Superoxide Dismutase from Escherichia coli B

A NEW MANGANESE-CONTAINING ENZYME*

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SUMMARY

Superoxide dismutase, which catalyzes the disproportionation of univalently reduced oxygen (\(\text{O}_2^- + \text{O}_2^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2\)) and which has previously been demonstrated in a variety of mammalian sources, has now been purified from Escherichia coli. Whereas the mammalian enzyme is blue and contains copper and zinc, the bacterial superoxide dismutase is red-purple and was found to contain manganese. The molecular weight of the E. coli enzyme was found to be 39,500 by sedimentation equilibrium and was shown, by gel electrophoresis in the presence of sodium dodecyl sulfate, to be composed of two subunits of equal size. Electron paramagnetic resonance spectrometry and chemical analysis demonstrated between 1.6 and 1.8 atoms of manganese per molecule of enzyme. The enzyme contains no significant amounts of copper or zinc. The ultraviolet and visible absorption spectra of the enzyme are presented, as are the results of amino acid analysis.

MATERIALS AND METHODS

Cytochrome c, type III, and xanthine were obtained from Sigma. E. coli B harvested in late log phase were purchased from Miles Laboratories, Elkhart, Indiana. Microgranular diethylaminoethyl cellulose (DE-32) and microgranular carboxymethyl cellulose (CM-52) were obtained from Reeve Angel Company, New York, New York. Ammonium sulfate, enzyme grade, was a product of Mann. Xanthine oxidase, purified from raw cream by a procedure which avoided exposure to proteolytic agents (7), was generously provided by Drs. F. Brady and K. V. Rajagopalan. All other materials were obtained from commercial sources at the highest available states of purity.

Superoxide dismutase was assayed, and units were defined as previously described (3). Ultracentrifugal analyses were performed at pH 7.3 in 0.10 M KCl, 0.05 M potassium phosphate, on a Beckman model E analytical ultracentrifuge by the method of Yphantis (8). Assessments of the purity of the enzyme by disc gel electrophoresis were performed essentially as described by Davis (9). The molecular weight of the subunits of the enzyme was estimated by disc gel electrophoresis on 10% acrylamide gels, in the presence of sodium dodecyl sulfate, as described by Weber and Osborn (10). The following molecular weight standards were used to calibrate the gels: phosphorylase a, 94,000; human transferrin, 77,000; bovine serum albumin, 68,000; catalase, 60,000; ovalbumin, 43,000; pepsin, 35,000; chymotrypsinogen A, 26,000; and ribonuclease, 13,600. Electron paramagnetic resonance spectroscopy was performed on a Varian E-9HF EPR spectrometer, with a 9.5 GHz microwave bridge assembly, which was operated at a modulation frequency of 100 kHz.

Manganese was determined quantitatively on the basis of the intensity of its EPR signal and by the colorimetric method of Srivastava, Pandya, and Zaidi (11). Zinc assays were performed as described by Malmstrom (12), and copper assays were performed by the method of Felsenfeld (13).

RESULTS

Purification of Enzyme—Frozen E. coli, 500 g, was thawed overnight at 5° and then suspended in 2500 ml of 0.05 M potassium phosphate, 1 X 10^{-4} M EDTA, at pH 7.8. The suspended cells were disrupted by sonication of 400-ml batches at 0° in a Rosett cell for 3 min. A Brancon conifer was used at a power setting of 125 watts. The suspension was clarified by centrifugation for 60 min at 13,200 X g, and after the addition of KCl to a final concentration of 0.10 M the supernatant solution was

