Synthesis of Either Fe- or Mn-Superoxide Dismutase with an Apparently Identical Protein Moiety by an Anaerobic Bacterium Dependent on the Metal Supplied*

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Superoxide dismutase of Propionibacterium shermanii, an anaerobe that produces an iron superoxide dismutase, was purified from cells grown in iron-free conditions. The enzyme isolated was found to contain manganese and to have spectral and catalytic properties very similar to those of typical Mn-superoxide dismutases. Its electrophoretic mobility, molecular weight, and subunit size were identical with those of the Fe-enzyme. Amino acid compositions were practically indistinguishable in either case. The NH₂-terminal sequence was found to be identical. The catalytic activity of an apoprotein sample prepared from the purified holoenzyme was restored by adding either Mn(II) or Fe(II). Only the metal/protein ratio varied from approximately 1 per subunit in the case of the Fe-enzyme to approximately 2 for the Mn-enzyme. It is concluded that this bacterium can accommodate either Fe or Mn on identical, or very slightly dissimilar, proteins forming active sites with the properties found in specific metalloenzymes.

Superoxide dismutases, the enzymes that catalyze dismutation of superoxide (O₂⁻) into O₂ and H₂O₂, are considered as an essential natural defense against the deleterious effect of "activated" oxygen in cells (1). They are nearly ubiquitous metalloenzymes, containing either copper, manganese, or iron as the active metal cofactor. The reason why some bacteria have Fe-superoxide dismutase, while other bacteria contain Mn-superoxide dismutase or both is not known. Moreover, in spite of the high level of general homology (2), metal binding properties very similar to those of typical Mn-superoxide dismutases. Its electrophoretic mobility, molecular weight, and to have spectral and catalytic properties depending on the metal supply and utilization of that aim proteins that are practically indistinguishable from each other.

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The incorporation of iron into superoxide dismutase was verified as occurring as well when iron salts were added to the synthetic medium used for the iron-lacking culture. Therefore, synthesis of either Mn- or Fe-superoxide dismutase was not dependent on the medium, but only on metal availability.

The questions arise whether: (i) the Mn-enzyme synthesized by iron-deprived cells has properties typical of Mn-superoxide dismutases already described; (ii) the Fe- and Mn-enzymes have comparable activity; (iii) the two enzymes consist of an identical protein moiety, as suggested by the same electrophoretic mobility, molecular weight, and subunit size.

(i) The optical and ESR spectra of the Mn- and Fe-enzymes are shown in Figs. 3 and 4. They are typical of Mn- and Fe-superoxide dismutases already described such as those of E. coli (16, 17) which have different molecular properties. In particular, they indicate for the Mn-enzyme of P. shermanii the presence of Mn(III) (16), which is unique to superoxide dismutase.

(ii) The catalytic rate constants were measured at pH 7.4 by the comparison procedure of Forman and Fridovich (18) and gave \( k = 8.5 \times 10^7 \text{M}^{-1} \text{s}^{-1} \) for both the Fe- and Mn-enzyme. These values are comparable to those reported for typical Fe- and Mn-superoxide dismutases (16-20).

(iii) The amino acid composition of the two proteins is shown in Table I, together with those of Mn- and Fe-superoxide dismutases already described such as those of E. coli (16, 17) which have different molecular properties. In particular, they indicate for the Mn-enzyme of P. shermanii the presence of Mn(III) (16), which is unique to superoxide dismutase.

The same elution volume was measured for either protein by gel exclusion chromatography (Fig. 2), indicating a comparable molecular weight of approximately 73,000. This value was confirmed by ultracentrifuge analysis (±5000). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of enzyme samples purified from either cell culture and denatured by 10 min of boiling in the presence of β-mercaptoethanol gave a single band corresponding to a subunit molecular weight approximately half that of the native enzyme (±32,000). Metal analysis of purified proteins gave a content of 0.13% iron and 0.02% manganese for the enzyme isolated from iron-containing cultures, and of 0.28% manganese and <0.01% iron from the enzyme purified from iron-deprived cells. Thus, Mn is present in the superoxide dismutase synthesized in the absence of iron as approximately 1.8 Mn per subunit, that is nearly twice the metal/protein ratio of the Fe-superoxide dismutase.

Fig. 1. Activity and protein-stained polyacrylamide gel electrophoresis of P. shermanii superoxide dismutases. From the left: superoxide dismutase from Fe-containing cultures, stained for activity (a) and protein (b); superoxide dismutase from Fe-deprived cultures, stained for activity (c) and protein (d). One and 10 μg of protein were applied to each gel for activity and protein staining, respectively.

Fig. 2. Gel exclusion chromatography (Sephadex G-100) of both P. shermanii superoxide dismutases (○) and of other proteins as indicators of molecular size (□, ovalbumin; ▲, chymotrypsin; Δ, lysozyme; and ■, cytochrome c). Column length, 80 cm; column diameter, 5 cm. 0.05 M K phosphate buffer, pH 7.8.

