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Properties of the Apoprotein and Role of Copper and Zinc in Protein Conformation and Enzyme Activity of Bovine Superoxide Dismutase†

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ABSTRACT: Different conditions of metal removal from hemocuprein (bovine superoxide dismutase) have been tested to obtain a selective detachment of copper and zinc without inducing irreversible changes in the protein. Electron paramagentic resonance (epr), circular dichroism (CD), and optical spectra, electrophoretic behavior, and superoxide dismutase activity have been used to evaluate the reconstitution capacity of the different types of apoprotein. It has been observed that complete removal of zinc prevents copper from recombination at the native site and leads to the irreversible loss of the properties of the holoprotein. Zinc appears to contribute neither to the ultraviolet CD and absorption spectra of the bovine enzyme, nor to the enzymatic activity. Addition of copper to apoprotein restores the spectral, electrophoretic, and catalytic properties of the holoprotein to an extent proportional to the residual zinc left in the protein after metal depletion. The bovine enzyme contains six cysteic acid residues per 33,000 molecular weight. Since 4 SH groups per mole of protein are titrated in the absence of zinc, it appears that one disulfide bridge is present in the bovine enzyme. One tryptophan per mole is found. Only one lysine residue per 33,000 molecular weight is released by carboxypeptidase B after complete removal of zinc. The bovine enzyme copper is reversibly reduced by ferrocyanide. Addition of stoichiometric amounts of H_2O_2 in the absence of oxygen leads to disappearance of the copper epr signal; the original intensity is slowly recovered by exposure to air. It is proposed that copper is directly involved in the catalytic activity of the bovine enzyme, while zinc stabilizes the protein conformation which provides for a native copper site. The two equivalent copper sites could be located on two subunits. These are not identical but probably of equal size and held together by the single disulfide bridge.

McCord and Fridovich (1969) have shown that an enzymatic activity, namely superoxide dismutase activity, is associated with human and bovine erythrocuprein, the copper and zinc containing protein of red blood cells. This activity is lost by removing copper and partially restored by incubating the apoprotein in the presence of excess cupric ion. Most recently the bovine protein, to which we shall refer as the bovine enzyme, has been further characterized (Bannister *et al.*, 1971; Wood *et al.*, 1971; Keele *et al.*, 1971) and the amino acid composition has been reported. On the basis of electron paramagnetic resonance (epr) and circular dichroism (CD) spectra under various conditions evidence was also presented (Rotilio *et al.*, 1971) for the presence of three to four nitrogen ligands around the copper and for the accessibility of the copper site for water. It became apparent from these studies that reversible and selective removal of the metals, so that protein denaturation is avoided, might lead to decisive new information. The subsequent paper reports the properties of the apoprotein prepared by different procedures: the results obtained will be discussed with a view to the possible role of the two types of metal ions in catalyzing superoxide dismutation and maintaining the native conformation of the protein. Data on the primary structure of the protein will also be reported.

Materials and Methods

Protein and Other Chemicals. All chemicals were reagent grade and were used without further purification. Ultra Pure guanidine-HCl was obtained from Mann Research Laboratories. The bovine enzyme was purified from cattle blood according to McCord and Fridovich (1969). The molecular weight was assumed to be 33,000 (McCord and Fridovich, 1969). Protein concentration was determined by measuring absorbance at 258 nm, with an extinction coefficient (ϵ_{1cm}^{mM}) of 10.3 for the holoprotein and 5.8 for the apoprotein. These values were obtained from dry weight determinations and nitrogen analyses carried out with a Coleman nitrogen analyzer. The nitrogen content was found to be 16%.

Amino Acid Analyses. Amino acid analyses after hydrolysis with 6 N HCl were performed with a Bio-Cal BC-200 instrument, using a single column system. Total cystine–cysteine content was determined as cysteic acid after 6 N HCl hydrolysis in the presence of 0.21 M dimethyl sulfoxide (Spencer and Wold, 1969). Sulfhydryl groups were determined

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TABLE I: Metal Removal from Bovine Enzyme under Different Conditions.[a]

Dialysis Conditions	pH	Dialysis Time (hr)	Temp (°C)	Copper (g-atom)	Zinc (g-atoms)
I, 0.05 M acetate buffer + 10^{-3} M EDTA	3.8	12	30	0.2	0.12
II, 0.05 M Tris-HCl + 0.05 M NaCN	8.0	72	30	0.38	2.2
	8.4	72	30	0.16	0.3
III, 0.05 M Tris-HCl + 0.05 M NaCN after reduction with excess ferrocyanide	8.0	18	4	0.35	2.2

[a] The copper and zinc contents before treatment were 1.85 and 2.2 g-atoms per mole of protein, respectively.

