Biochemistry **1984**, *23*, 4324–4329

Holland, P. C., Clark, M. G., & Bloxham, D. P. (1973) *Biochemistry 12*, 3309–3312.

Izbicka-Dimitrijević, E., & Gilbert, H. F. (1982) *Biochemistry 21*, 6112–6118.

Jacoby, W. B. (1958) *J. Biol. Chem. 232*, 89–94.

Karush, F. H., Klinman, N. R., & Marks, R. (1964) *Anal. Biochem. 9*, 100–114.

Kornblatt, J. A., & Rudney, H. (1971) *J. Biol. Chem. 246*, 4417–4423.

Kosower, E. M., & Kosower, N. S. (1969) *Nature* (*London*) *224*, 117–120.

Kosower, N. S., Kosower, E. M., & Wertheim, B. (1969) *Biochem. Biophys. Res. Commun. 37*, 593–596.

Laemmli, U. K. (1970) *Nature* (*London*) *227*, 680–685.

Levinthal, C., Singer, E. R., & Fetherolf, K. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1230.

Lynen, F. (1953) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 12*, 683–691.

MacQuarrie, R. A., & Bernhard, S. A. (1971) *J. Mol. Biol. 55*, 189–192.

Malthouse, J. P. G., & Brocklehurst, K. (1980) *Biochem. J. 185*, 217–222.

Manboodiri, M. A. A., Favilla, J., & Klein, D. C. (1981) *Science* (*Washington, D.C.*) *213*, 571.

Middleton, B. (1973) *Biochem. J. 132*, 717–730.

Muakiyama, T., & Takahashi, K. (1968) *Tetrahedron Lett. 56*, 5907–5908.

Nikkel, H. J., & Foster, J. F. (1971) *Biochemistry 10*, 4479.

Peters, R. A. (1963) in *Biochemical Lesions and Lethal Synthesis*, pp 40–58, Macmillan, New York.

Pontremoli, S., Traniello, S., Wood, W. A., & Horecker, B. L. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 286–293.

Raaka, B. M., & Lowenstein, J. M. (1979) *J. Biol. Chem. 254*, 6755–6762.

Rippa, M., Bellini, T., Signorini, M., & Dallocchio, F. (1981) *J. Biol. Chem. 256*, 451–455.

Staack, H., Binstock, J. F., & Schulz, H. (1978) *J. Biol. Chem. 253*, 1827–1831.

Stoops, J. K., & Wakil, S. (1981) *J. Biol. Chem. 256*, 5128–5133.

Stoops, J. K., Arslanian, M. J., Aune, K. C., & Wakil, S. J. (1978) *Arch. Biochem. Biophys. 186*, 348–359.

Takeuchi, S., & Maeda, A. (1977) *Biochem. J. 81*, 971–976.

White, H., & Jencks, W. P. (1976) *J. Biol. Chem. 251*, 1688–1699.

Wilson, J. M., Wu, D., Motiv-De Grood, R., & Hupe, D. J. (1980) *J. Am. Chem. Soc. 102*, 359–363.

Zahler, W. L., & Cleland, W. W. (1968) *J. Biol. Chem. 243*, 716–719.

Asymmetric Active Site Structures in Yeast Dicopper Dizinc Superoxide Dismutase. 1. Reconstitution of Apo-Superoxide Dismutase[†]

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ABSTRACT: The configuration of the metal binding sites in yeast dicopper dizinc superoxide dismutase ($\mathrm{Cu_2,Zn_2SOD}$) has been probed during metal titration of apoSOD. Cobalt can be substituted for zinc during the metal reconstitution to yield a copper–cobalt derivative that is equally as active as $\mathrm{Cu_2,Zn_2SOD}$, and the incorporated cobalt provides spectral accessibility to the zinc binding site. The absorption, circular dichroism (CD), and magnetic circular dichroism (MCD) spectra of the coordinated cobalt are consistent with a tetrahedral coordination geometry. However, cobalt can be incorporated into only one of the two vacant zinc binding sites, suggesting structural differences between these sites. Analogous titration of apoSOD with zinc results in the coordination of 2 equiv of zinc, with preferential incorporation of zinc into the site to which cobalt binds. Asymmetry is also suggested for the two copper binding sites, which can be distinguished by CD and visible absorption spectra during copper titration of apoSOD and $\mathrm{Zn_2SOD}$. In addition, interaction between the two active sites is indicated by the capacity of zinc incorporated at the higher affinity zinc binding site to determine the spectral properties of copper coordinated at the active site in both subunits.

