Further Characterization of Bovine Superoxide Dismutase and Its Isolation from Bovine Heart*

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SUMMARY

Amino acid analysis, electron resonance spectroscopy, metal analysis, and analysis for subunit structure have been performed on bovine superoxide dismutase. The enzyme consists of two subunits of identical molecular weight held together by disulfide bonds.

Superoxide dismutase has been purified from bovine heart by a procedure which was previously developed for the isolation of this enzyme from bovine erythrocytes. The heart enzyme is judged to be identical with the erythrocyte enzyme on the basis of response to an unusual purification procedure, specific enzymatic activity, content of copper and zinc, molecular weight, amino acid composition, ultraviolet absorption spectrum, and electron paramagnetic resonance spectrum. The widespread distribution of superoxide dismutase in mammalian tissues is taken as one indication of its importance to the survival of the cells which constitute these diverse tissues.

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Material and methods

Cytochrome c, type III, and xanthine were products of Sigma. Microgranular diethylaminoethyl cellulos (DEAE) was a product of the Reeve Angel Company, New York. Milk xanthine oxidase, purified from raw cream by a procedure not involving treatment with proteolytic agents (11), was a gift of F. O. Brady and K. V. Rajagopal. Spectrophotometric assays were performed with a Gilford model 2000 absorbance indicator equipped with a photoelectric indicator equipped with a thermostatted sample compartment. Absorption spectra were obtained with a Cary model 15 recording spectrophotometer. Electron paramagnetic resonance was investigated with a Varian model E-9 HP (Varian Associates, Palo Alto, California). Measurements of sedimentation equilibrium were performed at pH 7.3 in 0.10 M KCl-0.05 M potassium phosphate utilizing a Beckman model E ultracentrifuge. Amino acid analyses were performed with a Beckman model 120 amino acid analyzer. Samples were prepared by dialysis against deionized water. Lyophilized aliquots were then sealed under vacuum in 1 ml of 6 M HCl. The sealed tubes were hydrolyzed at 110°C for periods of 24, 48, and 72 hours. For those residues which suffer destruction upon hydrolysis maximal values were obtained by extrapolation to zero time. For those which are released slowly the 72-hour values were used. Tryptophan was determined by the method of Spies and Chambers (12).

The molecular weight of the subunits of the enzyme was estimated by sieving gel electrophoresis on 10% acrylamide gels in the presence of sodium dodecyl sulfate, with and without β-mercaptoethanol, as described by Weber and Osborn (13). Molecular weight standards used to calibrate the gels were 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. Zinc was quantitated by the method of Malmstrom (14), copper by the method of Felsenfeld (15), and manganese by a modification of the method of Srivastava, Pandya, and Zaidi (16). Samples (20 to 50 μl) were wet ashed by refluxing for 30 min with 0.2 ml of concentrated sulfuric acid. After
cooling, 2 drops of 30% H2O2 were added and the refluxing repeated. The sample was then diluted with 2.0 ml of the citrate-borate acid buffer (pH 7.0), and the pH of the solution was adjusted to pH 7.0 with 10.0 N NaOH under a glass electrode. The solution was then diluted to 10.0 ml with water. The remainder of the procedure was as reported by Srivastava et al. (16).

The extent of hemoglobin contamination of a heart tissue homogenate was determined in order to eliminate the possibility that a significant fraction of the enzyme purified from heart was contributed by occluded erythrocytes. A measured aliquot of the supernatant from the homogenate was applied to a column of Sephadex G-75 (2.5 X 40 cm) and was eluted with 0.1 M KCl. The hemoglobin separated completely from the lower molecular weight myoglobin. The total amount of hemoglobin and the second consisted of reduction of the heme proteins by the addition of solid sodium dithionite, followed by reading the absorbance at 555 nm. In both cases results were compared with those obtained with standard solutions of commercially prepared crystalline bovine hemoglobin from Pentex.

The human and bovine erythrocyte superoxide dismutases used for spectral and amino acid composition comparisons were prepared by the previously published procedure (1). The human enzyme was generously supplied by Dr. Herbert Evans.

**Assay of Superoxide Dismutase**—The instability of its substrate dictates an indirect assay for this enzyme. The method used depends upon the ability of superoxide dismutase to compete with ferricytochrome c for superoxide anions generated by the aerobic xanthine oxidase system and thus to inhibit the reduction of cytochrome c by this system (1). The standard assay was performed in 3.0 ml of a solution containing 1 X 10^-5 M ferricytochrome c, 5 X 10^-5 M xanthine, 1 X 10^-4 M EDTA, and 0.05 M potassium phosphate at 25° and pH 7.8. Sufficient xanthine oxidase (6 X 10^-3 u) was added to produce a rate of reduction of cytochrome c of 0.025 absorbance per min at 550 nm. Under these specified conditions one unit of superoxide dismutase is that amount which halved the rate of reduction of cytochrome c.