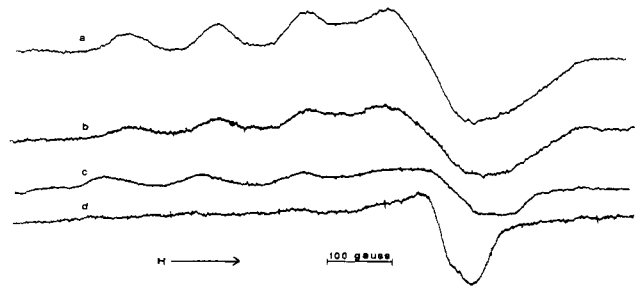

FIGURE 1: Reconstitution ability of differently prepared apoprotein samples as tested by epr. Curve a: bovine enzyme (1% in water). The solution (0.3 ml) contains 0.210 μmole of Cu^{2+} and 0.280 μmole of Zn. Curve b: to a sample of type II apoprotein (1% in water, 0.3 ml) containing 0.03 μmole of Cu^{2+} and 0.26 μmole of Zn, 0.11 μmole of $Cu(ClO_4)_2$ was added; the sample was kept 1 hr at room temperature and then dialyzed overnight against water before freezing for the spectrum. Curve c: a sample of type II apoprotein (1% in water, 0.3 ml) containing 0.026 μmole of Cu^{2+} and 0.05 μmole of Zn was treated as in curve b. Curve d: a sample of type I apoprotein (1% in water, 0.3 ml) containing 0.02 μmole of Cu^{2+} and 0.05 μmole of Zn was treated as in curve b. Previous addition of Zn^{2+} to the full complement had no effect. Modulation amplitude, 10 gauss; microwave power, 6 mW; microwave frequency, about 9.15 GHz; temperature, −150°.

by the method of Boyer (1954). The tryptophan content was estimated on the short column of the amino acid analyzer after alkaline hydrolysis in 4 N $Ba(OH)_2$ according to Davis *et al.* (1970) and by the spectrophotometric method of Edelhoch (1967). Amides were measured according to Hoare and Koshland (1967) using 1-ethyl-3-dimethylaminopropylcarbodiimide as activating agent and β-alanine ethyl ester as nucleophile. Carbohydrates were assayed according to the method of Dubois *et al.* (1958). N-Terminal amino acids were analyzed either by the cyanate method of Stark and Smyth (1963) or after dansylation according to the technique of Gros and Labouesse (1969). Dansylamino acids were separated on polyamide thin-layer plates with the solvent systems suggested by Woods and Wang (1967). Digestions with carboxypeptidases A and B (Worthington, diisopropyl fluorophosphate treated) were performed at 37° in 0.01 M ammonium bicarbonate, generally in the presence of 3×10^{-4} M sodium dodecyl sulfate. At various times aliquots of the reaction mixture, to which an internal standard of norleucine had been added in order to evaluate the recoveries, were removed, acidified with few drops of glacial acetic acid, and analyzed with the automatic amino acid analyzer. In every case blanks of the carboxypeptidases and of the bovine enzyme were subtracted (Ambler, 1967). Protein concentrations were determined after amino acid analysis on an aliquot of the digestion mixture which had been subjected to acid hydrolysis.

Preparation of Apoprotein. The apoprotein was prepared in three different ways: (I) according to McCord and Fridovich (1969) and Bannister *et al.* (1971), that is, by overnight dialysis at room temperature against 0.05 M acetate buffer (pH 3.8) containing 10^{-3} M EDTA; (II) according to Carrico and Deutsch (1970), by 72-hr dialysis at room temperature against 0.05 M Tris-HCl containing 0.05 M KCN at pH 8; (III) by reducing the copper with an excess of potassium ferrocyanide and dialyzing 18 hr at 4° against 0.05 M Tris-HCl containing 0.05 M KCN at pH 8.