Fig. 3. Optical spectra of P. shermanii superoxide dismutases (SOD). Protein concentration was 0.25 mg/ml and 14.2 mg/ml for UV and visible spectra, respectively. 0.05 M phosphate buffer, pH 7.80.
oxidase dismutases from E. coli, which are established to be different proteins, in spite of their high level of homology. In the case of E. coli superoxide dismutases, clearcut differences can be seen in the half-Cys and Met content and significant differences (≥25%) in the Leu and Lys content. No such differences could be observed in the case of P. shermanii proteins. Furthermore, the NH₂-terminal sequence was found to be identical for both proteins, namely Ala-Val-Tyr-Thr-Leu-Pro-Asp-Leu-Pro-Tyr-Asp-Tyr. This sequence is homologous to that of other Fe- or Mn-superoxide dismutases (2), nevertheless it is different from any other reported sequence, by at least 3 residues from the closest one, and by 5–7 from most of them. In a large series of NH₂-terminal sequences of this class reported recently (2), this is the average difference in the first 12-residue segment between any two members, including Mn-superoxide dismutases from the same bacterium, i.e. E. coli (4 differences).

An obvious way to verify the strong indication that the same protein can accommodate in P. shermanii either Fe or Mn is to prepare the apoprotein from the purified holoenzyme and then show that either Mn or Fe ions can restore the catalytic activity. However this approach proved very difficult with this protein. Dialysis against EDTA at low pH, in the presence of variable amounts of urea or guanidine, did remove the metals, but the protein precipitated upon raising the pH back to neutrality. Diethyldithiocarbamate produced metal-free derivatives unable to reconstitute the holoprotein. CN⁻ was totally ineffective. o-Phenanthroline, in the presence of ascorbate, gave a substantial metal removal and this apoprotein was able (Table III) to recover catalytic activity upon addition of either Fe(II) or Mn(II).

**CONCLUSIONS**

From these results it appears that P. shermanii can produce, as a function of the metal supplied, either Fe or Mn-superoxide dismutases, which reproduce spectral properties and catalytic efficiency of typical Fe- and Mn-superoxide dismutases. This seems to exclude a strict species specificity of Mn- and Fe-superoxide dismutase, as well as a dependence of the type of metal on special conditions of growth other than metal availability. As a matter of fact, no certain rule has been provided so far to predict the presence of either enzyme in different bacteria. E. coli contains both enzymes, but only the Mn-enzyme is inducible by oxygen (4). Mn- and Fe enzymes are present in both Gram-positive and Gram-negative bacteria, although Gram-negative bacteria tend to contain Fe-superoxide dismutase and most Gram-positive bacteria contain only Mn-SOD (15). Only one rule seemed to apply: no Mn-superoxide dismutase has so far been reported in anaerobes, but even this generalization is apparently contradicted by the synthesis of a typical Mn-superoxide dismutase in iron-deprived cultures of the anaerobe P. shermanii.

Another relevant result of the present work is the strong indication of identical protein moieties for either Fe- or Mn-superoxide dismutase of P. shermanii. Only the full amino acid sequence can give unequivocal evidence in this regard, but all the data reported here agree on substantial identity. In particular, Mn- and Fe-superoxide dismutases with identical electrophoretic mobility (Fig. 1) have been reported only in case of artificial metal substitution. Moreover, the apoenzyme of P. shermanii could be reconverted into an active holoenzyme by addition of either Mn(II) or Fe(II). This result is not the rule with other Fe- or Mn-superoxide dismutases, which have a rather strict metal specificity and, when they bind other metals, do not produce enzymatic active derivatives (3, 24). Of the two naturally produced P. shermanii superoxide dismutases, the Mn-superoxide dismutase had a higher metal/protein ratio. Nevertheless, addition of Mn to the purified Fe-protein did not lead to binding of extra Mn. In turn, addition of excess iron to the Mn-protein did not lead to either iron binding or manganese loss. Conformational effects of Fe- or Mn-binding may play a role in the properties of the protein moiety, as also suggested by the significantly less stability.

**TABLE III**

| Protein sample          | Activity % |
|-------------------------|------------|
| Holoprotein             | 100        |
| Apoprotein *             | 34         |
| Apoprotein + Fe(II) *    | 77         |
| Apoprotein + Mn(II) *    | 98         |

*The purified holoenzyme was dialyzed for 24 h at 4 °C against 1 mm o-phenanthroline and 10 mm ascorbate in 0.05 M acetate buffer, pH 5.5. A longer dialysis time led to precipitation.

*The apoprotein was dialyzed for 24 h at 4 °C against 1 mm FeSO₄ or MnSO₄ at pH 5.5 and then exhaustively against many changes of 0.05 M phosphate buffer, pH 7.5.
observed in the case of the Mn protein, for example, to denaturation at slightly alkaline pH.

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