the Mn-enzyme with either manganese sulfate or ferrous ammonium sulfate up to an activity level similar to that for the native Mn-SOD and the native Fe-SOD, respectively. The same result has been reported with the reconstitution using an apoprotein obtained from the Fe-enzyme [Yamakura, F., Matsumoto, T., & Terauchi, K. (1990) *Free Radical Res. Commun.* (in press)]. These results suggest the possibility that both types of SODs are composed of a single apoprotein synthesized in cells grown in either the Fe-rich medium or the Mn-rich medium. The amino acid sequence of Fe-SOD was deduced by analyses of peptide fragments derived from limited hydrolysis of apoprotein with lysylendopeptidase. Alignment of the peptide sequences with published amino acid sequences of Fe- and Mn-SOD suggests that the amino acid sequence of *Methylomonas* SOD resembles closely that of the Mn-SOD, except that a few amino acid residues are substituted for the Fe-SOD-specific amino acid residues from the other amino acid residues. We discuss possible candidates of amino acid residues which may weaken a specificity of the metals to exhibit the enzyme activity of *Methylomonas* SOD.

Three classes of superoxide dismutase (SOD)[1] have been distinguished according to their bound metals: an iron-containing SOD, a manganese-containing SOD, and a copper/zinc-containing SOD. The Mn- and Fe-SODs appear to be closely related both in structural and in evolutionary terms but have no resemblance to Cu/Zn-SOD (Fridovich, 1974; Steinman, 1983; Bannister et al., 1987). Recently, complete amino acid sequences have been determined by protein chemical analyses or gene base sequence analyses on the Mn-SOD from *Escherichia coli* (Steinman, 1978), *Bacillus stearothermophilus* (Brock & Walker, 1980), *Thermus thermophilus* (Sato et al., 1987), *Halobacterium cutirubrum* (Salin et al., 1988), *Saccharomyces cerevisiae* (Ditlow et al., 1982), and human liver (Barra et al., 1984) and on the Fe-SOD from *Photobacterium leiognathi* (Barra et al., 1987), *Escherichia coli* (Schinina et al., 1987), *Pseudomonas ovalis* (Isobe et al., 1987), and *Anacytis nidlans* (Landenbach et al., 1989). Comparison of the amino acid sequences showed considerable structure homology of the Fe- and Mn-SOD. The X-ray diffraction studies of Mn-SOD for *T. thermophilus* (Stalling et al., 1985) and *B. stearothermophilus* (Parker & Blake, 1988a) and of Fe-SOD from *P. ovalis* (Ringe et al., 1983; Stoddard et al., 1990) and *E. coli* (Stalling et al., 1983) have further demonstrated that these isozymes have a very similar three-dimensional fold of polypeptide chain and a similar active-site structure. The X-ray studies also identified the same metal ligands for both Mn- and Fe-SODs.

Previous reconstitution experiments, however, indicated that the metal requirement of the Mn- and Fe-enzyme was highly specific; namely, Fe-substituted Mn-SODs or Mn-substituted Fe-SODs retained little or no enzyme activity (Ose & Fridovich, 1976; Brock & Harris, 1977; Puget et al., 1977; Yamakura & Suzuki, 1980). This indicates that the metal ligand environments or the active-site geometries of these isozymes must be somewhat different. Recently, the result of X-ray analysis at 2.4 Å on the Mn-SOD from *T. thermophilus* (Stalling et al., 1985) and *B. stearothermophilus* (Parker & Blake, 1988a) has shown that the metal was surrounded by a group of aromatic amino acid residues. By comparison of these amino acid residues in the Mn-SODs with the amino acid residues of Fe-SODs from *P. ovalis* and *E. coli*, which were located at the same positions of the Mn-SOD in the aligned amino acid sequence, Parker et al. (1987), Parker and Blake (1988b), Isobe et al. (1988), Yamakura et al. (1989), and Carlioz et al. (1988) proposed that the conversion of glycine-77,[2] phenylalanine-84, and glutamine-155 in Mn-SODs to glutamine-77, tyrosine-84, and alanine-155 might account for the metal specificity of Mn- and Fe-SODs.

On the other hand, some anaerobic bacteria produce either iron- or manganese-type SODs depending on the metal condition of the medium, in which the metals may occupy the

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[1] Abbreviations: SOD, superoxide dismutase; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

[2] In this paper, amino acid residues are numbered by the positions in the aligned sequence in Figure 6.

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same site of the same protein (Meier et al., 1982; Gregory & Dapper, 1983; Martin et al., 1986; Pennington & Gregory, 1986). These SODs are believed to be a new SOD family, named "cambialistic SOD" (Martin et al., 1986). In a previous paper (Yamakura et al., 1990), we showed evidence that an obligate methylotrophic bacterium, *Methylomonas* J, had an Fe-SOD with low activity or a Mn-SOD with high activity, depending on the condition of the metals in the media. We also showed an identity of the amino-terminal sequence of these SODs and succeeded in reconstitution of Fe-SOD with manganese and iron (Yamakura et al., 1990). The Fe-reconstituted enzyme showed similar activity to the purified Fe-SOD, and the Mn-reconstituted enzymes showed similar activity to the purified Mn-SOD. In this report, we describe the reconstitution of Mn-SOD by Mn and Fe and present further evidence which suggests an identity of the protein moiety of the enzyme. These results suggest that *Methylomonas* SOD might be the same class of SOD which is capable of accepting either Fe or Mn for its activity. We also show the total amino acid sequence of the *Methylomonas* SOD. By comparison of the sequence with the sequences of other Fe- and Mn-SODs, we discuss the possible alternation of the amino acids residues, which may cause the reduction of the metal specificity for the activity of *Methylomonas* SOD.