Other Methods. Metal analyses were performed by atomic absorption spectroscopy using a Hilger & Watts Atomspek Model H 1170. Starch gel electrophoresis was carried out according to Poulik (1957). Superoxide dismutase activity was tested with the standard assay of McCord and Fridovich (1969). Epr and CD spectra were obtained as previously described (Rotilio *et al.*, 1971). Optical absorption spectra were obtained in a Beckman DK2A ratio recording spectrophotometer. pH measurements were made at room temperature in a pH 4 radiometer equipped with a G-200 B glass electrode.

Results

Metal Content and Reconstitution of the Apoprotein. Table I shows the metal content of typical apoprotein samples obtained by different treatments. Treatment I removes both metals, while treatment II removes only copper. With treatment II, the pH is critical for the extent of zinc ion removal. The visible absorption spectrum of the native bovine enzyme was restored (figure not shown) by addition of Cu(II) only in apoprotein samples of type II and III. Addition of Cu(II) to type I apoprotein gave an absorption band centered at 750 instead of at 680 nm. Figure 1 shows some results obtained in epr controls on type I and II apoprotein samples. It appears that the original epr signal is recovered only in the apoprotein sample containing the full amount of zinc. The extent of reconstitution of samples treated as in II was related to the residual zinc content of the apoprotein. The reconstitution was slow, taking several minutes at room temperature. Epr experiments have also shown (Figure 2) that the holoprotein is not irreversibly denatured on standing at pH values between 3 and 4. Copper-free and fully metal-free apoproteins have different electrophoretic behavior (Figure 3). Addition of copper to copper-free bovine enzyme was able to restore the electrophoretic pattern of the holoprotein, while addition of both metals to the fully metal-depleted apoprotein was not.

Amino Acid Composition of the Bovine Enzyme. Our data on amino acid composition of the bovine enzyme are not reported here, since they are in fairly good agreement with the data already published (Bannister *et al.*, 1971, Keele *et al.*, 1971). However, a few points deserve special mention. There were 6 residues of cysteic acid found and two tyrosines. Both tyrosines were titrated with a pK of 11.5, either in holoprotein or in apoprotein samples, independent of the residual metal

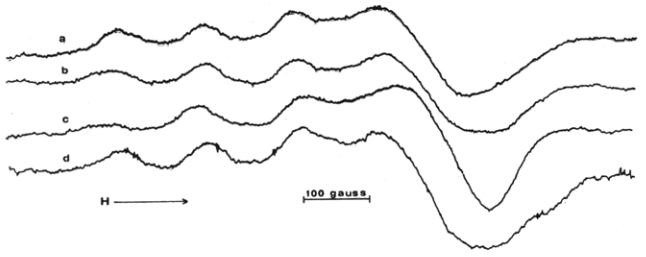

FIGURE 2: Epr spectra of the bovine enzyme at various pH values. A 1% solution (1 ml) of the protein in water (pH 6, curve a) was used. The pH of the solution was lowered by small additions of HCl to pH 3.43 (curve b) and 2.95 (curve c). After standing 1 hr at room temperature at the last pH value the pH was readjusted to 6.8 with KOH (curve d). Instrumental settings as in Figure 1. The amplification of curve d was 1.5 times higher.