$\mathrm{Cu_2,Zn_2SOD}$,[1] which catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide, is a dimer of 32 000 daltons (Fridovich, 1975). The two subunits have identical primary structures (Jabusch et al., 1980; Johansen et al., 1979; Lerch & Amner, 1981; Steinman et al., 1974), and each contains one copper and one zinc atom at the active site (Fridovich, 1975). Copper is the functional metal in catalysis and undergoes alternate oxidation and reduction during the dismutation reaction (Klug-Roth et al., 1973). In contrast, zinc confers structural stability to the protein and appears to direct the conformation around the active site (Cass et al., 1979; Forman & Fridovich, 1973; Lippard et al., 1977). Within each subunit, the copper and zinc lie in close proximity while in the dimeric enzyme, the two active sites are located on opposite sides of the molecule and are separated by a distance approaching 34 Å (Richardson et al., 1975). Investigations of the active site of $\mathrm{Cu_2,Zn_2SOD}$ have been performed almost exclusively on the enzyme isolated from

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[1] Abbreviations: SOD, superoxide dismutase; CD, circular dichroism; MCD, magnetic circular dichroism; OP, 1,10-phenanthroline; apoSOD, apo-superoxide dismutase; $\mathrm{Cu_2,Zn_2SOD}$, superoxide dismutase containing 2 equiv of copper and zinc/mol of dimer; M_xSOD, derivative of SOD reconstituted with x equiv of metal/mol of dimer.

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bovine erythrocytes. Nonetheless, comparative sequence analyses (Dunbar & Johansen, 1981) and ^{1}H NMR (Hill et al., 1980) and electron paramagnetic resonance (EPR) (Beem et al., 1974) studies have revealed substantial homology between the active site structures of Cu_2,Zn_2SOD isolated from a variety of sources.

It has generally been considered that the two active sites of the dimeric enzyme have identical structures (Bailey et al., 1980; Fielden et al., 1974; Richardson et al., 1975; Tainer et al., 1982). However, it has been shown recently that the stabilities of both the copper and zinc binding sites differ between the two subunits on the basis of a study of the kinetics of metal dissociation (Dunbar et al., 1982). These observations suggest asymmetry in the active site structures. The physical properties of yeast apoSOD, prepared by removal of copper and zinc with 1,10-phenanthroline, have been examined previously (Dunbar et al., 1982). At $5 \leq pH \leq 7.5$, the metal-free protein exhibits a low-wavelength ultraviolet circular dichroic spectrum very similar to that of the native protein. Furthermore, apoSOD has a sedimentation coefficient of 2.5 S, which, although slightly lower than that of Cu_2,Zn_2SOD, is consistent with a dimeric structure. Reconstitution of apoSOD with copper and zinc restores the sedimentation coefficient to that of the native enzyme. In the absence, then, of gross conformational changes accompanying the preparation of apoSOD, we have explored the apparent nonequivalence between the two active site structures by spectral analyses of the binding sites during metal titration of apoSOD. Cobalt has been employed as a spectroscopic probe of the conformation of the native zinc binding sites.

Materials and Methods

Yeast Cu_2,Zn_2SOD was obtained from Pharmacia (Uppsala, Sweden), and 1,10-phenanthroline was from Sigma Chemical Co. (St. Louis, MO). Spectroscopically pure $CoSO_4$, $ZnSO_4$, and $CuCl_2$ were obtained from Johnson-Matthey Co. Ltd., England. The metal salts were dissolved in distilled water, and concentrations of the stock solutions were determined by atomic absorption analyses. All other reagents were analytical grade, and buffers were rendered metal-free by extraction with 0.01% dithizone in CCl_4 (Thiers, 1957). Glass-distilled water was used throughout.

ApoSOD was prepared by dialysis of 250 mg (25 mg·mL^{-1}) of the native enzyme against 25 mM 1,10-phenanthroline, pH 3.2, for 24 h at room temperature (Dunbar et al., 1982). Excess reagent was subsequently removed by gel filtration of the protein on a column of Sephadex G-25 (superfine). ApoSOD was eluted from the column with 10 mM NH_4HCO_3, pH 8.0, and lyophilized. The absorption spectrum of apoSOD reveals no anomalous ultraviolet absorption at 327 nm characteristic of OP, nor did the reconstituted enzymes show absorbance at 346 nm characteristic of metal–OP complexes (Vallee et al., 1958), indicating the absence of any protein-bound reagent.

Metal reconstitution was carried out at a protein concentration of 10–15 mg·mL^{-1}. The lyophilized apoSOD was dissolved in 20 mM sodium acetate, pH 5.0, and clarified by centrifugation. Prior to reconstitution, an aliquot of apoSOD was taken for estimation of protein concentration from the absorbance at 280 nm and the absorptivity of 1.3 mg·mL^{-1}·cm^{-1} calculated from the UV absorption spectra and corresponding quantitative amino acid analyses of purified apoSOD preparations. Visible absorption spectra of the reconstituted derivatives were recorded at room temperature on a Cary 219 spectrophotometer, and the CD and MCD spectra were measured on a Cary 61 spectropolarimeter equipped with a Varian V-4145 superconducting magnet. MCD measurements were carried out at a magnetic field strength of 40 kG, and the spectra are corrected for natural CD. MCD is expressed as $[\theta]_M$ in units of deg·cm^2·dmol^{-1}·G^{-1} and CD in units of deg·cm^2·dmol^{-1}.