Materials and Methods

Cell Culture. *Methylomonas* J was grown on a methylamine medium at 30 °C for 2 days. The medium contained (mg/L) the following: methylamine hydrochloride, 5000; K_2HPO_4, 1360; $NaH_2PO_4 \cdot 2H_2O$, 2490; EDTA, 202; $MgSO_4 \cdot 7H_2O$, 290; $CaCl_2 \cdot 2H_2O$, 66; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 186; $ZnSO_4 \cdot 7H_2O$, 11; $CuSO_4 \cdot 5H_2O$, 0.4; $Co(NO_3)_2 \cdot 6H_2O$, 0.25; $Na_2B_4O_7 \cdot 10H_2O$, 0.18. Iron and manganese contents of the medium were controlled by the addition of 10 mg/L $FeSO_4 \cdot 7H_2O$ for the iron-rich medium and 8.5 mg/L $MnSO_4 \cdot 4H_2O$ and 0.08 mg/L $FeSO_4 \cdot 7H_2O$ for the manganese-rich medium.

Purification of Fe-SOD from Methylomonas J. The cells were grown on the iron-rich medium for the purification of Fe-SOD from *Methylomonas* J. Wet cells (368 g) that had been stored at −30 °C were homogenized with 1500 mL of 20 mM potassium phosphate buffer, pH 7.8, and then sonicated. These and subsequent operations were carried out at 4 °C. The sonicated preparation was centrifuged at 15000*g* for 30 min, and the precipitate was suspended in an additional 1100 mL of the same buffer and again sonicated and centrifuged. The two supernatants were combined and fractionated with $(NH_4)_2SO_4$, and the 45–95%-saturated fraction was dissolved in the same buffer containing 0.1 mM $FeSO_4$ and dialyzed extensively against 2.5 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM $FeSO_4$. The solution was applied to a DEAE-cellulose (Whatman DE-32) column (5 cm × 23 cm) equilibrated with 2.5 mM potassium phosphate buffer, pH 7.8. After the column was washed with the same buffer and 20 mM potassium phosphate buffer, pH 7.8, successively, SOD activity was eluted with a 28 mM sample of the same buffer. The fractions containing SOD activity were pooled and dialyzed against 2.5 mM potassium phosphate buffer, pH 6.1. The dialyzed solution was then applied to a hydroxylapatite column (2.8 cm × 8 cm) equilibrated with the same buffer as the dialyzed buffer. The active fractions were eluted from the column with a linear gradient (2–150 mM) of the buffer, pH 6.1, and were pooled and concentrated by ultrafiltration (YM-30, Amicon Corp.). The concentrated solution was applied to a Sephadex G-100 column (4 cm × 46 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.8, containing 0.1 M KCl and eluted with the same buffer. The active fractions were pooled and brought to 1.8 M $(NH_4)_2SO_4$ concentration by addition of the salt. The solution was then applied to a butyl-Toyopearl column (2 cm × 8.5 cm; TOSOH, Tokyo) equilibrated with 50 mM potassium phosphate buffer, pH 7.8, containing 1.8 M $(NH_4)_2SO_4$. The column was washed with the same buffer and 50 mM potassium phosphate buffer, pH 7.8, containing 1.3 M $(NH_4)_2SO_4$. The active fractions were eluted from the column with the same buffer containing 1.15 M $(NH_4)_2SO_4$. The typical results of the purification procedure are summarized in Table IA. The specific activities of the purified enzymes ranged from 60 to 88.5 units (mg of protein)$^{-1}$ (mol of Fe)$^{-1}$ (mol of dimer)$^{-1}$ in several preparations.

Table I: Purification of Fe-SOD and Mn-SOD from *Methylomonas* J

purification step	total protein (mg)	total units	sp act. (units/mg)	recovery (%)
(A) Fe-SOD				
crude extracts	32510	13980	0.43	
45–95% $(NH_4)_2SO_4$	14750	16960	1.15	121
DE-32	200	3350	16.7	24
hydroxylapatite	87.9	2390	27.2	17
Sephadex G-100	30.2	2530	83.9	18
butyl-Toyopearl	12.9	1178	92.0	8.4
(B) Mn-SOD				
crude extracts	5080	33900	6.7	
40–90% $(NH_4)_2SO_4$	1460	39200	26.8	117
DE-32	55.6	22500	405	67
Sephadex G-100	22.0	19000	864	57
isoelectrofocusing	6.0	7580	1260	23
second DE-32	3.8	8360	2200	25