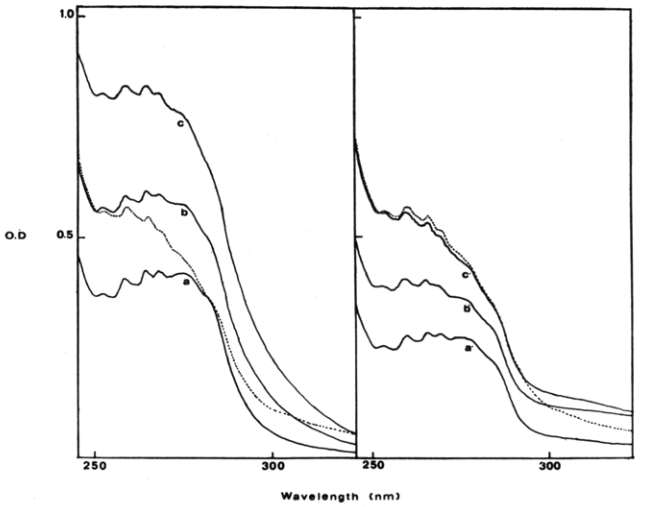

FIGURE 4: Optical spectra of the bovine enzyme and derivatives which show the reconstitution ability of differently prepared apoprotein and the contribution of copper and zinc to the near-ultraviolet optical spectrum. Dotted line: bovine enzyme (0.185% $\simeq 5.5 \times 10^{-5}$ M). (a) Type I apoprotein (0.24% $\simeq 7 \times 10^{-5}$ M) containing 10% copper and 5% zinc with respect to holoprotein. (b) To the solution of a $Cu(ClO_4)_2$ was added corresponding to half the average copper content of the holoprotein. Addition to this solution of increasing amounts of $Zn(CH_3COO)_2$ corresponding to the average zinc content of holoprotein did not change the spectrum. (c) To the solution of b $Cu(ClO_4)_2$ was added corresponding to the average copper content by the holoprotein. (a′) Type II apoprotein (0.16% $\simeq 5 \times 10^{-5}$ M) containing 9% copper and 90% zinc with the copper and zinc content of the holoprotein taken as 100%. Addition of zinc to zinc-depleted samples of type II apoprotein did not change the spectrum. (b′) To the solution of a′ $Cu(ClO_4)_2$ was added corresponding to half the average copper content of the holoprotein. (c′) To the solution of b′ $Cu(ClO_4)_2$ was added corresponding to the average copper content of the holoprotein.

content. Two methionines were found, in agreement with Bannister *et al.* (1971), but at variance with the reported absence of this amino acid in the bovine enzyme (Keele *et al.*, 1971). A tryptophan residue was found per 33,000 molecular weight, either by alkaline hydrolysis or spectrophotometric determination. The carbohydrate content was found to be about 0.1%, which excludes the presence of significant amounts of carbohydrate in the bovine enzyme. Amides were determined and an average value of 26 groups per 33,000 molecular weight was obtained, with a total of 53 dicarboxylic acid residues.

End Group Analysis. N-Terminal group determinations on holoprotein and various types of apoprotein gave negative results with both methods applied in agreement with the results obtained by Bannister *et al.* (1971) by the fluorodinitrobenzene method. After digestion with carboxypeptidase A and/or B, neither in the absence nor in the presence of sodium dodecyl sulfate, was there observed release of any amino acid. However lysine was released when the apoprotein was digested with carboxypeptidase B in the presence of sodium dodecyl sulfate. The amount of lysine released varied between about 0.5 and 1.0 mole per mole of protein, depending on the different types of apoprotein preparations used in the various experiments. There was less lysine released in the samples containing zinc. After 4-hr digestion with carboxypeptidase B, a sample of type I apoprotein released 1.07 moles of lysine/33,000 g of protein, while a sample of type II apoprotein containing the full amount of zinc released only 0.4. In the experiments in which a high amount of lysine was released, a slow release of valine was also observed. In the absence of the dodecyl sulfate, with the same preparations of apoprotein, the amount of lysine released was only one-half that liberated in the presence of the dodecyl sulfate.

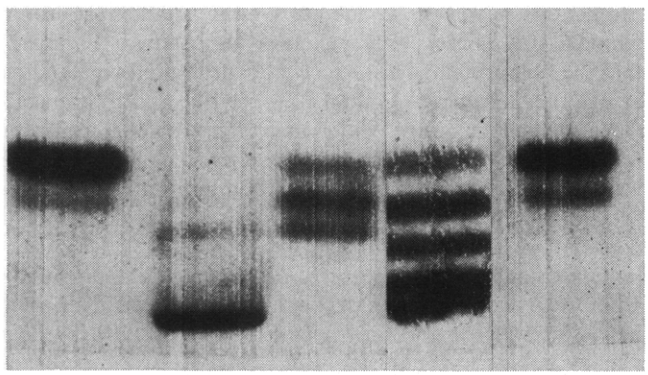

FIGURE 3: Reconstitution ability of differently prepared apoprotein samples as tested by starch gel electrophoresis, From left to right: the bovine enzyme, type I apoprotein, the same incubated with excess Cu^{2+} and Zn^{2+}, type II apoprotein (no zinc removed), the same incubated with excess Cu^{2+}.