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TABLE I

| Purification stage       | Protein concentration<sup>a</sup> | Volume | Total protein | Specific activity | Total units | Yield (%) | Purification fold |
|--------------------------|----------------------------------|--------|---------------|-------------------|-------------|-----------|------------------|
| Supernatant from         |                                  |        |               |                   |             |           |                  |
| Sonicate                 | 95                               | 3,100  | 294,500       | 1.8               | 516,000     | 100       |                  |
| Heat step                | 76                               | 2,950  | 224,200       | 2.2               | 491,700     | 95        | 1.2              |
| Streptomycin sulfate     | 76                               | 3,300  | 250,800       | 2.2               | 401,700     | 95        | 1.2              |
| 50% (NH₄)₂SO₄            | 47                               | 4,000  | 188,000       | 2.7               | 500,000     | 97        | 1.6              |
| 75% (NH₄)₂SO₄            | 30                               | 127    | 3,800         | 8.3               | 317,500     | 62        | 46               |
| precipitate redissolved  |                                  |        |               |                   |             |           |                  |
| Dialysis and centrifugation |                                |        |               |                   |             |           |                  |
| After chromatography on  |                                  |        |               |                   |             |           |                  |
| CM-52                    | 20                               | 150    | 3,000         | 100               | 300,000     | 58        | 56               |
| DE-32                    | 1.93                             | 31     | 163,300       | 61               | 61,000      | 122       | 100              |

*Protein concentration on all fractions, other than those obtained as column effluents, was measured in terms of absorbance at 280 nm assuming that ε<sub>280</sub> = 10.0. The concentration of protein in column effluents was determined by the method of Murphy and Kies (14).

Fig. 1. Chromatography on CM-52. The E. coli preparation after dialysis for 96 hours, as described in the text, was applied to a column, 2.5 × 28 cm, of CM-52 equilibrated at 4°C with 2 mM potassium acetate, pH 5.5, and was then eluted with this buffer until 85 fractions (7 ml) had been collected. At this point, 1000 ml of a gradient (0.002 to 0.20 M) in this buffer was applied. --- o, absorbance at 280 nm; ----, conductivity; -- - - - - , superoxide dismutase activity of the fractions collected. The fractions that were pooled for further manipulation are so indicated.

Fig. 2. Chromatography on DE-32. The superoxide dismutase obtained by chromatography on CM-52 was concentrated and then dialyzed against 5 mM potassium phosphate, pH 7.8, as described under "Purification of Enzyme." It was then applied to a column, 2.5 × 25 cm, of DE-32, which had been equilibrated at 4°C with 5 mM potassium phosphate, pH 7.8, and was eluted with this buffer. --- o, absorbance at 280 nm; ----, superoxide dismutase activity of the fractions collected.

Fig. 3. Equilibrium sedimentation of E. coli superoxide dismutase. Superoxide dismutase at a concentration of 0.3 mg per ml in 0.05 M potassium phosphate, 0.1 mM EDTA, 0.10 M potassium chloride, pH 7.8, was equilibrated at a rotor speed of 35,609 rpm. The ultracentrifuge was equipped with interference optics, and the fringe displacement is here plotted as a function of the square of the distance from the center of rotation.

Heated in 200-ml batches to 60° for 3 min and then chilled. Denatured proteins were removed by centrifugation. Streptomycin sulfate was added to the supernatant to a final concentration of 2.5% and, after incubation at room temperature for 30 min, the suspension was clarified by centrifugation. Solid ammonium sulfate was added to bring the supernatant to 50% saturation and, after stirring for 1 hour at room temperature, the mixture was centrifuged. Solid ammonium sulfate was added to this supernatant to bring it to 75% saturation with respect to this salt, and after stirring at room temperature for 1 hour, the precipitate was collected by centrifugation, suspended in 2 mM potassium acetate buffer, pH 5.5, and then dialyzed at 4°C for 96 hours against 20 volumes of this buffer. During this dialysis, the buffer was replaced every 12 hours. The precipitate that formed during dialysis was removed by centrifugation, and the supernatant was adsorbed onto a column, 2.5 × 28 cm, of CM-
a large peak of protein had been eluted, and 1000 ml of a gradient fate with and without fi-mercaptoethanol, and the molecular column was washed with 2 mM potassium acetate, pH 5.5, until into its component subunits by exposure to sodium dodecyl sulfate. The position of the stained protein bands on the gels was recorded with a Gilford model 2000 spectrophotometer equipped with a gel scanner. It is apparent that sodium conditions. The spectrum in the ultraviolet (solid line) was obtained with a solution containing 0.7 mg per ml of the enzyme in 0.05 M potassium phosphate at pH 7.8, while the spectrum in the visible region (segmented line) was obtained from a solution containing 7.0 mg per ml of the enzyme in the same buffer.

