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ISOLATION OF Mn-SOD AND LOW ACTIVE Fe-SOD FROM *METHYLOMONAS* J; CONSISTING OF IDENTICAL PROTEINS

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Cultures of *Methylomonas* J, an aerobic methylotrophic bacterium, were grown both in Mn-rich and Fe-rich media. Crude extracts of the cultures from the Mn-rich and Fe-rich medium showed a specific activity of 12.2 and 0.6 units/mg by a cytochrome c-xanthine oxidase method and 19.4 and 1.3 units/mg by an ESR method, respectively. We isolated Mn-SOD and Fe-SOD from the bacteria grown in the Mn-rich and Fe-rich mediums, respectively. Specific activity and metal contents of the Mn-enzyme were 2,250 units/mg/g-atom Mn and Mn = 0.98 and Fe = 0.12 (g-atoms/mol dimer), while those of the Fe-enzyme were 61 units/mg/g-atom Fe and Mn = 0.02 and Fe = 1.08. No difference of physicochemical properties of the Fe- and Mn-enzymes were detected. Furthermore, enzyme activity was restored by dialysis of an apoprotein obtained from the Fe-enzyme with either manganese sulfate or ferrous ammonium sulfate.

KEY WORDS: Fe-SOD, Mn-SOD, *Methylomonas* J, identical protein moiety.

INTRODUCTION

Three classes of superoxide dismutase (SOD) have been distinguished according to the bound metals; an iron containing SOD, a manganese containing SOD, and a copper, zinc containing SOD.[1] The Mn- and Fe-SOD appear to be closely related both in structural and evolutionary terms, but have no resemblance to Cu, Zn-SOD.[1] Recent studies on the complete amino acid sequence of Fe- and Mn-SODs and on the x-ray crystallography of the Fe- and Mn-SODs suggest that the Fe- and Mn-SODs have very similar amino acid sequences, tertiary folds of the peptide chain, and identical ligands to the metal cofactor. However, previous reconstitution experiments indicated the metal requirement of the Mn- and Fe-enzyme was highly specific. Recently, some differences in amino acid residues, which is located near metals, have been proposed as the site account for the metal specificity.[2-4]

On the other hand, some anaerobic bacteria produce both iron and manganese types of SOD by controlling of the metal condition of the medium, in which the metals may occupy the same site of the same protein.[5-8] Although some of them showed different activity between Fe- and Mn-type SODs, these SODs are believed to be a new family of SOD.

In this report, we describe the presence of the Fe-SOD with low activity and

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Mn-SOD with high activity in *Methylomonas* J. We also describe some evidence which suggests the identity of the protein moiety of the SODs. Therefore, the *Methylomonas* J superoxide dismutase is capable of accepting either Fe or Mn as a cofactor and this is the first example of an isolation of this kind of SOD from aerobic bacterium.

MATERIALS AND METHODS

Methylomonas J was grown in a medium as described elsewhere.[9] Methylamine hydrochloride was used as the sole carbon and energy source. For the iron-rich medium, 7 mg of $FeSO_4\ 7H_2O$ and no manganese per liter of medium were added. For the Mn-rich medium, 8.5 mg of $MnSO_4\ 4H_2O$ and 0.08 mg of $FeSO_4\ 7H_2O$ were added. Superoxide dismutase activity was tested by the method of cytochrome c-xanthine oxidase[10] and by an EPR method using 5,5-dimethyl-1-pyrroline-N-oxide as a spin trap reagent, as described previously.[11] The isolation of the Fe- and Mn-SOD from *Methylomonas* J grown in the Fe- and Mn-rich mediums were described elsewhere.[9] Electrophoresis was performed on polyacrylamide gels as described by Davis.[12] Gels were stained for SOD activity as described by Beauchamp and Fridovich.[13] Protein concentration was estimated by the method of Lowry *et al.*[14] Metals were determined by atomic absorption spectrometry with a Hitachi Z-9000 atomic absorption spectrophotometer. The amino terminal sequences of the purified proteins were determined with an Applied Biosystem 470A sequenator with a 120A PTH analyzer. Inactivation of Fe- and Mn-SOD by H_2O_2 was carried out according to the method described previously.[15] ApoSOD was prepared from the purified Fe-enzyme by treatment of the enzyme for 24 hr, with 50 mM Tris-HCl buffer, pH 7.1, containing 5 M guanidine hydrochloride, 1 mM *o*-phenanthroline, and 10 mM dithiothreitol. The holoenzyme was prepared by treatment of 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 $MnSO_4 4H_2O$ for Mn-reconstitution.