SH Content of the Bovine Enzyme. No free SH group was titrated in native bovine enzyme. In the presence of 6 M guanidine hydrochloride and of a tenfold excess of pCMB,[1] an absorbance difference corresponding to about 1 equiv of pCMB mercaptide was observed. The number of groups titrated in the apoprotein varied depending on the presence of residual zinc: copper-free samples containing the full complement of zinc behaved in the same way as holoprotein, while four SH groups per mole were titrated in the samples fully depleted of both metals.

Effect of Metals on Ultraviolet Absorption and CD Spectra of the Bovine Enzyme. Figure 4 shows the effect of metal removal and readdition on the ultraviolet absorption spectrum of the bovine enzyme. The native protein has a maximum at 258 nm, centered on a distinct peak of the phenylalanine fine structure. The apoprotein absorbs much less in this region. Only the addition of copper is able to restore the original ultraviolet-absorption spectrum to an extent proportional to the residual zinc. Similar results were ob-

[1] Abbreviation used is: pCMB, *p*-chloromercuribenzoate.

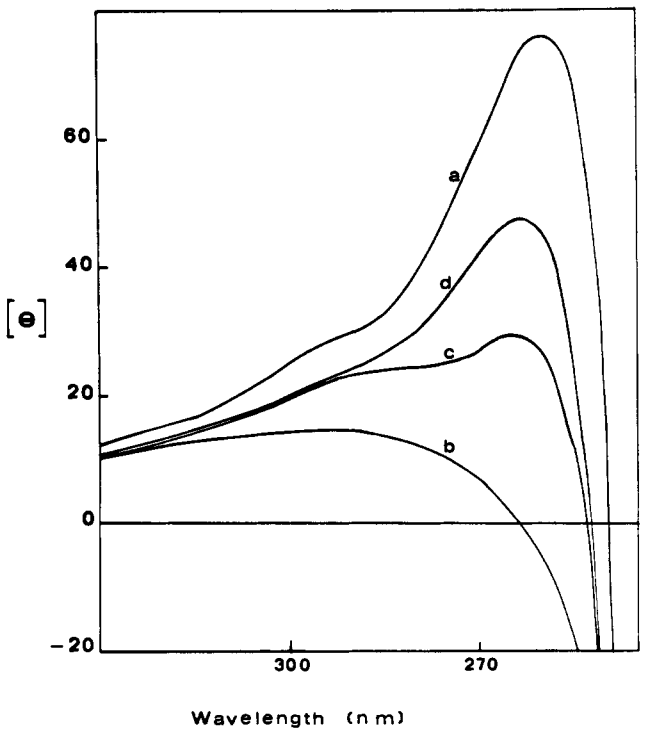

FIGURE 5: CD spectra of the bovine enzyme and derivatives which show the contribution of copper and zinc to the ellipticity bands in the near-ultraviolet. Curve a: Bovine enzyme; curve b: type II apoprotein (same preparation used for Figure 5); curves c and d: analogous to b′ and c′ of Figure 5.