Purification of Mn-SOD from Methylomonas J. The wet cells (46 g) of *Methylomonas* J grown on the manganese-rich medium were homogenized with 250 mL of 10 mM potassium phosphate buffer, pH 7.0, and sonicated. The sonicated solution was centrifuged at 11000*g* for 20 min, and the precipitate was resuspended in a further 250 mL of the same buffer and again sonicated and centrifuged. The two supernatants were combined and fractionated with solid $(NH_4)_2SO_4$, and the 40–95% saturated fraction was suspended in a minimum volume of the same buffer. After the fraction was dialyzed extensively against the same buffer, the solution was applied to a DEAE-cellulose (Whatman DE-32) column (2.5 × 31 cm) equilibrated previously with 10 mM potassium phosphate buffer, pH 7.0. After the column was washed with 800 mL of the same buffer, SOD was eluted with a linear gradient of NaCl (0–0.15 M) in the same buffer. The fractions containing SOD activity were collected and concentrated by ultrafiltration. The concentrated solution was passed through a Sephadex G-100 column (3 × 48 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The active fraction was pooled and dialyzed against 1% glycine solution, and fractionated by using LKB 8100 Ampholine electrofocusing equipment and a carrier ampholite of pH 4–6. The fractions containing the enzyme activity were passed through a Sephadex G-50 column (3 × 35 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.8, to remove the carrier ampholite and applied on a DEAE-cellulose (Whatman DE-32) column (2.5 × 8 cm) previously equilibrated with the buffer, pH 7.8. The enzyme was eluted with a linear gradient of NaCl (0–0.15 M) in the same buffer. The typical results of the purification procedure are summarized in Table IB.

Analytical Methods. SOD activity was measured by inhibition of xanthine/xanthine oxidase induced reduction of

cytochrome *c* (McCord & Fridovich, 1969). SDS-polyacrylamide gel electrophoresis with and without urea was carried out according to the method of Laemmli (1970) with a few modifications. Reversed-phase HPLC was carried out with a Nihon Bunko HPLC system on both a TSK ODS-120T column (4.5 × 250 mm, TOSOH, Tokyo) and a phenyl-5PWRP column (7.5 × 75 mm, TOSOH) with 40-min gradient of acetonitrile (30–60%, v/v) in 0.1% trifluoroacetic acid. In the case of molecular weight determination, a Hitachi 655 HPLC system was used with a TSK SW-3000 column (7.5 × 300 mm, TOSOH) using 10 mM potassium phosphate buffer, pH 7.0. Protein concentration was estimated by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Metals were determined by atomic absorption spectrometry with a Hitachi Z-9000 atomic absorption spectrophotometer. Amino acid compositions of the enzymes were determined by using a Hitachi 835 amino acid analyzer according to the method of Murayama and Sugawara (1981). Samples containing 1.2 nmol of protein were hydrolyzed with constant-boiling HCl in vacuo at 110 °C for 22, 48, and 72 h.

Preparation of Apoproteins and Reconstituted Proteins from Mn–SOD and Fe–SOD. All procedures were carried out at 4 °C. ApoSOD was prepared from the purified Mn–enzyme and by treatment of the enzyme for 24 h, with 50 mM Tris-HCl buffer, pH 7.1, containing 5 M guanidine hydrochloride, 1 mM *o*-phenanthroline, and 10 mM dithiothreitol. The solution was dialyzed against 50 mM Tris-HCl buffer, pH 7.1, containing 1 mM EDTA for 24 h and then against 50 mM Tris-HCl buffer, pH 7.1, for 24 h. A metal-reconstituted enzyme was prepared by treatment of the apoenzyme with 50 mM acetate buffer, pH 5.5, containing 1 mM $(NH_4)_2Fe(SO_4)_2$ for Fe reconstitution and 50 mM glycine–NaOH buffer, pH 9.1, containing 1 mM $MnSO_4 \cdot 4H_2O$ for Mn reconstitution for 24 h. The solution was dialyzed against 50 mM Tris-HCl buffer, pH 7.1, with 1 mM EDTA and then without EDTA for 24 h successively to remove excess metals bound to the proteins. Preparation of an apoprotein from the Fe–SOD and reconstitution of a Fe– or Mn–protein from apoFe–SOD were carried out with the same method as described above (Yamakura et al., 1990).

Amino Acid Sequence Determination. Purified Fe–SOD (1 mg) was denatured in 0.25 M Tris-HCl buffer (pH 8.5) containing 1 mM EDTA and 6 M guanidine hydrochloride at room temperature for 2 h, and the apoprotein was recovered by reversed-phase HPLC on a phenyl-5PWRP column (4.6 × 75 mm, TOSOH) with a 30-min gradient of acetonitrile (20–75%, v/v) in 0.1% trifluoroacetic acid. After lyophilization, the apoprotein was redissolved in 50 μL of 4 M guanidine hydrochloride, diluted to 500 μL with 0.1 M NH_4HCO_3 (pH 8.0), and digested with 20 μg of lysylendopeptidase (Wako Pure Chemical Industries, Tokyo) at 37 °C for 5 h. The resulting fragments were separated by directly applying the digestion mixture to a Lichrosorb Rp-8e column (4.6 × 250 mm; Merck, Dalmstadt, FRG), followed by elution with a 60-min gradient of acetonitrile (10–60%) in 0.1% trifluoroacetic acid. Where necessary, the isolated fragments were further purified by rechromatography using 0.08% heptafluorobutyric acid in place of trifluoroacetic acid. The yield of each peptide fragments was as follows: L1 (74%), L2 (57%), L3 (69%), L4 (82%), L5 (74%), L6 (87%), L7 (42%), L8 (76%), L9 (65%), L10 (22%), and L11 (66%). This procedure allowed us to isolate 11 fragments, which apparently covered the total sequence of *Methylomonas* J SOD (Figure 5). The amino acid sequences of these fragments were determined by an automated sequence analyzer (Model 470A; Applied Biosystems, Foster City, CA) according to the protocol provided by the supplier, entirely to the C-terminal amino acids in each fragment. The amino acid composition of peptides was determined on an automated amino acid analyzer (Model 800; Jusco Ltd., Tokyo) equipped with a postcolumn *o*-phthalaldehyde derivatization system. Peptides were hydrolyzed at 110 °C with 6 N HCl for 24 h in evacuated sealed tubes.

