RECONSTITUTION OF IRON-SUPEROXIDE DISMUTASE

Fumiyuki Yamakura and Koji Suzuki

Department of Chemistry, School of Medicine
Juntendo University, Narashino, Chiba 275, JAPAN

Received August 4,1976

Summary: Iron-free superoxide dismutase has been prepared by incubation with sodium carbonate buffer (pH 11.0) containing 10 mM dithiothreitol and 2 mM EDTA under anaerobic conditions and subsequent dialysis to return the pH to 7.8. Reconstituted enzyme was obtained from the apoenzyme by incubation under the same conditions, with addition of 12 mM ferrous ammonium sulfate instead of EDTA. The apoenzyme has no visible absorption and no significant amount of activity. The reconstituted enzyme had the same visible absorption and activity as the native enzyme. Four sulfhydryl groups per molecule of apoenzyme reacted rapidly with 5,5'-dithiobis-(2-nitrobenzoic acid) while holoenzyme reacted with the reagent very slowly.

Superoxide dismutase (EC 1.15.1.1), which catalyzes the reaction $O_2^- + O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2$, is widely distributed among oxygen-metabolizing organisms (1) and some obligate anaerobes (2). With a few exceptions (3,4), SOD was obtained as the Mn or Fe-containing enzyme from procaryotes and mitochondria, whereas SOD from cytosoles of eukaryotes was found to contain Cu and Zn (1). Since McCord and Fridovich (5) reported the preparation of the apo- and reconstituted enzymes of Cu, Zn-SOD, many studies have been carried out on the properties of apo- and reconstituted SOD (1,6,7). Recently, Ose and Fridovich (8) have extended the studies of the reversible removal of Mn atom from Mn-SOD of Escherichia coli and the replacement of Mn with Co,Zn,

Abbreviation: SOD, superoxide dismutase

or Ni, but they failed in the reversible removal of Fe atom from Fe-SOD of Escherichia coli using the same technique which had succeeded with the Mn-SOD.

Fe-SOD from Pseudomonas ovalis has a molecular weight of 40,000 and is composed of two subunits of equal size (9). The crystalline enzyme contains 1.1 g atoms of Fe and 4 mol of sulfhydryl groups per mol of enzyme (9). In this communication, we describe the preparation of apo- and reconstituted enzyme of Fe-SOD from Pseudomonas ovalis. The results have led us to conclude that Fe is essential for enzymatic activity of Fe-SOD. Furthermore, the results of quantitative studies indicate that 4 sulfhydryl groups of holoenzyme might be buried in enzyme molecule and/or chelated to Fe atoms.

MATERIALS AND METHODS

Fe-SOD was isolated from Pseudomonas ovalis as described previously (9). Enzymatic activity was measured by the method of McCord and Fridovich (5) with a few modifications (9). For the studies of the enzyme stability , the enzymatic activity was estimated by the method of Nishikimi et al. (10). Protein was determined by the micro-biuret method of Itzhaki and Gill (11), using bovine serum albumin as standard. Iron was determined by the method of Massey (12) except that batho-phenanthroline was used as a colorimetric reagent. Polyacrylamide gel electrophoresis was performed at 5° C in accordance with the method of Davis (13). Mobilities of the apo- and reconstituted SOD on polyacrylamide gels (6, 7.5, 9, and 10.5 %) were analyzed according to the method of Hedrick and Smith (14). SOD activity on polyacrylamide gel was located by the photochemical method of Beauchamp and Fridovich (15). Sulfhydryl groups were determined spectrophotometrically using 5,5'-dithiobis-(2-nitrobenzoic acid) as described by Ellman (16). Optical absorption spectra were measured with a Hitachi Model 124 Spectrophotometer.

RESULTS AND DISCUSSION

Purified Fe-SOD was treated at various pH's at 30° C for 5 min and the activity of the enzyme was measured. As shown in Fig. 1, the enzyme was completely inactivated at pH 10.9.