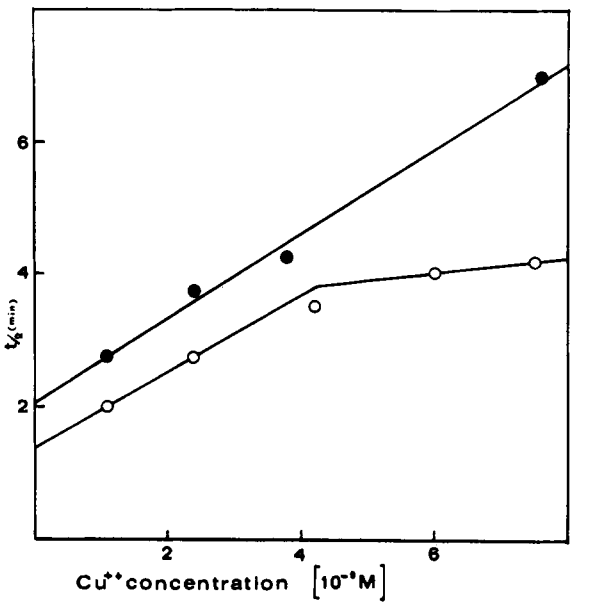

FIGURE 6: Superoxide dismutase activity of native protein and of protein reconstituted with different amounts of copper from a sample of type II apoprotein containing 6% copper and 50% zinc taking the copper and zinc content of the holoprotein as 100%. The standard incubation mixture was modified as follows: 2×10^{-5} M ferricytochrome *c* (Sigma); 10^{-4} M xanthine; 10^{-4} M EDTA; 40 μg of xanthine oxidase (Boehringer); buffer to 3 ml. The extent of inhibition of cytochrome *c* reduction was calculated from the half-time of the reduction. (●) Bovine enzyme expressed as $[Cu^{2+}]$; (○) apoprotein (0.4 μg ≃ 4.10^{-9} M) in the presence of increasing amounts of $Cu(ClO_4)_2$.

tained by investigating the effect of the metals on the near-ultraviolet CD spectra of protein (Figure 5). The optical activity present in the holoprotein in the 310- to 255-nm region is absent in the apoprotein either in the absence of both metals or in the presence of zinc alone and is restored only by addition of copper to the zinc protein. No effect of copper removal was observed on the CD spectrum at shorter wavelengths.

Role of Metals in the Bovine Enzyme Activity. Figure 6 shows the results obtained in measurements of dismutase activity performed with different concentrations of the bovine enzyme and type II apoprotein reconstituted to a different extent with copper. The data clearly indicate that the activity is dependent on the recombination with copper, and that the fraction of the copper which is in excess over the residual zinc of the apoprotein is not effective in restoring any activity. Zinc alone does not appear to sustain any activity as the copper-free samples which contained zinc were not active. Type I apoprotein did not recover the catalytic activity after addition of stoichiometric amounts of either or both metals.

Effect of H_2O_2 and Ferrocyanide. When H_2O_2 was added anaerobically in amounts stoichiometric to the copper, the intensity of the copper signal was decreased; the original intensity was slowly recovered by exposure to air (Figure 7); this effect is specific for the native copper, as the copper signal which appears in recombination experiments in the absence of zinc (see Figure 1, d) did not disappear under the same conditions under which the signal of the native enzyme did. Ferrocyanide reduced the bovine enzyme copper when added in slight excess. The ferrocyanide-reduced protein was stable to air and was slowly reoxidized only after dialysis of the reduced sample.

Discussion

The enzymic activity of the bovine enzyme has been extensively investigated (McCord and Fridovich, 1971) and data on its physicochemical properties have also been reported (Bannister *et al.*, 1971; Wood *et al.*, 1971; Keele *et al.*, 1971). The results presented above are in agreement with these previous investigations, in that they confirm the involvement of copper in the superoxide dismutase activity (McCord and Fridovich, 1971) and the amino acid composition. The present investigation was mainly addressed to: (a) establishing the conditions necessary to obtain an apoprotein capable of reconstituting a protein with properties of the native one which we hoped might allow a deeper insight into the relative role of the two types of metal ions; (b) investigating further the primary structure of the protein with a view to the subunit composition.

(a) Zinc is present in the bovine enzyme in amounts equimolar to copper. The copper is more suited as a probe for the native state of the protein as the state of the copper site is clearly reflected in specific epr and visible absorption spectra; furthermore, dismutase activity of the bovine enzyme

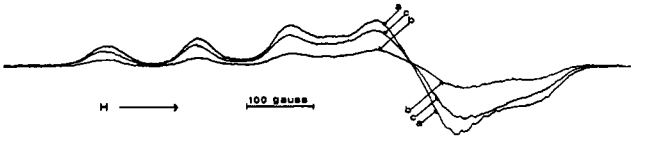

FIGURE 7: Epr spectra of the bovine enzyme in the presence of H_2O_2. The protein, 1.6% (≃5×10^{-4} M), was dissolved in 0.5 ml of water in an anaerobic cell. Curve a: before addition of H_2O_2. Curve b: 1 min after addition of 10^{-3} M H_2O_2. Curve c: the solution as in b shaken 5 min in air. Instrumental settings as in Figure 1.