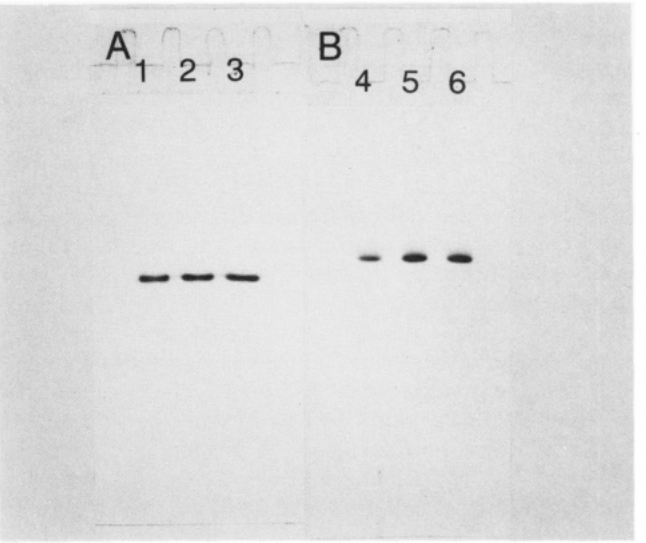

FIGURE 1: Analyses of Fe–SOD and Mn–SOD isolated from *Methylomonas* J by SDS–polyacrylamide gel electrophoresis with and without urea. Fe–SOD (lanes 1 and 4, 1.5 μg), Mn–SOD (lanes 3 and 6, 1.5 μg), and equimolar mixture of both SODs (lanes 2 and 5, 1.5 μg) were subjected to SDS–polyacrylamide gel electrophoresis (A) and SDS–urea–polyacrylamide gel electrophoresis which contained 4.4 M urea in the gel (B). The proteins were visualized by staining with Coomassie Blue.

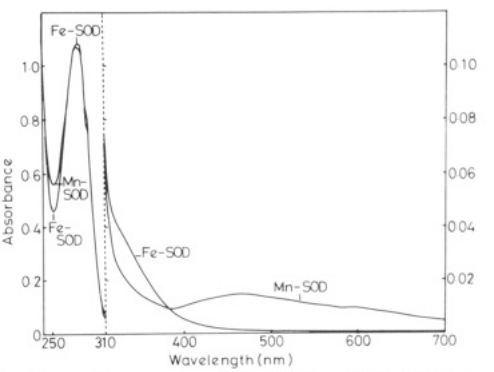

FIGURE 2: Absorption spectra of Fe–SOD and Mn–SOD from *Methylomonas* J. The spectra were obtained with solutions containing 19 μM Fe–SOD and 18.6 μM Mn–SOD for the ultraviolet region and the visible region, respectively. The buffer used was 10 mM potassium phosphate buffer, pH 7.0.

Results

Properties of Mn– and Fe–SOD from Methylomonas J. The purified Mn– and Fe–SODs gave single bands on SDS–polyacrylamide gel electrophoresis with and without urea (Figure 1). They also gave single protein bands on a polyacrylamide disc gel electrophoresis (Yamakura et al., 1990). Molecular weights of the native Fe– and Mn–enzyme were determined by using an HPLC system as described in the text and were shown to be 42000 ± 2000 for each enzyme. Figure 2 shows absorption spectra of Fe–SOD and Mn–SOD from *Methylomonas* J. Each spectrum resembles the spectrum of Fe–SOD and Mn–SOD isolated from other sources (Yama-

Table II: Activities and Metal Contents of Purified Fe- and Mn-SOD from *Methylomonas* J

sample	sp act.[a] [units (mg of protein)$^{-1}$ (mol of Fe or Mn)$^{-1}$ (mol of dimer)$^{-1}$]	metal content (mol/mol of dimer)			
		Fe	Mn	Cu	Zn
Fe-SOD	88.5 ± 2.9	1.04 ± 0.04	0.02 ± 0.001	0.05 ± 0.004	0.75 ± 0.039
Mn-SOD	2250 ± 125	0.12 ± 0.07	0.98 ± 0.08	0.02 ± 0.04	0.16 ± 0.02

[a] Activities were measured by the method of McCord and Fridovich (1969). Values are given as means ± SD.

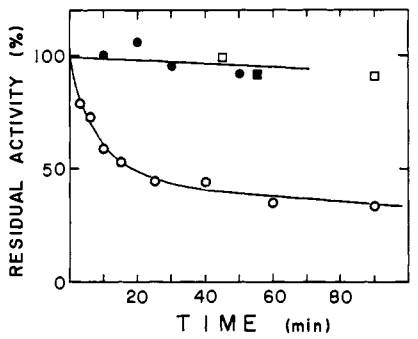

FIGURE 3: Inactivation of Fe-SOD by H_2O_2. Reaction mixtures contained 1.2 mg/mL Fe-SOD or 0.07 mg/mL Mn-SOD, 20 mM H_2O_2, and 25 mM sodium pyrophosphate buffer, pH 7.8, incubated at 25 °C. At intervals, aliquots were withdrawn and assayed for residual SOD activity by using an assay mixture containing xanthine/xanthine oxidase/cytochrome *c* (McCord & Fridovich, 1969) with 1.7 μg of catalase. (○) Fe-SOD with 20 mM H_2O_2; (●) Mn-SOD with 20 mM H_2O_2; (□) Fe-SOD without H_2O_2; (■) Mn-SOD without H_2O_2.