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Molecular Weight—E. coli superoxide dismutase at a concentration of 0.3 mg per ml was equilibrated in a centrifugal field, and the resultant distribution of protein was analyzed by the method of Yphantis (8). When in fringe displacement was plotted as a function of the square of the distance from the center of rotation, a straight line was obtained. This is shown in Fig. 3. The slope of this line and an assumed partial specific volume of 0.720 gave a molecular weight of 39,500. The linearity of the data in Fig. 3 indicates a high degree of homogeneity with respect to sedimentation properties. Polyacrylamide disc gel electrophoresis at pH 8.9 in Tris-HCl buffer did, however, expose the presence of an impurity which was estimated to represent approximately 5% of the total protein.

Subunit Weight—E. coli superoxide dismutase was dissociated into its component subunits by exposure to sodium dodecyl sulfate with and without β-mercaptoethanol, and the molecular weight of these subunits was estimated by disc gel electrophoresis in the presence of these agents (10). Proteins of known subunit

Fig. 4. Determination of subunit molecular weight. E. coli superoxide dismutase was subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate as described by Weber and Osborn (10). The gels were calibrated in terms of molecular weight by using the protein standards listed in the text under identical experimental conditions. The position of the stained protein bands on the gels was recorded with a Gilford model 2000 spectrophotometer equipped with a gel scanner. It is apparent that sodium dodecyl sulfate was able to cause the dissociation of the enzyme in the absence of β-mercaptoethanol (E + SH).

![Molecular weight (x10^4) vs. Absorbance](image)

Fig. 5. Absorption spectrum of E. coli superoxide dismutase. The spectrum in the ultraviolet (solid line) was obtained with a solution containing 0.7 mg per ml of the enzyme in 0.05 M potassium phosphate at pH 7.8, while the spectrum in the visible region (segmented line) was obtained from a solution containing 7.0 mg per ml of the enzyme in the same buffer.

52 which had been equilibrated with the dialysis buffer. The column was washed with 2 mM potassium acetate, pH 5.5, until a large peak of protein had been eluted, and 1000 ml of a gradient (0.002 to 0.20 M) of this buffer were then applied. Fig. 1 presents the results of this elution. The fractions that contained the superoxide dismutase activity were pooled, as indicated in Fig. 1, and were concentrated by ultrafiltration over a PM-10 membrane (Amicon Corporation, Cambridge, Massachusetts). The resultant red-purple solution, which was dialyzed against several changes of 5 mM potassium phosphate, pH 7.8, for 60 hours at 4°C, was placed on a column, 2.5 × 25 cm, of DE-32 which had been equilibrated with this buffer. The enzyme was eluted with 5 mM potassium phosphate, pH 7.8, with the results shown in Fig. 2. The fractions containing the highest specific activity were pooled, as indicated in Fig. 2, and concentrated by ultrafiltration. The results of this purification procedure are summarized in Table I. The yield was 16 mg of superoxide dismutase with a specific activity of 3800 units per mg. This may be compared with the specific activity of 3000 units per mg observed with the enzyme from bovine erythrocytes (3).

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![Absorbance vs. Centimeters](image)

| Amino acid | Amino acid content | Residues per mole of enzyme (nearest integer) |
|------------|-------------------|---------------------------------------------|
| Lysine     | 32.9              | 29                                           |
| Histidine  | 12.3              | 12                                           |
| Arginine   | 10.4              | 10                                           |
| Aspartic acid | 41.9          | 42                                           |
| Threonine  | 18.5              | 19                                           |
| Serine     | 26.0              | 22                                           |
| Glutamic acid | 36.7            | 37                                           |
| Proline    | 15.4              | 15                                           |
| Glycine    | 26.4              | 26                                           |
| Alanine    | 47.1              | 47                                           |
| Valine     | 20.4              | 20                                           |
| Methionine | 3.1               | 3                                            |
| Isoleucine | 13.7              | 14                                           |
| Leucine    | 37.5              | 38                                           |
| Tyrosine   | 11.8              | 12                                           |
| Phenylalanine | 18.0            | 18                                           |
| Total residues | 364              | 364                                          |