appears to depend on the presence of copper (McCord and Fridovich, 1969) and the ultraviolet-absorption spectrum is drastically modified by metal removal (Bannister *et al.*, 1971). We have taken into consideration a number of properties of the native protein to test if differently prepared apoprotein samples may be capable of specific recombination. The apoprotein prepared by treatment at acid pH in the presence of EDTA (type I) is almost completely metal free (see Table I). However the recombination experiments indicated that addition of stoichiometric amounts of copper, either in the presence or in the absence of zinc, was not able to restore the features of the native protein, namely absorption, CD, and epr spectra, bovine enzyme activity, electrophoretic pattern, and reaction of the copper with H_2O_2. This observation calls for a reevaluation of the results previously reported for this type of apoprotein (McCord and Fridovich, 1969; Wood *et al.*, 1971).

This fact cannot be explained by simply assuming that the acid treatment denatures the protein irreversibly. In fact the typical epr signal of copper of the native bovine enzyme is maintained through the full range of pH 3–10. Below pH 3 the spectrum changes, and the maximum of the optical absorption in the visible shifts to shorter wavelengths (figure not shown); these changes are reversible and probably reflect a modification of the symmetry of the copper site which favors the removal of the metal.

The explanation for the failure of type I apoprotein in recombination can be found in the results of recombination tests carried out with the type II apoprotein. Contrary to the statement of Bannister *et al.* (1971) we have found that the copper site of the bovine enzyme has a high affinity toward cyanide (Rotilio *et al.*, 1972). Furthermore, zinc removal depends critically on pH, probably because of the pH dependence of the CN^- concentration (Rotilio *et al.*, 1972). Taking advantage of this feature one may obtain apoprotein samples almost copper-free and with different amounts of residual zinc. In this way it was possible to show that the extent of recovery of the native copper site in terms of spectroscopic features and catalytic activity was related to the amount of residual zinc. Addition of zinc to zinc-depleted samples had no effect on the extent of recombination. Copper added in excess over the residual zinc was bound to a site clearly different from the native one (see Figure 1, c). However, the cyanide treatment performed as in II (Table I) leads to the irreversible denaturation of some protein. On the other hand, reduction of the copper before dialysis against the cyanide solution prevents this denaturation. Under these conditions one may work at a lower temperature and less time is needed for copper removal while no zinc is lost (treatment III). The effect of zinc removal on the copper recombination capacity of the bovine enzyme might be related to the different reactivity of sulfhydryl groups in the presence and in the absence of zinc. Two SH groups per zinc atom are titratable in the apoprotein fully depleted of metals, while neither in the holoprotein nor in the copper-free protein which still contains zinc are there SH groups detected. Since six cysteic acid residues are determined by amino acid analysis, only one disulfide bridge is probably present in the bovine enzyme. It may be suggested that SH groups exposed by zinc removal induce an irreversible change of protein conformation, possibly by interchange with the disulfide bridge, and thus prevent recombination with copper at the native site. The fact that the zinc-free samples gave four SH residues when titrated with pCMB, while the zinc-containing samples displayed the absorbance difference corresponding only to 1 equiv in the presence of 6 M guanidine hydrochloride on a tenfold excess of pCMB, indicates that the protein conformation is constrained by the presence of zinc against gross changes by guanidine, as already shown in other zinc proteins (Trotman and Greenwood, 1971). The symmetry of the copper site appears to be strictly dependent on the constraint induced by zinc, as indicated by the identity of the epr spectra of the protein in the presence and absence of guanidine (Rotilio *et al.*, 1971). Another indication of the structural role of zinc is the different susceptibility to carboxypeptidase digestion displayed by zinc-containing and zinc-depleted samples of the bovine enzyme. However, evidence for involvement of copper in the conformational stability of the bovine enzyme has also been obtained. No C-terminal amino acid is liberated in the holoprotein, while removal of copper leads to partial digestion by carboxypeptidase B. Moreover, copper restores the near-ultraviolet optical and CD spectra of the protein while zinc has no effect (Figures 4 and 5). It should be recalled that zinc was claimed (Bannister *et al.*, 1971) to restore the original ultraviolet-absorption spectrum of apoprotein samples obtained according to procedure I to a greater extent than copper. The spectral features due to the presence of copper are very pronounced and extend over a wide wavelength range. Tryptophanyl, tyrosyl, phenylalanyl, and cystinyl residues are probably involved in such changes. Since these amino acids appear not to be part of the copper site (Rotilio *et al.*, 1971) the great sensitivity of their environment to the removal of copper would be an indication of the local conformational effects brought about by the particular type of copper binding present in the bovine enzyme (Rotilio *et al.*, 1972). The role of copper in stabilizing a definite protein conformation is also indicated by the profound changes of the electrophoretic pattern brought about by copper removal (Figure 3). The main conclusion which arises from these results is that both copper and zinc are necessary to the catalytic activity although in a different way. Copper is possibly directly involved in the process of the dismutation, as suggested by the dependence of the dismutase activity on the extent of recombination of copper with the protein still containing zinc and by reduction of copper in the presence of stoichiometric amounts of hydrogen peroxide, the product of dismutation.