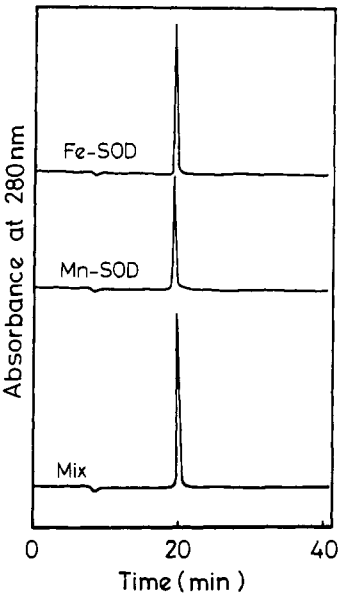

FIGURE 4: Comparison of an HPLC chromatogram of Fe-SOD, Mn-SOD, and a mixture of both SODs. HPLC chromatography was carried out by using a TSK ODS-120T column as described under Materials and Methods. The flow rate was 1.0 mL/min. The amount of samples applied to the column was 17.3 μg of Fe-SOD, 14 μg of Mn-SOD, and 22.4 μg of an equimolar mixture of both SODs, respectively.

kura, 1976; Steinman, 1983; Yamakura et al., 1984). Table II shows metal contents and specific activities of the purified preparations. In order to correct for the influence of different metal contents on the apparent activity of each SOD preparations, we expressed specific activities of SOD as in McCord-Fridovich (1969), units per milligram of protein per mole of Fe or Mn per mole of dimer. The specific activity of Mn-SOD was about 25 times larger than that of Fe-SOD. Although contaminated manganese in the Fe-SOD preparation was less than 0.02 mol/mol of dimer, there could be a slight possibility that the activity of Fe-SOD could be attributed to the manganese in the Fe-SOD preparation. In order to check this possibility, inhibition studies of Mn-SOD and Fe-SOD by hydrogen peroxide and sodium azide were carried out. The Mn-enzyme was not inactivated by 20 mM H_2O_2 after 50-min incubation. The Fe-enzyme was inactivated to about 34% of its original activity by 20 mM H_2O_2 after 90-min incubation (Figure 3). Since it has been reported that Fe-SOD is generally inactivated by H_2O_2 and Mn-SOD is not (Steinman, 1983; Bannister et al., 1987), these results suggest that at least about 70% of the activity of the Fe-enzyme is derived from the iron of the enzyme. Sodium azide also inhibits the enzyme activity of Mn- and Fe-SOD to a different extent. A 40% and 70% inhibition was measured with 1 and 20 mM azide, respectively, for Fe-SOD. On the other hand, 10% and 40% inhibition was measured with 2 and 20 mM azide, respectively, for Mn-SOD. These values resembled the extent of the inhibition of Fe-SOD and Mn-SOD from *E. coli* (Misra & Fridovich, 1978). This evidence also supports the idea that the activity of Fe-SOD is mostly attributed to the iron in the enzyme.

Identity of the Protein Moiety of Mn- and Fe-SOD. We reported in a previous paper that the N-terminal amino acid sequences of Mn- and Fe-SOD from *Methylomonas* J were identical over 32 amino acid residues (Yamakura et al., 1990). This suggested a possibility that both SODs consisted of an identical protein moiety. In order to confirm this, we compared the mobility on electrophoresis, chromatographic behavior, and amino acid compositions of Mn- and Fe-SOD. As shown in Figure 1, Mn-SOD, Fe-SOD, and a mixture of them migrated as single protein bands with the same mobility on polyacrylamide gel electrophoresis in the presence of SDS or SDS and urea. From this experiment, the subunit molecular weights of both SODs were estimated to be 21 000 ± 2000. Figure 4 shows elution profiles of Mn-SOD, Fe-SOD, and a mixture of them from an octadodecylsilicate column in reverse-phase HPLC. Each sample showed a symmetrical single peak with retention times of 19.2 min for Fe-SOD, 19.3 min for Mn-SOD, and 19.7 min for mixture of the SODs (Figure 4). The same result was obtained by using a phenyl-5PWRP column (data not shown). Table III shows the amino acid composition of Mn- and Fe-SOD. No significant difference was observed between these amino acid compositions. This evidence strongly suggests that Fe- and Mn-SOD consisted of the same protein moiety.

Metal Replacement Studies of Fe-SOD and Mn-SOD. We studied the reconstitution of Mn-SOD with Fe and Mn, in order to be further assured that the SODs consisted of same protein moiety. The apoprotein and metal-substituted proteins from Mn-enzyme were prepared by the methods described in the text. The apoprotein from Mn-SOD was reconstituted with Mn and Fe, and the metal contents and specific activity of each preparation were determined (Table IV). Mn-reconstituted apoMn-SOD showed similar activity to native Mn-SOD. Fe-reconstituted apoMn-SOD showed similar activity to native Fe-SOD. Each enzyme contained almost 1 atom of metal per dimer. The Fe- and Mn-reconstituted enzymes from apoFe-SOD had also been prepared, and the results are reported in a previous paper (included in Table IV).