30 mg of the Fe-SOD, which had a specific activity of 3,200 and contained 1.12 g atoms of Fe per mol of enzyme, was dialyzed

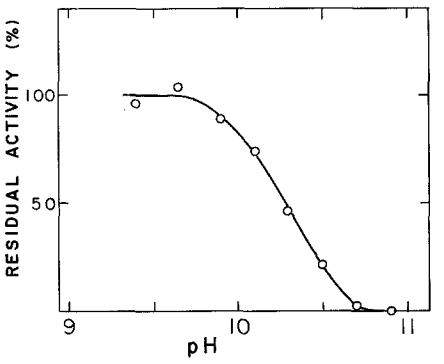

Fig. 1 Effect of pH on the stability of SOD. The enzyme (34 μg/ml) was incubated for 5 min at 30° C with 0.1 M sodium carbonate buffer, at various pH's as indicated. Residual activities were measured by the assay method of Nishikimi et al. (10).

against distilled water overnight. The following procedures were carried out under anaerobic conditions using N_2 gas substitution: The enzyme was incubated for 25 min at 30° C with 0.2 M sodium carbonate buffer containing 2 mM EDTA and 10 mM dithiothreitol, which pH was adjusted to pH 11 by addition of 1 N NaOH. This solution was dialyzed against 0.2 M sodium carbonate buffer (pH 9.2) containing 1 mM EDTA and 1 mM dithiothreitol for 5- 12 hr at 4° C, and subsequently against 50 mM potassium phosphate buffer (pH 7.8) containing 0.5 mM dithiothreitol for 18 hr at 4° C. The apo-SOD was diluted ten fold with distilled water and applied to a DEAE-cellulose (Whatman DE-32) column (0.9 X 4 cm), previously equilibrated by 2.5 mM potassium phosphate buffer (pH 7.8), and then eluted by 50 mM of this buffer containing 1 mM dithiothreitol. This preparation was employed in the reconstitution study.

The apo-SOD was incubated for 25 min at 30° C with the same alkaline buffer as above, with addition of 12 mM ferrous ammonium sulfate instead of 2 mM EDTA. Then this enzyme solution was dialyzed against 0.2 M sodium carbonate buffer (pH 9.2) containing

Fig. 2 Polyacrylamide gel electrophoreses of native, apo-, and reconstituted SOD. Native SOD (a): 44 μg, apo-SOD (b): 40 μg, reconstituted SOD (c): 39 μg, and reconstituted SOD (d): 4 μg were each subjected to polyacrylamide gel electrophoresis at pH 8.9 The gels (a,b,c) were stained for protein by Amido black 10 B; the achromatic zone indicates the enzymatic activity (d).

1 mM dithiothreitol and 1 mM ferrous ammonium sulfate for 5-12 hr at 4° C. After dialysis of the reconstituted enzyme against two changes of 50 mM potassium phosphate buffer (pH 7.8), it was recovered by using a DEAE-cellulose column as described above.

Fig. 2 shows the electrophoretic patterns of the apo- and reconstituted SOD on polyacrylamide gels. One major protein band and one minor were observed in both the apo- and reconstituted SOD. The protein bands of the reconstituted SOD coincided with the zones of the enzymatic activity, which were located by the photochemical method described in the text. Major protein bands of the apo- and reconstituted SOD have the same mobility as the native enzyme (Fig. 2). It appeared that the major and minor protein bands of the apo- and reconstituted SOD were "charge" isomers, according to the analytical method of Hedrick and Smith (15).

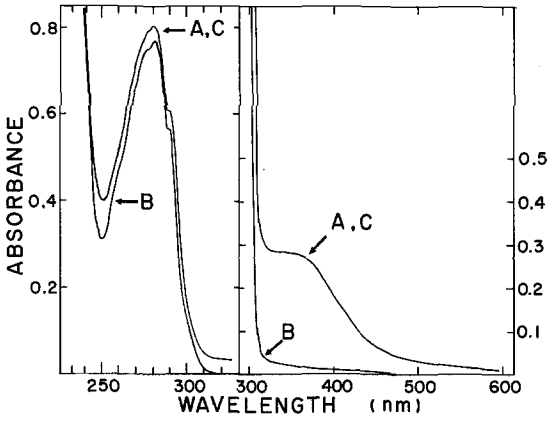

Fig. 3 Absorption spectra of native, apo-, and reconstituted SOD. All protein concentrations were 1 X 10^{-5}M for ultraviolet regions and 1 X 10^{-4}M for visible regions. A: native SOD; B: apo-SOD; C: reconstituted SOD.

Fig. 3 shows the optical absorption spectra of apo- and reconstituted SOD. In the visible range from 320 to 600 nm, the broad absorption normally observed in the native SOD disappeared in the apo-SOD and reappeared in the reconstituted SOD. Although no significant absorptional change was observed in the ultra-

violet region, a small decrease in the absorption at 250 nm was observed in the apo-SOD. This decrease was restored in the reconstituted SOD (Fig. 3).