(b) Keele *et al* (1971) reported that bovine superoxide dismutase was dissociated into two subunits of equal size by exposure to sodium dodecyl sulfate in the presence of β-mercaptoethanol and suggested that their association involves at least one disulfide bridge. Our data on the primary structure together with SH titration after removal of zinc indicate that only one disulfide bridge is present in the bovine enzyme, and at the same time support the notion that the two subunits are not identical. First, tryptophan has been detected in amounts very close to one residue per mole of protein. Second, only one C-terminal amino acid per mole of protein was found. This suggests that the bovine enzyme is composed of two subunits, probably of equal size but not identical. However, the difference between subunits does not appear to be essential for the function, as the copper sites seem equivalent (Rotilio *et al.*, 1971) and the reaction with Koshland's reagent for tryptophan does not affect the enzymatic activity (Keele *et al.*, 1971).

Acknowledgments

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Studies of the Metal Sites of Copper Proteins. Symmetry of Copper in Bovine Superoxide Dismutase and Its Functional Significance†

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ABSTRACT: The two equivalent copper sites of bovine superoxide dismutase have been shown to be rhombic, on the basis of the electron paramagnetic resonance (epr) spectrum at 35 GHz. Three distinct g values were calculated, that is $g_z = 2.265$, $g_y = 2.108$, $g_x = 2.023$, which indicate a high degree of rhombic distortion. The bovine enzyme copper reacted with anions, such as CN^-, F^-, N_3^-, giving reversibly rise to axial types of epr spectra. The reaction with CN^- has been studied in detail. It has been demonstrated that this ligand binds to copper in the ratio of 1 mole/g-atom of metal, *via* its carbon end. In the presence of cyanide a superhyperfine pattern appeared on the hyperfine line at lowest magnetic field. It has been assigned to three nitrogen atoms of the protein. It is suggested that the rhombic site of the bovine enzyme copper can be described as composed of three nitrogen atoms as strong ligands and of a fourth weaker ligand, which can be easily exchanged with solvent anions. It is also suggested that this capability of binding anions as the fourth in plane ligand can be related to the demonstrated involvement of copper in the superoxide dismutase activity of the bovine enzyme.

Evidence has been presented (Rotilio *et al.*, 1971) for the presence of three to four nitrogen atoms as planar ligands for copper in bovine superoxide dismutase (the bovine enzyme). Moreover, interaction of copper with a water molecule was suggested on the basis of modification of the electron paramagnetic resonance (epr) and circular dichroism (CD) spectra of the bovine enzyme in alkaline solution after deuterium exchange. In the accompanying paper (Rotilio *et al.*, 1972) the conditions of reversible copper removal have been studied and in these experiments evidence was obtained that this metal is directly involved in the superoxide dismutase activity as previously pointed out by McCord and Fridovich (1969). In the present paper the symmetry properties of the bovine enzyme copper were investigated, both in the native protein and in the presence of coordinating anions which obviously modify the environment of the copper. The results obtained give more precise information about the planar ligands of the metal and suggest a possible role for the typical symmetry displayed by the copper coordination in this protein.

Materials and Methods

Chemicals were purchased from commercial sources and were used without further purification. [^{13}C]KCN of 63.2% enrichment was obtained from Merck Sharpe and Dohme,

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