**Purification of Enzyme**—The purification procedure which was used in the isolation of superoxide dismutase from bovine erythrocytes (1) was applicable, with minor modifications, to the purification of the enzyme from heart. Fresh bovine heart, obtained from a local abattoir, was washed, trimmed of fat and connective tissue, and stored at -20° until needed. After thawing overnight at 4°, 1,200 g of heart muscle was diced and homogenized at high speed in a Waring blender with 2,500 ml of 0.05 M potassium phosphate, 1 X 10^-4 M EDTA, pH 7.8. Unless otherwise specified all subsequent steps were performed at 4°. The homogenate was clarified by centrifugation at 13,700 x g for 1 hour. The supernatant was treated with 0.25 volume (475 ml) of ethanol and 0.05 volume (95 ml) of chloroform under continuous stirring. After 15 to 30 min the mixture was centrifuged at 25,400 x g for 15 min and the supernatant was warmed to room temperature. Solid dibasic potassium phosphate (300 g per liter) was then added slowly while the solution was stirred. This procedure resulted in the settling out of an ethanol-rich upper phase. This upper phase was collected and was centrifuged at 25,400 x g for 15 min. The supernatant was pooled in an ice-salt bath to 0°, and 0.75 volume of acetone (chilled to -20°) was added with stirring. The rubbery precipitate was collected by centrifugation at 13,700 x g for 1 hour. The supernatant was treated with 0.25 volume (475 ml) of ethanol and 0.05 volume (95 ml) of chloroform under continuous stirring. After 15 to 30 min the mixture was centrifuged at 25,400 x g for 15 min and the supernatant was warmed to room temperature. Solid dibasic potassium phosphate (300 g per liter) was then added slowly while the solution was stirred. This procedure resulted in the settling out of an ethanol-rich upper phase. This upper phase was collected and was centrifuged at 25,400 x g for 15 min. The supernatant was pooled in an ice-salt bath to 0°, and 0.75 volume of acetone (chilled to -20°) was added with stirring. The rubbery precipitate was collected by centrifugation at -20°, was suspended in 0.005 M potassium phosphate, pH 7.8, and was stirred in this buffer for 24 hours. The suspension was clarified by centrifugation at 4°, 1,200 g for 1 hour. The supernatant was treated with 0.25 volume (475 ml) of ethanol and 0.05 volume (95 ml) of chloroform under continuous stirring. After 15 to 30 min the mixture was centrifuged at 25,400 x g for 15 min and the supernatant was warmed to room temperature. Solid dibasic potassium phosphate (300 g per liter) was then added slowly while the solution was stirred. This procedure resulted in the settling out of an ethanol-rich upper phase. This upper phase was collected and was centrifuged at 25,400 x g for 15 min. The supernatant was pooled in an ice-salt bath to 0°, and 0.75 volume of acetone (chilled to -20°) was added with stirring. The rubbery precipitate was collected by centrifugation at -20°, was suspended in 0.005 M potassium phosphate, pH 7.8, and was stirred in this buffer for 24 hours. The suspension was clarified by centrifugation at 4°, 1,200 g for 15 min. The supernatant was dialyzed against several changes of 0.005 M potassium phosphate, pH 7.8, over a period of 24 hours and was adsorbed onto a column of DE-32 (2.5 X 36 cm) which had been equilibrated with this buffer. A linear gradient of potassium phosphate ranging from 0.005 to 0.20 M, pH 7.8, was applied to a column, 2.5 X 40 cm, of DE-32 equilibrated with 0.005 M potassium phosphate, pH 7.8, and elution with this buffer was continued up to Fraction 62, at which point 1000 ml of a gradient, linear in potassium phosphate from 0.005 to 0.20 M, and at pH 7.8, was applied. Fractions were assayed for absorbance at 280 nm (• • • •) and for superoxide dismutase activity (ΔΔΔΔ). There were no peaks of activity aside from that one indicated. The conductivity of the effluent is indicated by the broken line.
Results

Purification—The pattern of elution of materials absorbing in the ultraviolet and of superoxide dismutase activity from the DE-32 column is shown in Fig. 1. The solid points in Fig. 1, which indicate absorbance at 280 nm, show that several protein components were well separated from the peak of activity (open triangles) which coincided with one of the minor protein peaks. The dashed line in this figure represents the conductivity of the effluent solution. Fig. 2 presents the results of gel-exclusion chromatography on Sephadex G-75 of the active component obtained from the DE-32 column. The symmetry of this peak suggested that the product was essentially homogeneous at this point, as did its specific activity of 3660 (1). This product was a pale blue-green when viewed at a concentration of 10 mg per ml. The results of this purification scheme are summarized in Table I.