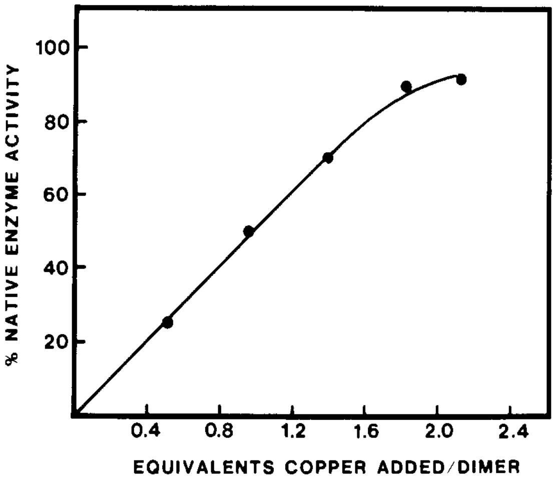

FIGURE 1: Regeneration of enzyme activity upon the incremental addition of $CuCl_2$ to Zn_2SOD in 20 mM sodium acetate, pH 5.0. The native enzyme activity was 7500 units·mg^{-1}.

The reconstituted protein samples were chromatographed on Pharmacia PD-10 columns packed with Sephadex G-25 and eluted with 10 mM sodium acetate, pH 5.0. The metal content of the eluted protein was determined on a Perkin-Elmer 603 atomic absorption spectrometer, and the protein concentration was estimated by amino acid analyses. Amino acid analyses were carried out on a Durrum D-500 analyzer after hydrolysis of the protein in 6 M HCl for 24 h at 110 °C. Enzyme activity was determined by the pyrogallol procedure of Marklund & Marklund (1974).

Results

Metal Reconstitution of ApoSOD. Dialysis of Cu_2,Zn_2SOD against 1,10-phenanthroline, pH 3.2, yields apoSOD containing less than 0.02 equiv of copper and zinc per dimer and the protein exhibits negligible dismutase activity. Reconstitution of apoSOD with 2 molar equiv of zinc, by the incremental addition of 12.5 mM $ZnSO_4$ over a period of 1–2 h, resulted in the incorporation of a 2 ± 0.18 mol of zinc/mol of dimer. The stoichiometry of incorporation was determined by atomic absorption analyses after gel filtration of the reconstituted protein. Subsequent titration of Zn_2SOD with copper yielded an increase in enzyme activity that was a linear function of the number of copper equivalents added (Figure 1). The fully reconstituted Cu_2,Zn_2SOD exhibited greater than 90% of the dismutase activity of the native enzyme.

Analogous reconstitution of apoSOD, in which cobalt was substituted for zinc, was also characterized by a linear increase in enzyme activity during titration with up to 2 equiv of copper. The dismutase activity of the copper–cobalt derivative exceeded 90% that of native Cu_2,Zn_2SOD.

Spectral Properties of Cobalt-Reconstituted SOD. ApoSOD reconstituted with 1 equiv of cobalt at pH 5.0 exhibits a visible absorption spectrum characterized by maxima at 565 and 585 nm and a shoulder at 540 nm (Figure 2). During titration of apoSOD with 2 equiv of cobalt, no changes were detected in the spectral pattern per se. However, the titration curve, as indicated by the aborptivity at 585 and 565 nm, was biphasic, appearing linear up to the addition of 1 equiv of cobalt per dimer. Continued titration with the second cobalt equivalent did not substantially increase the absorbance

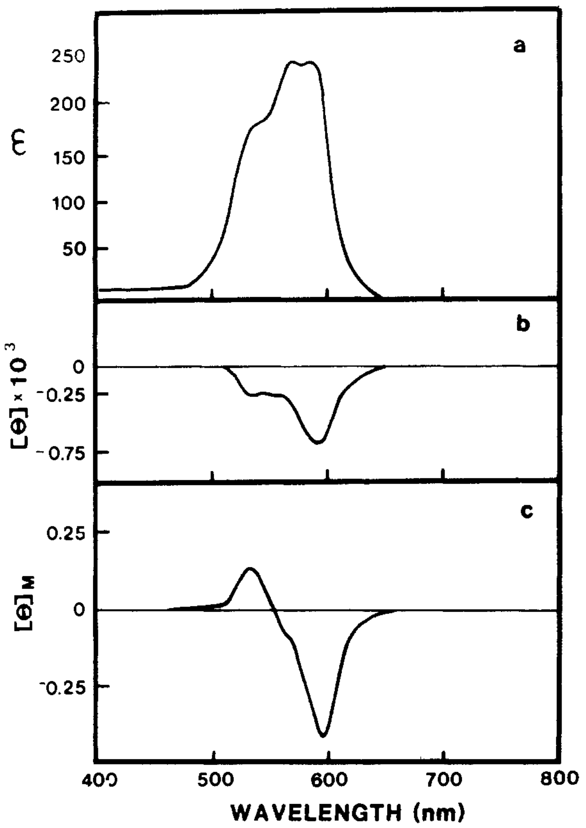

FIGURE 2: (a) Visible absorption, (b) circular dichroic, and (c) magnetic circular dichroic spectra of $\mathrm{Co_1SOD}$ in 20 mM sodium acetate, pH 5.0. The MCD spectrum was recorded at a magnetic field strength of 40 kG.