RESULTS

Superoxide Dismutase in the Crude Extracts from Methylomonas J

The crude extracts from *Methylomonas* J were subjected to polyacrylamide gel electrophoresis and to staining of the enzymatic activity. (a) and (b) in Figure 1 show the activity zones with the same protein amount of crude extracts from *Methylomonas* J grown in Fe-rich medium and Mn-rich medium, respectively. The activity zone in Figure 1 (a) was much weaker than that in (b). Table I shows SOD activity of the crude extracts measured by a cytochrome c-xanthine oxidase method and a spin trapping method using ESR as described in the text. The specific activities of the crude extracts from *Methylomonas* J grown in Fe-rich medium showed to be about one fifteenth to one twentieth of that grown in Mn-rich medium.

Criteria of Purity and Properties of Mn- and Fe-SOD

The purified Mn- and Fe-SODs gave single bands on polyacrylamide gel electrophoresis with the same mobilities (Figure 1 c, d). Molecular weights of the native Fe-

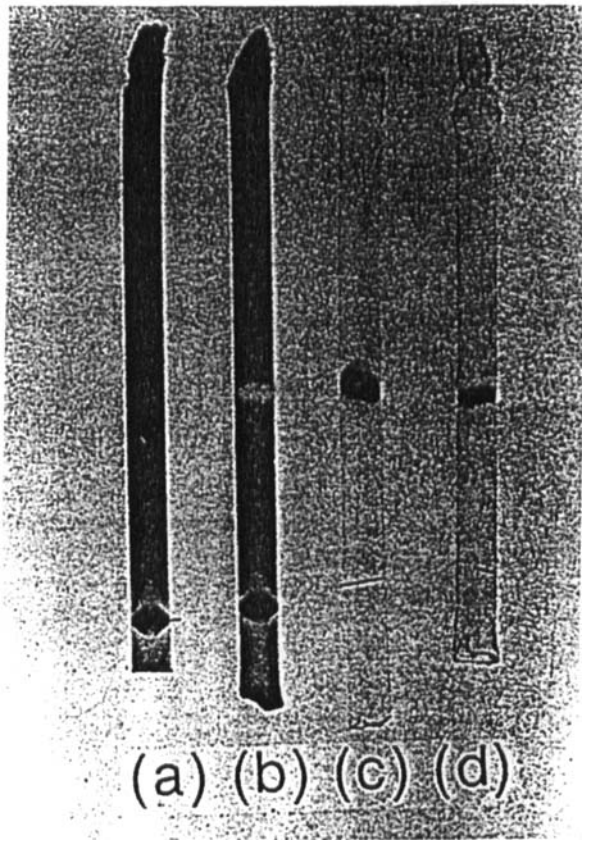

FIGURE 1 Polyacrylamide gel electrophoresis of the crude extracts and purified enzymes from *Methylomonas* J grown in Fe- and Mn-rich medium. 100 μg proteins of the crude extracts from the bacteria grown in Fe-rich medium (a) and Mn-rich medium (b), 30 μg of the purified Fe-SOD (c), and 18 μg of the purified Mn-SOD were subjected to polyacrylamide gel electrophoresis. The gels were stained with nitroblue tetrazolium (a,b) and Coomassie brilliant blue (c,d) for activity and protein, respectively.

and Mn-enzyme determined by high performance liquid chromatography were shown to be both 42,000. Table II shows metal contents and specific activity of the purified preparations. Specific activity of the Mn-SOD was about 40 times larger than that of the Fe-SOD. The Mn-enzyme was not inactivated by 20 mM of H_2O_2 after 50 min incubation. The Fe-enzyme was inactivated to about 40 % of its original activity by 20 mM of H_2O_2 after 90 min incubation. These results suggest that at least 60 % of the activity of the Fe-enzyme is derived from the iron of the enzyme.