Contamination by Erythrocyte Enzyme—Assays for hemoglobin in the homogenate of heart tissue yielded, by two independent methods, values of 5.1% and 4.3% of the total protein. Calculations based on the higher value indicate that sufficient blood was present at the initial step in Table I to contribute 3.9 g of hemoglobin. This amount of blood would contain 1.6 mg of superoxide dismutase (1). Since a 50% loss can be expected, the product represented in Table I could contain maximally 0.8 mg of the erythrocyte enzyme. Thus the product must represent at least 91% of the heart enzyme.

Molecular Weight—The purified heart enzyme was brought to sedimentation equilibrium at 35,600 rpm. The pattern of interference fringes which was obtained was analyzed by the method of Yphantis (19). The plot of ln fringe displacements as a function of the square of the distance from the center of rotation defined a straight line and from the slope of this line and assuming a partial specific volume of 0.72, a molecular weight of 32,500 was obtained.

Subunit Weight—Bovine erythrocyte superoxide dismutase was dissociated into its component subunits by exposure to sodium dodecyl sulfate in the presence of β-mercaptoethanol, and the molecular weight of these subunits was estimated by disc gel electrophoresis in the presence of these agents (13). Proteins of known subunit weight were used as standards, as described under "Materials and Methods." The superoxide dismutase was found to be composed of subunits having a molecular weight of approximately 16,300. As shown in Fig. 3 both sodium dodecyl sulfate and β-mercaptoethanol were required to dissociate superoxide dismutase. In the absence of β-mercaptoethanol a molecular weight of approximately 35,000 was obtained. We may conclude that bovine superoxide dismutase is composed of two subunits of equal size, the association of which does involve at least one disulfide bridge.

Ultraviolet Absorption Spectrum—The ultraviolet absorption spectrum of the superoxide dismutase isolated from bovine heart was compared with that of the previously described erythrocyte enzyme (1). Both enzymes were at approximately 1.5 mg per ml and as shown in Fig. 4 their spectra were identical.
Electron Paramagnetic Resonance Spectrum and Copper Content—Because of its content of copper(II), superoxide dismutase may be expected to display a characteristic EPR spectrum. Indeed, EPR spectra were used by Carrico and Deutsch to help establish the identity of erythrocuprein, hepatocuprein, and cerebrocuprein (9). Fig. 5 compares the EPR spectra of the superoxide dismutase from bovine heart with that from bovine erythrocytes. Their similarity is obvious. Integration of the derivative EPR signal and determination of the area under the EPR absorption curve thus generated for the erythrocyte enzyme indicated that it contained 1.94 moles of copper(II) per 32,600 g of enzyme. The EPR spectrum obtained from the heart enzyme was not integrated.

When the copper content of the erythrocyte enzyme was determined by the biquinoline method (15), a result in perfect agreement with the EPR data was obtained. Thus, the erythrocyte superoxide dismutase contained 1.94 moles of total copper per mole (32,600 g) of enzyme. This agreement also indicates that all of the copper in the erythrocyte superoxide dismutase was copper(II). A second preparation of superoxide dismutase from bovine erythrocytes was prepared and was found to contain 1.84 moles of total copper per mole of enzyme as estimated by the biquinoline method. The heart enzyme, when similarly assayed, was found to contain 1.64 moles of copper per 32,600 g of enzyme.

Zinc and Manganese Content—Carrico and Deutsch (10) have reported that human erythrocuprein contains zinc as well as copper and in equivalent amounts. The superoxide dismutase from bovine erythrocytes and from bovine heart were accordingly analyzed for zinc by the dithizone method (14). The erythrocyte enzyme was found to contain 1.71 and 1.76 moles of zinc per 32,600 g of enzyme. It is evident that the superoxide dismutases from bovine heart and erythrocytes do contain equivalent amounts of zinc and copper, having two atoms of each per molecule of enzyme. Because the superoxide dismutase from Escherichia coli was found to contain manganese in place of either copper or zinc (20), it was considered of interest to assay the bovine super-

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**TABLE II**

**Amino acid composition of bovine heart and erythrocyte superoxide dismutase**

| Amino acid   | Erythrocyte enzyme | Heart enzyme |
|--------------|--------------------|--------------|
| Lysine       | 22                 | 22           |
| Histidine    | 16                 | 16           |
| Arginine     | 10                 | 9            |
| Aspartic acid| 35                 | 36           |
| Threonine    | 26                 | 25           |
| Serine       | 20                 | 21           |
| Glutamic acid| 24                 | 27           |
| Proline      | 14                 | 15           |
| Glycine      | 50                 | 54           |
| Alanine      | 21                 | 22           |
| Valine       | 28                 | 26           |
| Methionine   | 0                  | 0            |
| Isoleucine   | 17                 | 17           |
| Leucine      | 20                 | 18           |
| Tyrosine     | 2                  | 2            |
| Phenylalanine| 10                 | 9            |
| Tryptophan   | 0                  | 0            |

1 The abbreviation used is: EPR, electron paramagnetic resonance.

2 Nearest integer as residues per mole of enzyme, based on a molecular weight of 32,600.

3 Determined by the method of Spies and Chambers (12).
oxide dismutase for manganese. No manganese could be detected in either the heart or erythrocyte enzyme by the method of Srivastava et al. (16).