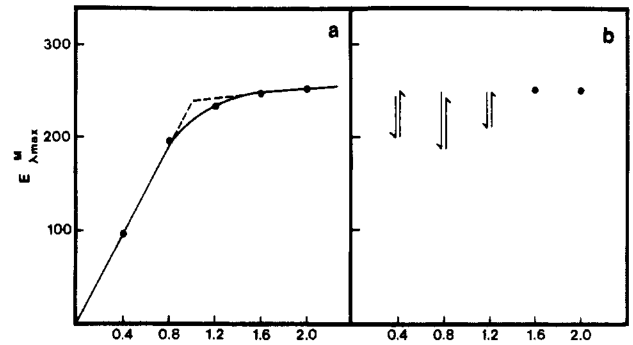

FIGURE 3: (a) Titration of apoSOD with $\mathrm{CoSO_4}$ in 20 mM sodium acetate, pH 5.0, and an initial protein concentration of 10 $\mathrm{mg{\cdot}mL^{-1}}$. The absorptivity corresponds to the peak of absorbance at 585 nm. (b) Copper titration of $\mathrm{Co_1SOD}$. The absorptivity was calculated from the new absorbance maximum at 600 nm and is corrected for the absorbance arising from overlap of the copper spectrum. The arrows represent the extent of the initial decrease (down arrow) and subsequent increase (up arrow) in the absorptivity upon the addition of copper to the protein.

(Figure 3). The absorptivity at 585 nm of the protein titrated with 1 and 2 equiv of cobalt per dimer is 210 and 250 $\mathrm{M^{-1}\,cm^{-1}}$ (per M_r 15 000), respectively.

The corresponding CD and MCD spectra of the cobalt-reconstituted SOD are shown in Figure 2. The increase in the molar ellipticity and magnetic ellipticity during the reconstitution yielded biphasic titration curves similar to that observed for the absorptivity, with no apparent changes in the shape of the spectra throughout the titration. The positive band at 540 nm and the intense negative band near 590 nm in the MCD spectrum are typical of that displayed by tetrahedrally coordinated cobalt complexes and a number of cobalt-substituted zinc metalloenzymes (Holmquist & Vallee, 1978).

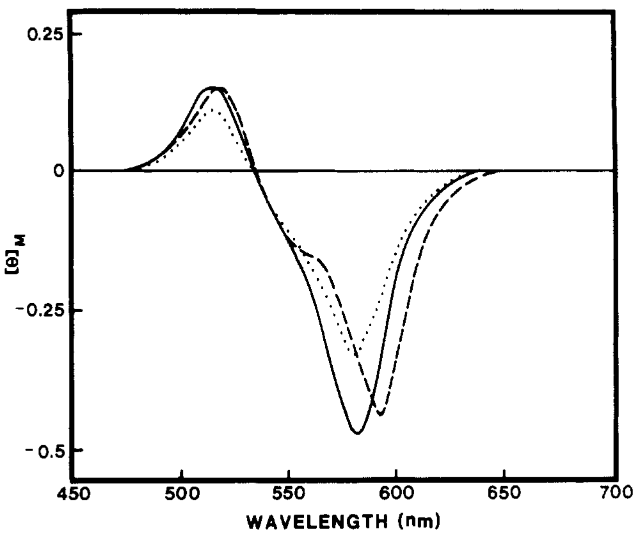

FIGURE 4: MCD spectrum of $\mathrm{Co_1SOD}$ (—) and at 5 min (···) and 2 h (– –) after the addition of 1 equiv of copper. Reconstitution of apoSOD was carried out in 20 mM sodium acetate, pH 5.0.

The absorption, CD, and MCD spectra of the cobalt-reconstituted SOD were unaltered by gel filtration of the protein to remove any excess or loosely bound metal. Atomic absorption analyses of the chromatographed protein samples indicated the incorporation of 1.02 ± 0.03 equiv of cobalt/mol of dimer for the protein titrated with 2 equiv of cobalt. The intercept of the tangents drawn to the titration curves was also consistent with 1 equiv of bound cobalt per dimer.

The incremental addition of 1 equiv of zinc to $\mathrm{Co_1SOD}$ results in the slow bleaching of the cobalt spectrum. Metal analyses revealed that the loss of absorbance was a consequence of the displacement of cobalt by zinc. Conversely, prior coordination of 1 equiv of zinc to apoSOD prevented the appearance of the characteristic cobalt spectrum upon the subsequent addition of cobalt, up to a 10-fold molar excess.