Amino Terminal Sequence

The amino acid compositions of the Mn- and Fe-enzymes were obtained from the samples of 24, 48, and 72 hr hydrolysis by 6N HCl. Virtually identical compositions were obtained for both type of SODs (data not shown). Figure 2 shows the amino terminal sequence of each of the enzymes. The amino-terminal sequences of the Fe-

TABLE I
Specific activity of SOD in the crude extracts from *Methylomonas* J grown in Fe- and Mn-rich mediums.

Growth condition	cytochrome c-xanthine oxidase method (units/mg protein)	ESR method (units/mg protein)
Fe-rich medium	0.56 ± 0.19	1.26 ± 0.77
Mn-rich medium	12.2 ± 1.7	19.4 ± 4.6

Values represent the mean ± S.D.

and Mn-enzymes were identical through the first 32 residues, indicating that it is highly possible that the same protein was used to form either enzyme.

Metal Replacement Studies

The specificity of metal replacement in the apoprotein from Fe-enzyme was tested. The apoprotein and metal-substituted proteins from the Fe-enzyme were prepared by the method described in the text. Table II shows the metal contents and specific activities of the Fe- and Mn-reconstituted proteins. The Fe-reconstituted protein showed a similar content of Fe and a three times larger activity than the purified Fe-enzyme, and Mn-reconstituted protein showed about two atoms of Mn per dimer of enzyme and about 22 times larger activity. The activity of the Mn-reconstituted protein was much closer to that of the purified Mn-enzyme than to that of the Fe-enzyme (Table II). These results also support the idea that the Fe-enzyme and Mn-enzyme consist of an identical protein.

DISCUSSION

Although a slight possibility that the Fe-SOD and Mn-SOD have somewhat different protein moiety remains, the data described in this text suggests that it is highly likely that both SODs consist of an identical protein. There are three other documented examples of SOD that accept Fe or Mn as cofactors, depending on growth conditions.[5,7,8] However, the ratio of the activity between the Fe-type and Mn-type of enzymes were different in some SODs. For instance, SOD from *Streptococcus mutans*[8] also showed high activity in Mn-enzyme and low activity in Fe-enzyme; the same as our results on *Methylomonas* J. Therefore, we speculate that bacterial Fe- and

TABLE II
Specific activities and metal contents of purified Fe- and Mn- SODs and metal reconstituated Fe-SODs

Sample	Specific activity (units/mg/g-atom metal)	Metal contents (g-atoms/mol dimer)			
		Fe	Mn	Cu	Zn
Fe-SOD	61	1.08	0.02	0.04	0.08
Mn-SOD	2,250	0.12	0.98	0.02	0.16
Reconstituted Fe-SOD					
with Fe	162	1.48	0.02	0.08	0.26
with Mn	1,355	0.08	2.28	n.d.	0.08

n.d., not detectable.

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Amino acid No.   1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16
      Fe-SOD :  Ala-Tyr-Thr-Leu-Pro-Pro-Leu-Asp-Tyr-Ala-Tyr-Thr-Ala-Leu-Glu-Pro-
      Mn-SOD :  Ala-Tyr-Thr-Leu-Pro-Pro-Leu-Asp-Tyr-Ala-Tyr-Thr-Ala-Leu-Glu-Pro-

                17  18  19  20  21  22  23  24  25  26  27  28  29  30  31  32
      Fe-SOD :  His-Ile-Asp-Ala-Gln-Thr-Met-Glu-Ile-His-His-Thr-Lys-His-His-Gln-
      Mn-SOD :  His-Ile-Asp-Ala-Gln-Thr-Met-Glu-Ile-His-His-Thr-Lys-His-His-Gln-
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FIGURE 2 Amino-terminal sequences of Fe- and Mn-SODs from *Methylomonas* J.