*a Duplicate samples were hydrolyzed for 24, 48, and 72 hours.
*b All calculations were based on a molecular weight of 39,500.
*c Values were extrapolated to zero time of hydrolysis.
*d Samples of 72 hours were used for calculations.
Each repetition was preceded by careful checking of the alignments.

from the native enzyme at a concentration of 4.5 mg per ml in water; while Spectrum C was that obtained from 0.1 mM MnCl₂ in 0.10 M HCl. These spectra, which were obtained with a flat cell assembly and of reduced flavins, quinones, and dyes (26) to carry out the univalent reduction of oxygen to this radical poses the possibility that superoxide radicals may also be generated within cells by nonenzymic oxidations. Indeed, although the quantitatively small amount of superoxide dismutase that we have been able to isolate and purify is composed of two subunits of equal size, the association of which does not involve disulfide bridges.

Spectral Properties—The color of the *E. coli* superoxide dismutase was obviously different from the color of the erythrocyte enzyme. Its absorption spectrum in the visible region confirmed these differences. Thus, as shown in Fig. 5, the *E. coli* enzyme had an absorption maximum at 473 nm with a molar extinction coefficient of 400. The erythrocyte enzyme, on the other hand, absorbed at 650 nm (3). The differences in spectral properties between the superoxide dismutases from *E. coli* and from erythrocytes extended to the ultraviolet region as well. Thus, as shown in Fig. 5, the *E. coli* enzyme exhibited the usual protein absorption maximum at 283 nm, whereas the erythrocyte enzyme had previously been seen to absorb maximally at 258 nm (3).

Amino Acid Analysis—*E. coli* superoxide dismutase was dialyzed at 4° against changes of de-ionized water for 1 week, and its concentration was then estimated on the basis of absorbance in the short ultraviolet. Aliquots were hydrolyzed under reduced pressure by heating to 110° in the presence of 6 N HCl for 24, 48, and 72 hours. After removal of the HCl under reduced pressure, the samples were assayed for their amino acids on a Beckman model 120 C amino acid analyzer. The results of this analysis are presented in Table II. The amino acid composition of the *E. coli* enzyme is compared with that of the human erythrocyte enzyme (15), striking differences are apparent. The erythrocyte enzyme contains no tyrosine and no methionine, whereas the *E. coli* enzyme contains 3 residues of methionine and 12 of tyrosine. Less pronounced differences occur in virtually all of the amino acids in these proteins.

**Manganese—** EPR spectroscopy of native *E. coli* superoxide dismutase failed to show the characteristic copper(II) signal observed with the bovine erythrocyte and heart superoxide dismutase⁴ or with human cytocuprein (16) which is, in fact, superoxide dismutase. Indeed, no EPR signal was observed with the native enzyme. Upon denaturation by boiling in 0.1 N HCl, however, the very characteristic signal of manganese(II) appeared. This is shown in Fig. 6. Spectrum A was obtained from 1.35 mg per ml of *E. coli* superoxide in 0.10 N HCl which had been exposed to 100° for 3 min; Spectrum B was obtained from the native enzyme at a concentration of 4.5 mg per ml in water; while Spectrum C was that obtained from 0.1 mM MnCl₂ in 0.10 N HCl. These spectra, which were obtained with a flat cell assembly at room temperature, were repeated three times. Each repetition was preceded by careful checking of the alignment of the flat cell in the magnetic field. The results obtained, which were perfectly reproducible, indicated that the *E. coli* superoxide dismutase contained 1.6 atoms of manganese per molecule of enzyme. Quantitative colorimetric analysis for manganese after wet-ashing of the enzyme by the method of Srivastava et al. (11) demonstrated 1.8 atoms of manganese per molecule of enzyme. Because erythrocyte superoxide dismutase contains both copper (4, 5, 16) and zinc (17), the *E. coli* enzyme was assayed for both of these metals. Copper could not be detected by the 2,2'-biquinoline method (13), and only insignificant amounts of zinc were found by the dithizone method (12).