Copper Titration of $\mathrm{Co_1SOD}$. Reconstitution of $\mathrm{Co_1SOD}$ with copper induces significant changes in the environment of the cobalt. Although overlap of the copper and cobalt spectra precluded analyses of these changes by circular dichroism, the influence of copper coordination was clearly observed in the cobalt absorption and MCD spectra. The arrows in Figure 3 indicate the time-dependent changes in the cobalt absorption maxima during copper titration of $\mathrm{Co_1SOD}$. The addition of 1 equiv of copper is accompanied by a rapid decrease in the absorbance, followed by a slow increase over a period of 1–2 h. Furthermore, the absorption maximum, as revealed by the difference spectrum of $\mathrm{Cu_1,Co_1SOD}$ vs. $\mathrm{Cu_1SOD}$, is shifted 15 nm from 585 to 600 nm. A shoulder near 425 nm also appears in the absorption spectrum. Parallel changes were detected in the MCD spectra, such that immediately upon the addition of each increment of copper there is a rapid decrease in the ellipticity. A shift occurs in the wavelength of the maxima from 590 to 600 nm and 530 to 537 nm, respectively, concomitant with the subsequent increase in the ellipticities (Figure 4). Increased resolution of the shoulder at 570 nm in the MCD spectra is also apparent upon coordination of copper to $\mathrm{Co_1SOD}$. These changes in the cobalt spectrum appeared to occur slowly, in comparison to the changes in the copper spectral properties that accompanied the concomitant coordination of copper to the protein. In contrast to the effects arising from the coordination of 1 equiv of copper to $\mathrm{Co_1SOD}$, the addition and incorporation of the

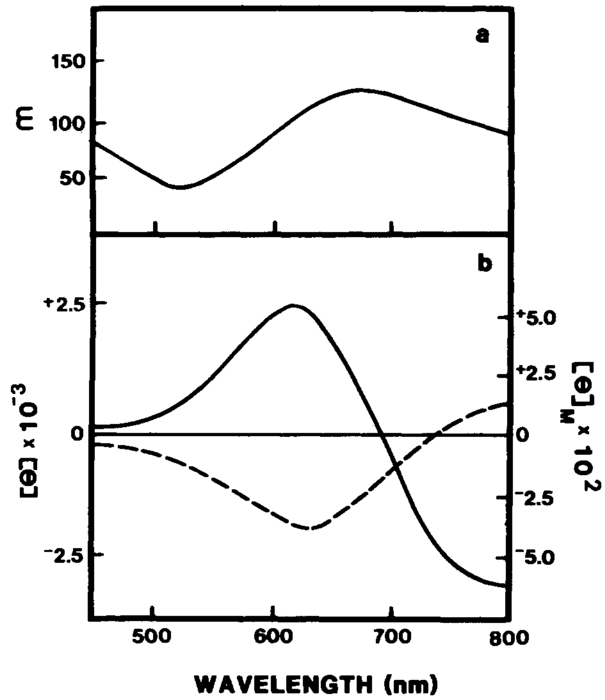

FIGURE 5: Visible absorption (panel a) and circular dichroic (—) and magnetic circular dichroic spectra (– –) (panel b) of native Cu_2,Zn_2SOD.

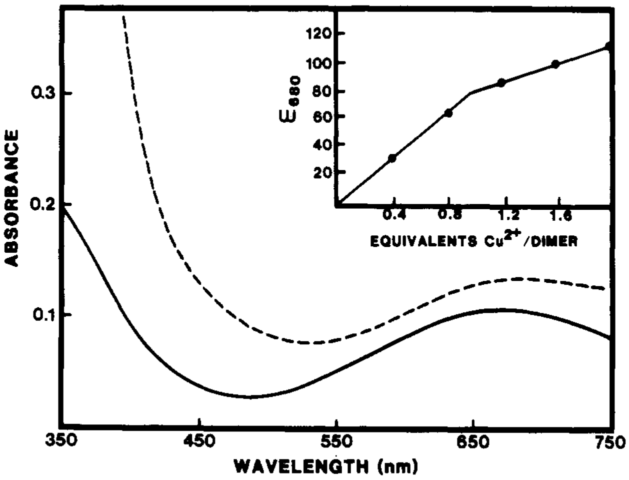

FIGURE 6: Copper titration of apoSOD in 20 mM sodium acetate, pH 5.0. The visible absorption spectra were recorded after the addition of 1 (—) and 2 (– –) equiv of copper per dimer. The initial protein concentration was 15 mg·mL^{-1}. (Inset) Increase in absorptivity during titration of apoSOD with $CuCl_2$.

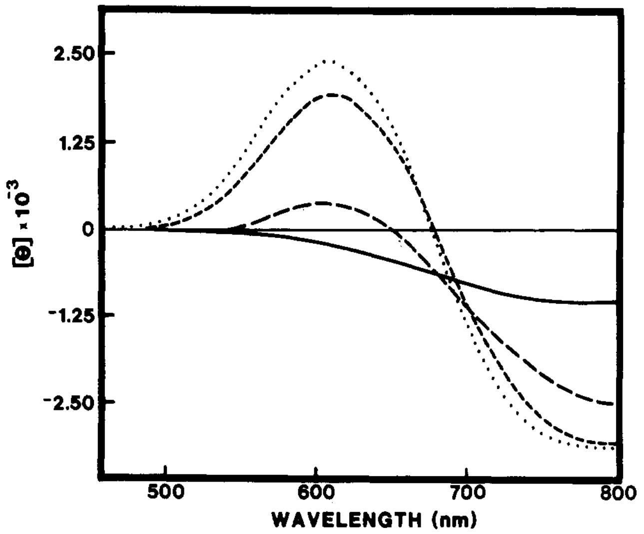

FIGURE 7: Circular dichroic spectra of copper and zinc derivatives of SOD: Cu_1SOD (—), Cu_2SOD (– –), Cu_1,Zn_1SOD (- - -), and Cu_2,Zn_2SOD (···). Metal reconstitution of apoSOD was carried out in 20 mM sodium acetate, pH 5.0.