Table III: Amino Acid Composition of Fe- and Mn-Superoxide Dismutases from *Methylomonas* J[a]

amino acid	Fe-SOD (residues/protein subunit)	Mn-SOD (residues/protein subunit)
Asx	22.6 ± 0.25	23.5 ± 1.58 (23)[e]
Thr[b]	14.3	13.7 (14)
Ser[b]	9.8	11.0 (10)
Glx	21.0 ± 0.52	20.6 ± 1.12 (20)
Pro	11.8 ± 0.69	10.7 ± 1.59 (12)
Gly	17.1 ± 0.48	18.5 ± 1.62 (17)
Ala	20.8 ± 0.47	20.7 ± 0.58 (20)
Val[c]	11.3	10.9 (11)
Met	0.8 ± 0.09	0.7 ± 0.20 (1)
Ile[c]	6.9	6.7 (7)
Leu[c]	19.9	19.8 (21)
Tyr	8.9 ± 0.34	8.8 ± 0.25 (9)
Phe	7.0 ± 0.15	6.8 ± 0.32 (7)
Lys	9.5 ± 0.26	9.4 ± 0.23 (10)
His	7.6 ± 0.23	7.6 ± 0.38 (8)
Arg	7.4 ± 0.87	7.4 ± 0.82 (7)
Trp	nd[d]	nd (5)

[a] The number of residues was calculated on the basis of a subunit molecular weight of 21 000. Values are given as means of 24, 48, and 72-h analyses ± SD. [b] Based on extrapolation to zero time of hydrolysis. [c] Based on values obtained after 72-h hydrolysis. [d] nd, not determined. [e] The values in parentheses are calucurated from the total amino acid sequence of the SOD from *Methylomonas* J (Figure 5).

The Fe-reconstituted enzyme from apoFe-SOD showed a similar content of Fe, and a similar activity to the purified Fe-SOD. The Mn-reconstituted enzyme showed about 2 atoms of manganese per dimer of enzyme and had about 60% activity of the purified Mn-SOD. These results support the idea that the organism synthesizes a single apoprotein which is capable of accepting either Fe or Mn at the active site, and which shows activity by using either of the metals.

Amino Acid Sequence of Fe-SOD from Methylomonas J. Identical amino acid sequences of the N-terminal 32 residues of the SODs from *Methylomonas* J were previously determined (Yamakura et al., 1990). To obtain the sequence information of *Methylomonas* SOD in further detail, the Fe-SOD was digested by lysylendopeptidase, and the fragments were isolated as described under Materials and Methods. After determination of the amino acid sequences of these peptides, we aligned the determined sequences to obtain a maximum homology with the published sequences of the Fe- and Mn-SODs from other bacteria. Figure 5 shows the sequence of *Methylomonas* SOD thus obtained together with the peptide fragments which were obtained by digestion with lysylendopeptidase. According to the sequence, *Methylomonas* SOD is composed of 202 residues and has a molecular weight of 22 366. Figure 6 shows the comparison of the amino acid sequence of the *Methylomonas* SOD with the amino acid sequences of other Fe- and Mn-SODs. Because the deduced sequence of *Methylomonas* SOD fit the amino acid composition (Table III), and the molecular weight value estimated by SDS-polyacrylamide gel electrophoresis, and because it contained no apparent gap as compared with other SOD sequences, we predicted that this represents the total sequence of the SOD from *Methylomonas* J. The amino acid sequence of *Methylomonas* SOD has a higher percentage of identity with that of the Mn-SODs isolated from bacteria (60.2-67%) than that of the Fe-SODs (43.5-47.7%) and the Mn-SOD isolated from mitochondria (41.3 and 48.7%).

Discussion

Of three types of SOD, Cu/Zn-SOD and Fe-SOD were inactivated by H_2O_2, whereas Mn-SOD was not (Steinman, 1983; Yamakura & Suzuki, 1986). We used this property to elucidate whether the small activity of the purified Fe-SOD from *Methylomonas* J is in fact attributed to the iron or to the manganese which might have been contaminated in our preparation. The inactivation study of Fe-SOD (Figure 3) showed that at least 70% of the enzyme activity is attributed to the iron that is incorporated in the active site of the enzyme. The remaining activity could be attributed to the modified form of Fe-SOD by H_2O_2, which might still contain iron, as has been shown by Bayer and Fridovich (1987) on the Fe-SOD from *E. coli*.

Although the possibility that the Fe-SOD and Mn-SOD have a somewhat different protein moiety has not been completely excluded, the data described in this text suggest that both SODs consist of an identical protein. The enzyme preparations, which were purified from *Methylomonas* J grown in a medium containing both Fe and Mn, always contained less than 1 mol of metals (Fe + Mn) per mole of dimer, similar to the metal contents of the native Fe- and Mn-SOD (Table II) (data not shown). This evidence supports the idea that each metal might occupy the same site or a mutually exclusive binding site of the enzyme. There are four other documented examples of SODs which were isolated from strict or facultative anaerobic bacteria and which accepted both Fe and Mn as cofactors (Meier et al., 1982; Gregory & Dapper, 1983; Martin et al., 1986; Pennington & Gregory, 1986). One of these SODs, which was isolated from *Streptococcus mutans*, showed higher activity when the apoprotein was bound with Mn than when it was bound with Fe. The *Methylomonas* SODs have about the same ratio of Mn/Fe-SOD activities (25:1) as the *Streptococcus* SODs. Furthermore, *Methylomonas* SOD is the first example of "cambialistic" SOD isolated from aerobic bacteria. Therefore, we speculate that bacterial SODs might generally show a variety of types of activity in regard to metal specificity, ranging from cases of a SOD which requires only Fe or Mn for the activity to cases which allow either of these metals to exhibit the activity with the same efficiency.