Mn-SODs might show a variety of types of activity in regard to metal specificity, ranging from cases of an SOD which requires only one metal for the activity to cases which allow either metal to exhibit the activity in same efficiency.

A few recent papers, including one by ourselves, have suggested that the metal specificity for the activity of Fe- and Mn-SOD could be attributed to the differences of a few amino acid residues, which were positioned in and close to the active site of the enzyme. This was determined by an analysis of the aligned amino acid sequence and structural data for x-ray diffraction study.[2-4] In this context, it would be interesting to know the primary structure of SOD from *Methylomonas* J to investigate further the structural attribution of the metal specificity of the Fe- and Mn-SOD activity. This study is now in progress in our laboratory.

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References

1. J.V. Bannister, W.H. Bannister and G. Rotilio (1987) Aspects of the structure, function, and applications of superoxide dismutase. *CRC Critical Reviews in Biochemistry*, **22**, 111–180.
2. T. Isobe, Y.-Il. Fang, D. Muno, T. Okuyama, D. Ohmori and F. Yamakura (1988) Difference between amino acid residues in the metal-ligand environments of iron- and manganese-superoxide dismutase. *Biochemistry International*, **16**, 495–501.
3. M.W. Parker and C.C.F. Blake (1988) Iron- and manganese-containing superoxide dismutases can be distinguished by analysis of their primary structures. *FEBS Letters*, **229**, 377–382.
4. A. Carlioz, M.L. Ludwig, W.C. Stallings, J.A. Fee, H.M. Steinman and D. Touati (1988) Iron superoxide dismutase nucleotide sequence of the gene from *Escherichia coli* K12 and correlations with crystal structures. *Journal of Biological Chemistry*, **263**, 1555–1562.
5. B. Meire, D. Barra, F. Bossa, L. Calabrese and G. Rotilio (1982) Synthesis of either Fe- or Mn-superoxide dismutase with an apparently identical protein moiety by an anaerobic bacterium dependent on the metal supplied. *Journal of Biological Chemistry*, **267**,. 13977–13980.
6. E.M. Gregory and C.H. Dapper (1983) Isolation of iron-containing superoxide dismutase from *Bacteroides fragilis*: Reconstitution as a Mn-containing enzyme. *Archives in Biochemistry and Biophysics*, **220**, 293–300.
7. C.D. Pennington and E.M. Gregory (1986) Isolation and reconstitution of iron- and manganese-containing superoxide dismutase from *Bacteroides thetaiotaomicron*. *Journal of Bacteriology*, **166**, 528–532.
8. M.E. Martin, B.R. Byers, M.O.J. Olson, M.L. Salin, J.E.L. Arceneaux and C. Tolbert. (1986) A *Streptococcus mutans* superoxide dismutase that is active with either manganese or iron as a cofactor. *Journal of Biological Chemistry*, **261**, 9361–9367.

9. T. Matsumoto, K. Terauchi, T. Isobe, K. Matsuoka and F. Yamakura. Manuscript in preparation.
10. J.M. McCord and I. Fridovich (1969) Superoxide dismutase as enzymic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry*, **244**, 6049–6055.
11. Y. Nakai, Y. Hashimoto, D. Ohmori and F. Yamakura (1989) Assay method of SOD in biological samples by ESR spectroscopy. *Journal of Pharmacobiological Dynamics*, **12**, s-10.
12. B.J. Davis (1964) Disc electrophoresis, II Method and application to human serum protein. *Annals of the New York Academy of Science*, **121**, 404–427.
13. C.O. Beauchamp and I. Fridovich (1971) Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. *Analytical Biochemistry*,**44**, 276–287.
14. O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
15. F. Yamakura and K. Suzuki (1986) Inactivation of *Pseudomonas* iron-superoxide dismutase by hydrogen peroxide. *Biochimica et Biophysica Acta*, **874**, 23–29.

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