Table I: Optical Activity of the Reconstituted Copper and Zinc Derivatives of Yeast Superoxide Dismutase

sample	CD ([θ] × 10^{-3}) 610 nm	CD ([θ] × 10^{-3}) 800 nm	MCD ([θ]$_M$ × 10^2) 610 nm
Cu_1SOD	-0.24	-0.61	-1.15
Cu_2SOD	+0.34	-2.25	-2.25
Cu_1,Zn_1SOD	+1.62	-1.95	-2.06
Cu_1,Zn_2SOD	+1.72	-1.95	-1.94
Cu_2,Zn_1SOD	+2.03	-2.60	-3.32
Cu_2,Zn_2SOD	+2.39	-2.80	-3.67
native Cu_2,Zn_2SOD	+2.45	-2.89	-3.80

second copper equivalent produced negligible changes in both the cobalt absorption spectra and MCD spectra.

Copper Titration of ApoSOD and Zn_2SOD. The visible absorption, CD, and MCD spectra of native yeast Cu_2,Zn_2-SOD are shown in Figure 5. The native enzyme exhibits a broad absorption band near 680 nm with a molar absorptivity of 127 M^{-1} cm^{-1}. The corresponding CD spectrum displays optically active bands at 800 and 610 nm (Figure 5). The MCD spectrum of the native enzyme contains a major negative band at 610 nm and a small positive band near 800 nm.

Titration of apoSOD with copper reveals a biphasic increase in the visible absorbance at 680 nm with the first equivalent of bound copper contributing approximately 75% of the extinction of the fully reconstituted Cu_2SOD (Figure 6). Metal analyses subsequent to gel filtration of apoSOD reconstituted with 1 and 2 equiv of copper revealed incorporation of 0.96 ± 0.04 and 1.93 ± 0.07 mol of copper/mol of dimer, respectively. In addition to the nonequivalent absorptivity exhibited by the two copper binding sites, distinct spectral changes accompany the titration of Cu_1SOD to Cu_2SOD: There is a shift in the absorption maximum from 680 nm to approximately 700 nm and an increase in the absorbance at wavelengths below 500 nm (Figure 6). Continued titration of Cu_2SOD to yield Cu_4SOD (not shown) is accompanied by enhanced broadening of the copper absorption band near 680 nm together with substantial increases in the absorbance at wavelengths below 500 nm.

Significant differences were also detected in the CD spectra of Cu_1SOD and Cu_2SOD (Figure 7). Although some optical activity is evident at 800 nm in Cu_1SOD, the greatest ellipticity appears to be associated with coordination of the second equivalent of copper. Compared to the native enzyme, neither Cu_1SOD nor Cu_2SOD displays significant dichroism at 610 nm. In contrast to the visible absorption and CD spectra, no differences between the two copper binding sites are detected by MCD. The magnitude of ellipticities in the MCD spectra increased linearly during titration of apoSOD with 2 equiv of copper per dimer (Table I).

The spectral properties of Cu_1SOD and Cu_2SOD are modified by the subsequent coordination of zinc to the protein. The addition of 1 equiv of zinc to Cu_1SOD induces a marked increase in the ellipticities in both the CD (Figure 7) and MCD spectra (Table I), indicative of conformational changes at the higher affinity copper binding site. There was no significant effect of zinc on the visible absorption spectrum of Cu_1SOD. However, changes in the visible absorption spectrum are observed upon the addition of 1 equiv of zinc to Cu_2SOD. The copper absorption maximum at 700 nm in Cu_2SOD is shifted to 680 nm, although there is no significant change in the absorptivity. A decrease in the absorbance at wavelengths below 500 nm is also apparent. These spectral changes appear to be associated with copper coordinated at the second, or lower affinity, binding site. It is suggested, then, that the coordination of 1 equiv of zinc has the capacity to modify the spectral properties at both copper binding sites.

Copper titration of Zn_2SOD generates absorption, CD, and MCD spectra comparable to those of the native enzyme. Nonetheless, the incorporation of 1 equiv of copper per dimer contributes approximately 70% of the total molar absorptivity and ellipticity of the fully reconstituted Cu_2,Zn_2SOD. In contrast, the magnetic molar ellipticity was directly proportional to the amount of protein-bound copper.