A few recent papers have suggested that active-site metals of Fe- and Mn-SODs were surrounded by a hydrophobic box of residues which includes a high percentage of aromatic residues and most of these amino acid residues, including four

Table IV: Specific Activities and Metal Contents of Metal-Reconstituted Mn-SODs and Fe-SODs[a]

sample	sp act. [units (mg of protein)$^{-1}$ (mol of Mn or Fe)$^{-1}$ (mol of dimer)$^{-1}$]	metal content (mol/mol of dimer) Fe	Mn	Cu	Zn
Mn-SOD					
apoMn-SOD	20.4 ± 4.1[b]	0.11 ± 0.012	0.01 ± 0.003	0.08 ± 0.003	0.47 ± 0.009
Fe reconstituted	85.5 ± 13.0	0.73 ± 0.073	<0.005	<0.005	0.11 ± 0.005
Mn reconstituted	1920 ± 210	<0.01	0.80 ± 0.05	0.04 ± 0.02	0.07 ● 0.01
Fe-SOD[c]					
apoFe-SOD	6.7 ± 2.2[b]	0.06 ± 0.009	<0.005	<0.005	0.06 ± 0.009
Fe reconstituted	162 ± 10	1.48 ± 0.06	0.02 ± 0.007	0.08 ± 0.006	0.26 ± 0.01
Mn reconstituted	1360 ± 129	0.08 ± 0	2.28 ± 0.08	<0.01	0.08 ± 0.02

[a] Values are given as means ± SD. [b] The activities of apoSOD are given as units per milligram of protein. [c] Yamakura et al. (1990).

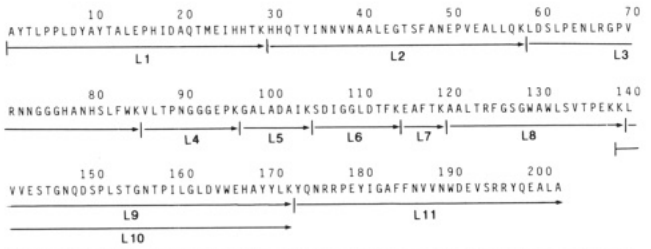

FIGURE 5: Schematic outline of the peptides obtained by lysylendopeptidase in the amino acid sequence of *Methylomonas* SOD. L1–L11 represents the peptide fragments obtained by the method described under Materials and Methods.

metal ligands, were conserved through Fe- and Mn–SODs. This has been suggested by analysis of the aligned amino acid sequence of Fe–SODs and Mn–SODs and structural data for X-ray diffraction studies. Among these, amino acid residues, however, glycine-77, phenylalanine-84, and glutamine-155 in the Mn–SODs were substituted for glutamine-77, tyrosine-84, and alanine-155 in the Fe–SODs, respectively (Parker et al., 1987; Isobe et al., 1988; Carlioz et al., 1988; Parker & Blake, 1988b; Yamakura et al., 1989; Stoddard et al., 1990; Figure 6). These amino acid residues have been proposed as possible sites which might be responsible for conferring the distinguishing properties of the Fe–SOD and Mn–SOD. We compare these three amino acid residues of the *Methylomonas* SOD with those of Fe–SODs from *P. ovalis* and *P. leiognathi* and Mn–SODs from *E. coli* and *B. stearothermophilus*. These SODs had been determined as the SODs which strictly required only iron or manganese, respectively, for their activity by reconstitution studies (Ose & Fridovich, 1976; Brock & Harris, 1977; Puget et al., 1977; Yamakura & Suzuki, 1980). Furthermore, we also chose Fe–SOD from *E. coli* and Mn–SOD from *Saccharomyces cerevisiae* and human liver as a noncambialistic SOD for comparison of the amino acid sequences with that of the *Methylomonas* SOD for the following reason: *E. coli* has both Fe–SOD and Mn–SOD within the cell, so that these enzymes do not need to be a cambialistic SOD and no eukaryotic organism has been reported to have a cambialistic SOD so far. Since the other bacterial SODs listed in Figure 6 have not been examined for the specificity of the metals to exhibit enzyme activity, we exclude the other bacterial SODs from the subject of the comparison. *Methylomonas* SODs hold glycine-77, phenylalanine-84, and glutamine-155, in common with Mn–SODs (Figure 6). The total sequence of *Methylomonas* SOD also has a higher homology with the bacterial Mn–SODs than with the bacterial Fe–SODs. These results might reflect the difference of the specific activity between Mn-type SOD and Fe-type SOD of *Methylomonas* J; namely, Mn-type SOD had a 25 times higher specific activity than that of the Fe-type SOD.

On the other hand, *Methylomonas* SOD must have some similarity in amino acid residues which are specific for the Fe–SODs from *P. ovalis*, *P. leiognathi*, and *E. coli* and/or some difference in amino acid residues specific for Mn–SODs from *E. coli*, *B. stearothermophilus*, *Saccharomyces*, and human liver, which may result in the small activity of Fe-type *Methylomonas* SOD. In order to evaluate this idea, we examined Fe–SOD-specific amino acid residues which are invariant in the Fe–SODs listed above but not found in Mn–SODs listed above. A total of 26 amino acid residues are applicable to the Fe–SOD-specific amino acid residues. Although most of these amino acid residues are not found in the aligned amino acid sequence of *Methylomonas* SOD, two of these amino acid residues are found in *Methylomonas* SOD, i.e., threonine-45 (located in the loop between the first and second α-helix of Fe/Mn–SODs), and glycine-69 (in the second α-helix). These residues have a possibility to give an Fe–SOD-like structure to the *Methylomonas* SOD. We also examined whether some amino acid residues are different from Mn–SOD-specific amino acid residues which are invariant in the Mn–SODs but not found in Fe–SOD. This residue also has a possibility to give an alternate structure of the *Methylomonas* SOD compared to the structure of the other Mn–