**Amino Acid Analysis**—The heart and erythrocyte superoxide dismutases were also compared on the basis of their amino acid analyses. The results, which are shown in Table II, indicate that their compositions were found to be identical within experimental error. Analysis of the erythrocyte enzyme for tryptophan by the method of Spies and Chambers (12) revealed only 0.1 residue of tryptophan per subunit weight of 10,000. Furthermore, no loss of activity resulted when the enzyme was treated with Koshland’s reagent (2-hydroxy-5-nitrobenzyl bromide) (21) under conditions which resulted in total inactivation of acetoacetate decarboxylase (22). Cystine was not quantitatively determined.

**DISCUSSION**

A number of criteria indicate that the superoxide dismutase from bovine heart is identical with that previously isolated from bovine erythrocytes. A purification procedure which was effective for the erythrocyte enzyme (1) was found to be similarly useful in the isolation of the heart enzyme, although the yield was significantly lower. The reasons for the lower yield are not clear, but may involve interactions between other protein components of the heart muscle. This isolation procedure contains a very unusual step, the migration of the enzyme into a largely alcoholic phase, which was rendered immiscible with the aqueous phase by salting out with potassium phosphate. The extraction of superoxide dismutase into this salted-out phase must reflect an unusual disposition of hydrophobic residues. The superoxide dismutases from bovine heart and from erythrocytes both responded to this unusual fractionation procedure, which leads to the conclusion that they have similar, if not identical structures. In addition, the enzymes isolated from these two tissues exhibited specific enzymatic activities, copper and zinc contents, EPR spectra, molecular weights, and amino acid compositions which were identical within the limits of precision of the analytical methods used.

The amino acid composition of bovine superoxide dismutase has not been previously published and merits some comment. As would be expected, the composition of the bovine enzyme corresponds closely to that of the human enzyme (4, 6, 9, 23), but with certain notable differences. The bovine enzyme contains 25 or 26 residues of threonine and two of tyrosine compared to only 16 residues of threonine and no tyrosine reported for the human protein. The presence of 2 tyrosine residues and the absence of tryptophan in the bovine enzyme permits a more thorough discussion and comparison of the ultraviolet absorption spectra of the human and bovine proteins. Both of these proteins are characterized by somewhat atypical ultraviolet absorption spectra. Molar absorptivities at 280 nm are low for both the human and bovine enzymes and were found to be 16,300 and 8,100, respectively, with a difference of 8,200 between the two. The bovine enzyme contains 2 tyrosine residues while the human enzyme contains none (6, 9, 23), yet in spite of this the bovine enzyme exhibits only half as much absorptivity at 280 nm (Fig. 6). To account for this discrepancy we suggest that the human enzyme contains 2 residues of tryptophan not present in the bovine enzyme. Fig. 3 shows that the bovine enzyme is composed of two subunits of molecular weight 16,300. (Although the subunits have not been shown to be identical, this is, for the present, assumed to be the case. Therefore, the amino acid substitutions are discussed in terms of pairs of residues.) In accord with the proposed substitutions is the shoulder characteristic of tryptophan which is evident on the absorption spectrum of the human enzyme at 290 nm but absent with the bovine enzyme. Fig. 6 makes this difference apparent. Spectra of the human enzyme have been published by others (6, 9, 10), but these earlier spectra were not obtained with a recording spectrophotometer and the fine structure evident in Fig. 6 was therefore not apparent.

There is, furthermore, quantitative support for our suggestion that the human enzyme has 2 tryptophan and no tyrosine residues, while the bovine enzyme has two tyrosine and no tryptophan. According to Wetlaufer (24), the predicted change in molar absorptivity for such substitutions would be 8400, which compares favorably with our observed difference of 8200.

Previous investigators of human superoxide dismutase (erythrocuprein) were unable to detect significant amounts of tryptophan (4, 6). Bannister, Salisbury, and Wood (25), however, by titrating fluorimetrically with N-homosuccinimide, concluded that the human enzyme does contain “at least one tryptophan residue per mole” of protein, lending support to our present suggestion that it actually contains two residues per mole.

If we tentatively assume that the catalytic activity of superoxide dismutase is essential to the survival of aerobic cells, then we need not be surprised to find it widely distributed in mammalian tissues. In this light its presence in bovine heart and erythrocytes is a fully expected finding.

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