Discussion

Reconstitution of yeast apoSOD with copper and zinc restores greater than 90% of the native enzyme activity, and the absorption, CD, and MCD spectra of the reconstituted protein are identical with those of the native enzyme. Cobalt may be substituted for zinc during the reconstitution to yield a derivative with an enzyme activity equal to that of Cu_2,Zn_2SOD. However, while 2 equiv of zinc per dimer are readily coordinated, titration of apoSOD with cobalt under identical conditions results in the incorporation of only 1 equiv of cobalt. The addition of excess cobalt fails to increase the concentration of protein-bound metal. The capacity to incorporate cobalt into only one of the two potential zinc binding sites provides strong evidence for asymmetry in the active site structures in the two subunits.

The MCD spectrum exhibited by Co_1SOD is consistent with a tetrahedral coordination geometry and suggests that at least one of the zinc binding sites in yeast Cu_2,Zn_2SOD is tetrahedral-like.[2] This observation is in accordance with the geometry of the zinc binding site concluded from the X-ray crystal structure of bovine Cu_2,Zn_2SOD (Richardson et al., 1975; Tainer et al., 1983). The inability to incorporate cobalt into the second zinc binding site suggests that this site has a very unique or distorted structure since cobalt can generally be incorporated into zinc sites of irregular geometry.

The visible absorption and MCD spectra of Co_1SOD reveal changes in the environment of the cobalt upon subsequent coordination of copper to the protein, although the tetrahedral-like coordination geometry appears unchanged. An interaction between the cobalt and copper may be expected from the proximity (6 Å) of the metal binding sites in each subunit. However, the time-dependent changes that are observed in the cobalt spectra suggest conformational changes at the active site subsequent to the coordination of copper. Alternatively, the copper-induced changes in the cobalt spectra could represent displacement of cobalt from a copper binding site followed by the incorporation into a zinc site. Such an explanation, however, requires that one of the copper sites be of identical geometry as the zinc site since the characteristics of the cobalt spectra did not alter during titration of apoSOD with cobalt. Furthermore, from the percentage change in the absorptivity of the cobalt–protein complex, approximately 50% of the incorporated cobalt (equal to 0.5 equiv per dimer) would have to be initially incorporated into the copper site, whereas the induced cobalt spectral changes were observed up to the addition of 1 equiv of copper per dimer. Copper-induced protein conformational changes, rather than displacement of cobalt from a copper binding site, therefore appears a more probable explanation.

Titration of Co_1SOD with 1 equiv of copper induces changes in the cobalt spectrum, while no effect is observed upon coordination of copper into the second copper binding site. The appearance of a shoulder at 425 nm in the visible absorption spectrum of Cu_1,Co_1SOD is consistent with both metals coordinated at the same active site (Pantoliano et al., 1982) and implies that copper can modify the environment of the metal in the neighboring zinc site, but copper coordinated at the lower affinity binding site in the second subunit (vide infra) does not significantly influence the active site structure on the opposite side of the molecule.

Cu_1SOD and Cu_2SOD are clearly distinguished by their visible absorption and CD spectra. It has been shown for bovine superoxide dismutase that coordination of copper into both the copper and adjacent zinc binding sites leads to loss of the copper EPR signal at room temperature (Valentine et al., 1979). During titration of yeast Cu_1SOD to Cu_2SOD, no loss is detected in the copper EPR, whereas there is a marked decrease in signal intensity in yeast Cu_4SOD (J. C. Dunbar, unpublished observations). It is suggested, then, that Cu_2SOD represents coordination of copper into two different subunits and that the variation in the visible absorbance and optical activity of the two copper binding sites is attributable to differences in the local protein environment. Asymmetry in the structure of the copper binding sites exists not only in the zinc-free Cu_2SOD but also in the Cu_2,Zn_2SOD. Evidence for the nonequivalent environments of the two copper atoms in the latter case is provided by the biphasic increase in the molar absorptivity and ellipticities during copper titration of Zn_2SOD. Furthermore, the absorption, CD, and MCD spectra of the reconstituted Cu_2,Zn_2SOD and the native enzyme are identical, suggesting that nonequivalent binding sites may also be a characteristic of the native protein.

NMR (Cass et al., 1979) and chemical modification (Lippard et al., 1977) have suggested a structural role for zinc in Cu_2,Zn_2SOD. More recently, EPR studies have revealed differences in the microenvironment of copper in bovine Cu_2SOD and Cu_2,Zn_2SOD (Pantoliano et al., 1982). The discrete spectra of Cu_2SOD and Cu_2,Zn_2SOD observed in the current experiments are also consistent with a role for zinc in determining the protein conformation around the active site. However, coordination of 1 equiv of zinc is capable of converting the spectra of Cu_2SOD to that of the native enzyme, of inducing changes in the chiroptical properties of Cu_1SOD, and of modifying the spectra arising from the lower affinity copper binding site during copper titration of Cu_1SOD to Cu_2SOD. Coordination of zinc into one subunit therefore not only appears to direct the structure around the active site at which it binds but apparently also induces conformational changes that embrace the active site structure in the alternate subunit.