Iron content and enzymatic activity of the apo- and reconstituted SOD are shown in Table I. Apo-SOD has no significant amount of Fe nor of activity, being respectively 7.5 % and 3 % of those amount in the native enzyme. Reconstituted SOD has about 1.1 g atoms of Fe per mol of enzyme, indicating that only one atom of Fe is combined to each molecule (2 subunits) of the apo-SOD by this reconstitution method. Activity of the reconstituted SOD was 106 % of that of the native enzyme.

Table I

Properties of apo- and reconstituted SOD

	Fe* (g atoms/mol)	Specific activity	$A_{350}:A_{280}$**
1. Native SOD	1.12 ± 0.06	3,200 ± 151	0.035
2. Apo-SOD	0.085 ± 0.022	100 ± 23	0.0025
3. Reconstituted SOD	1.13 ± 0.04	3,400 ± 380	0.035

* Atoms per mol ratio is calculated on the basis of a molecular weight of 40,000.
** Ratio of absorption at 350 nm to 280 nm.
Values are given as mean ± S.D.

Table II shows the reactivity of the sulfhydryl groups in apo- and holo-SOD with 5,5'-dithiobis-(2-nitrobenzoic acid). Four sulfhydryl groups of the native SOD reacted with this reagent only after denaturation by 0.4 % sodium dodecyl sulfate. In contrast, the sulfhydryl groups of the apo-SOD were able to react with the reagent without sodium dodecyl sulfate. Sulfhydryl

Table II

Quantitative analysis of the sulfhydryl groups of apo- and holo-SOD.

	No. of SH groups	
	- SDS	+ SDS (0.4%)
1. Native SOD	0.2	4.1
2. Apo-SOD	4.0	4.3
3. Reconstituted SOD	0.4	4.0

The enzyme (0.8-0.9 mg) was allowed to react with 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.1 M sodium phosphate buffer (pH 8.0) containing 0.1 mM EDTA at 35° C. Increase in the absorption at 412 nm was measured after 30 min.
SDS: sodium dodecyl sulfate

groups of the reconstituted SOD reacted with the reagent only after denaturation, as was the case with the native SOD (Table II). These results suggest that the 4 sulfhydryl groups of the Fe-SOD are masked in the native and reconstituted SOD, but in the apo-SOD these groups are exposed. It is not clear whether this difference in reactivity of the sulfhydryl groups between apo-SOD and holo-SOD was caused by the conformational change of the enzyme molecule, or by the release of Fe from the sulfhydryl groups, or by both. A study of the possibility of participation of the sulfhydryl groups in the binding of Fe to the enzyme is now underway in this laboratory.

ACKNOWLEDGEMENT

The authors thank Prof. T. Arai and Mr. K. Yazawa of Research Institute for Chemobiodynamics, Chiba University for the large scale cultivation of Pseudomonas ovalis.

REFERENCES

1. Fridovich, I. (1974) Adv. Enzymol. Relat. Areas Mol. Biol., 41, 35-97.

2. Hewitt, J., and Morris, J.G. (1975) FEBS Letters, 50, 315-318.
3. Puget, K., and Michelson, A.M. (1974) Biochem. Biophys. Res. Commun., 58, 830-838.
4. Lindmark, D.G., and Muller, M. (1974) J. Biol. Chem., 249, 4634-4637.
5. McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem., 244, 6049-6055.
6. Fee, J.A., and Phillips, W.D. (1975) Biochim. Biophys. Acta, 412, 26-38.
7. Beem, K.M., Rich, W.E., and Rajagopalan, K.V. (1974) J. Biol. Chem., 249, 7298-7305.
8. Ose, D.E., and Fridovich, I. (1976) J. Biol. Chem., 251, 1217-1218.
9. Yamakura, F. (1976) Biochim. Biophys. Acta, 422, 280-294.
10. Nishikimi, M., Rao, N.A., and Yagi, K. (1972) Biochem. Biophys Res. Commun., 46, 849-854.
11. Itzhaki, R.F., and Gill, D.M. (1964) Anal. Biochem., 9, 401-410.
12. Massey, V. (1957) J. Biol. Chem., 229, 763-770.
13. Davis, B.J. (1964) Ann. N.Y. Acad. Sci., 121, 404-427.
14. Hedrick, J.L., and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155-164.
15. Beauchamp, C.O., and Fridovich, I. (1971) Anal. Biochem., 44, 276-287.
16. Ellman. G.L. (1959) Arch. Biochem. Biophys., 82, 70-77.