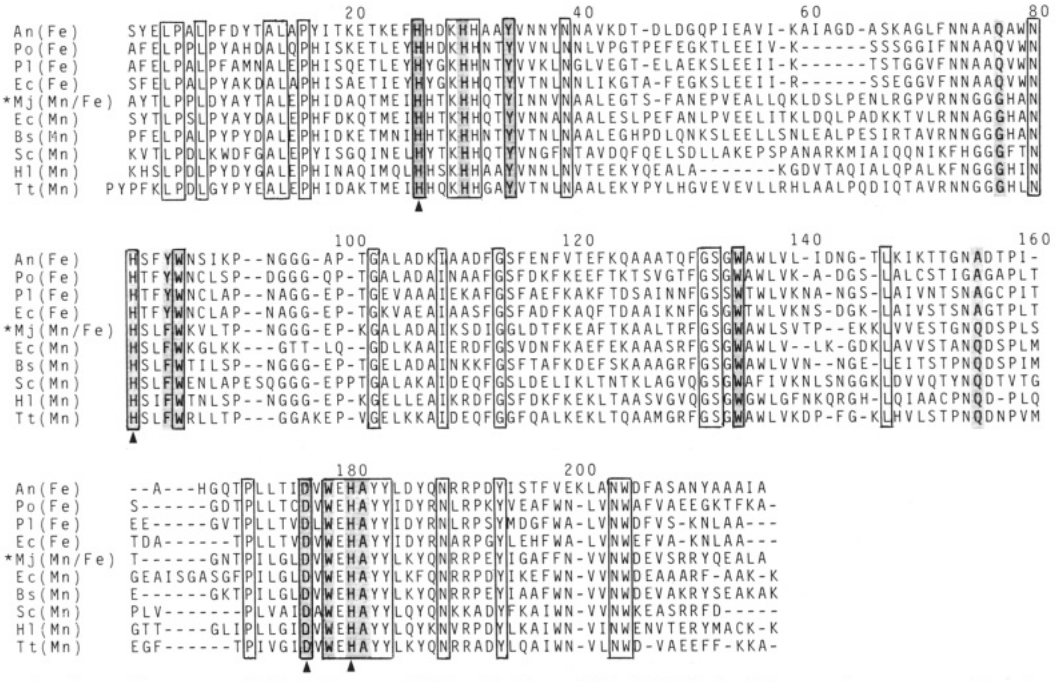

FIGURE 6: Aligned amino acid sequence of *Methylomonas* SOD with Fe- and Mn–SOD isolated from various sources. Gaps have been inserted to obtain maximal homologies among the sequences. Boxes indicate positions at which residues are identical. Shaded residues are constituents of a putative active site which include the metal ligands and residues within 7.3 Å of the metal found in the *T. thermophilus* Mn–SOD (Stalling et al., 1985) and *B. stearothermophilus* Mn–SOD (Parker & Blake, 1988a). Arrowheads indicate the ligands of the metals. An, *Anacystis nidulans*; EC, *Escherichia coli*; Po, *Pseudomonas ovalis*; Pl, *Photobacterium leiognathi*; MJ, *Methylomonas* J; Bs, *Bacillus stearothermophilus*; Sc, *Saccharomyces cerevisiae*; Hl, human liver; Tt, *Thermus thermophilus*.

SODs and could weaken the specificity of manganese to exhibit the enzymatic activity of the *Methylomonas* SOD. However, all of the Mn–SOD-specific amino acid residues are also found in the aligned sequence of *Methylomonas* SOD.

In addition to this difference, there are several amino acid residues in *Methylomonas* SOD [isoleucine-35 (in the first α-helix), isoleucine-112 (between the third and fourth α-helix), glycine-114 (between the third and fourth α-helix), glutamic acid-144 (in the first β-structure), and phenylalanine-198 (in the sixth α-helix)] which are invariant through the Fe- and Mn–SODs but different in the *Methylomonas* SOD. These substitutions could be other candidates which could weaken the specificity of the metals to exhibit the enzyme activity in *Methylomonas* SOD by giving a subtle difference in the structure of *Methylomonas* SOD compared to the structure of the other Fe–SODs and Mn–SODs.

Since a functional role of these amino acid residues is not clear at the present time, it is difficult to explain what kind of difference in structure of *Methylomonas* SOD might result from these amino acid substitutions, compared to the structure of the other Fe- or Mn–SODs. Possible explanations are given as follows: these amino acid substitutions might give rise to some subtle differences in the hydrophobicity of the metal–ligand environment and/or in the coordination geometry of the amino acid ligands in *Methylomonas* SOD. These subtle differences may affect the oxidation–reduction potential of the metals and/or the mechanism of the dismutation reaction, which could make the *Methylomonas* SOD show low specificity of metals for the activity. Further work, including X-ray crystallographic studies, physicochemical studies of the *Methylomonas* SOD, and determination of amino acid sequences of SODs which have low metal specificity, is required to clarify these hypothetical explanations.

ACKNOWLEDGMENTS

We thank Drs. J. Kajiwara, M. Enomoto, and K. Takagi of Nippon Kayaku Co. Ltd. for the determination of amino acid sequences of the digested peptides.

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