In the current study and from the kinetics of metal dissociation (Dunbar et al., 1982), we have been able to demonstrate asymmetry in both the copper and zinc binding sites in yeast SOD. Furthermore, interactions between the active sites are indicated by the capacity of zinc to determine the protein conformation both in the subunit in which it is bound and, additionally, at the active site on the opposite side of the molecule. Thus, it is conceivable that the observed asymmetry in the active site structures may be induced by the initial

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[2] It has been emphasized elsewhere that cobalt metalloenzyme spectra cannot be unambiguously interpreted on the basis of model Co(II) complex ions. To deemphasize assignments of formal geometry, the modes of metal coordination in the various SOD forms are referred to as tetrahedral- or pentacoordinate-like, implying an empirical description.

coordination of metals on one subunit leading to conformational changes at the second active site.

It has yet to be determined whether asymmetric active sites and subunit interactions also occur in bovine Cu_2,Zn_2SOD. Supportive evidence may be derived from sequence and NMR studies that have revealed substantial structural homology between the active sites of the yeast and bovine enzymes. In addition, Lawrence & Sawyer (1979) have concluded from potentiometric titrations that the copper binding sites are not equivalent in the bovine enzyme. More recently, Cocco et al. (1981) have proposed that the specific enzyme activity per mole of copper decreases upon coordination of copper into the second copper binding site. Evidence, then, is now accumulating that is consistent with asymmetry in the active structures of Cu_2,Zn_2SOD.

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Registry No. SOD, 9054-89-1; Co, 7440-48-4.

References

Bailey, D. B., Ellis, P. D., & Fee, J. A. (1980) *Biochemistry 19*, 591–596.

Beem, K. M., Rich, W. E., & Rajogopalan, K. V. (1974) *J. Biol. Chem. 249*, 7298–7305.

Cass, A. E., Hill, H. A., Bannister, J. V., & Bannister, W. H. (1979) *Biochem. J. 177*, 477–486.

Cocco, D., Calabrese, L., Rigo, A., Marmocchi, F., & Rotilio, G. (1981) *Biochem. J. 199*, 675–680.

Dunbar, J. C., & Johansen, J. T. (1981) *Bull. Eur. Physiopathol. Respir. 17*, 51–60.

Dunbar, J. C., Johansen, J. T., & Uchida, T. (1982) *Carlsberg Res. Commun. 47*, 163–171.

Fielden, E. M., Roberts, P. B., Bray, R. C., Lowe, D. J., Martner, G. N., Rotilio, G., & Calabrese, L. (1974) *Biochem. J. 139*, 49–60.

Forman, H. J., & Fridovich, I. (1973) *J. Biol. Chem. 248*, 2645–2649.

Fridovich, I. (1975) *Annu. Rev. Biochem. 44*, 147–159.

Hill, H. A., Lee, W. K., Bannister, J. V., & Bannister, W. H. (1980) *Biochem. J. 185*, 245–252.

Holmquist, B., & Vallee, B. L. (1978) *Methods Enzymol. 49*, 149–179.

Jabusch, J. R., Farb, D. L., Kerschensteiner, D. A., & Deutch, H. F. (1980) *Biochemistry 19*, 2310–2316.

Johansen, J. T., Overballe-Petersen, C., Martin, B., Hasemann, V., & Svendsen, I. (1979) *Carlsberg Res. Commun. 44*, 201–217.

Klug-Roth, D., Fridovich, I., & Rabani, J. (1973) *J. Am. Chem. Soc. 95*, 2786–2790.

Lawrence, G. D., & Sawyer, D. T. (1979) *Biochemistry 18*, 3045–3050.

Lerch, K., & Amner, D. (1981) *J. Biol. Chem. 256*, 11545–11551.

Lippard, S. J., Burger, A. K., Ugurbil, K., Pantoliano, M., & Valentine, J. S. (1977) *Biochemistry 16*, 1136–1141.

Marklund, S., & Marklund, G. (1974) *Eur. J. Biochem. 47*, 469–474.

Pantoliano, M. W., Valentine, J. S., & Nafie, L. A. (1982) *J. Am. Chem. Soc. 104*, 6310–6317.

Richardson, J. S., Thomas, K. A., Rubin, B. A., & Richardson, D. C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1349–1351.

Steinman, H. M., Nash, V. R., Abernathy, J. L., & Hill, R. L. (1974) *J. Biol. Chem. 249*, 7326–7338.

Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S., & Richardson, D. C. (1982) *J. Mol. Biol. 160*, 187–217.

Tainer, J. A., Getzoff, E. D., Richardson, J. S., & Richardson, D. C. (1983) *Nature (London) 306*, 284–287.

Thiers, R. C. (1957) *Methods Biochem. Anal. 5*, 273–335.

Valentine, J. S., Pantoliano, M. W., McDonnell, P. J., Burger, A. R., & Lippard, S. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4245–4249.

Vallee, B. L., Coombs, T. L., & Williams, R. J. P. (1958) *J. Am. Chem. Soc. 80*, 397.