**DISCUSSION**

The demonstrated ability of milk xanthine oxidase (2, 3, 18-21), liver aldehyde oxidase (19, 22), bacterial dihydroorotic dehydrogenase (19, 23), and diamine oxidase (24) to generate superoxide radicals indicates the likelihood that other oxidative enzymes can also do so. The ability of reduced ferredoxin (25) and of reduced flavins, quinones, and dyes (26) to carry out the univalent reduction of oxygen to this radical poses the possibility that superoxide radicals may also be generated within cells by nonenzymic oxidations. Indeed, although the quantitatively important sources of superoxide radical within living cells remain to be identified, it appears unavoidable that this radical will be found to be generated, to a greater or lesser extent, in all aerobic cells. In this case, the superoxide dismutase, which we suppose to be a defense against the damaging reactivities of this free radical, should be ubiquitous in aerobic cells. As one means of exploring the validity of these lines of thought, we have sought to isolate the superoxide dismutase of a common microorganism.

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⁴ B. B. Keele, Jr., J. M. McCord, and I. Fridovich, unpublished observations.
Superoxide dismutase was found in E. coli and has been isolated from that source. This result, although not conclusive in itself, is consistent with the position taken above.

The enzyme obtained from E. coli, although possessing a specific activity virtually identical with that of the mammalian enzyme, was nevertheless grossly different from it in many important respects. The molecular weight of the mammalian enzyme has been shown to be 37,600 (3), whereas that of the E. coli enzyme is 39,500. The mammalian enzyme is composed of two subunits whose association depends upon disulfide bonds, while the association of the subunits of the E. coli enzyme depends upon noncovalent interactions. The amino acid compositions of these enzymes bear no relation to each other, and their solubility properties reflect these differences. The mammalian enzyme contains no methionine and no tyrosine (15), and its spectrum, which is very atypical for a protein, is strongly influenced by the spectrum of phenylalanine (3). In contrast, the E. coli enzyme contains 3 eq of methionine and 12 of tyrosine and exhibited the usual protein ultraviolet absorption band near 280 nm. The E. coli enzyme was unable to survive the acetone precipitation that was successfully applied during the purification of the erythrocyte superoxide dismutase. Finally, and most strikingly, the mammalian enzyme contains copper and zinc (17), whereas the E. coli enzyme contains manganese.

Only a few proteins and only one other enzyme have thus far been found to contain tightly bound manganese. The pyruvate carboxylase from chicken liver mitochondria has been shown to contain 3.6 atoms of tightly bound manganese per molecule of enzyme (27). It was not possible to dissociate reversibly the manganese of pyruvate carboxylase, and we have also been unable, thus far, to accomplish this with the superoxide dismutase from E. coli. A hemagglutinin from jack bean, called concanavalin A, has also been found to contain manganese (28-30), and in this case it was possible to demonstrate that a reversible loss of hemagglutinin activity accompanied the reversible removal of manganese (28). Concanavalin A has been assayed for superoxide dismutase and contains no activity. Another manganese-protein has recently been isolated from peanuts (31). It has been reported that E. coli possesses a specific transport system for the uptake of manganese (32).

Since superoxide dismutase probably functions as an oxidoreductase, alternately being oxidized and reduced by its radical substrate, the metal ions associated with these enzymes may oscillate between two valence states during the course of the catalytic action. EPR studies on the mammalian superoxide dismutase have indicated that all of the copper in the resting enzyme can be accounted for as Cu(II). Similar studies performed on the E. coli enzyme, however, do not lead to conclusive results as to the valence state of the manganese in the resting enzyme. The fact that no manganese signal could be observed in the native enzyme may be explained in terms of the metal being present as either Mn(II) or Mn(III). Since Mn(III) is not a paramagnetic species, it would not be expected to give a signal. It could readily be reduced upon denaturation of the enzyme, however, giving rise to the characteristic Mn(II) signal. Although Mn(II) is a paramagnetic species, it need not produce a signal if it is tightly bound to protein and thereby immobilized within its ligand field. Such an effect on the EPR signal of bound Mn(II) has been observed in the case of concanavalin A (30). One additional consideration, that of the visible chromo-
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