Asymmetric Active Site Structures in Yeast Dicopper Dizinc Superoxide Dismutase. 2. pH-Dependent Incorporation of Cobalt into the Metal Binding Sites†

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ABSTRACT: The incorporation of cobalt into the native zinc binding sites in yeast apo-superoxide dismutase (apoSOD) is pH dependent. Only 1 equiv of cobalt per dimer is incorporated into apoSOD at pH 5.0, while at pH 7.0 2 equiv of cobalt is bound. The cobalt binding sites in Co_2SOD reconstituted at pH 7.0 can be distinguished by their visible absorption, circular dichroic, and magnetic circular dichroic spectra. The spectral properties are consistent with a high-affinity site of tetrahedral-like geometry, while a pentacoordinate complex is suggested for the lower affinity cobalt binding site. Zinc displaces cobalt from both binding sites, while the incorporation of 2 equiv of copper into Co_2SOD has no effect on the spectral properties of the cobalt. The regeneration of enzyme activity accompanying reconstitution of apoSOD with zinc and copper is also pH dependent. Maximal activity is attained by reconstitution at pH 5.0, while there is a significant decrease in the percent of native enzyme activity regained as the pH of reconstitution is increased to pH 7.0. The lower enzyme activities at the higher pH of reconstitution appear to be correlated with the coordination of zinc into the apparent lower affinity pentacoordinate-like binding site.

Cu_2,Zn_2SOD[1] is comprised of two subunits of identical amino acid sequence, with one copper and one zinc ion coordinated at the active site of each subunit (Fridovich, 1975). The metals may be effectively removed from the enzyme, and reconstitution of the resultant apoSOD with zinc and copper restores both the enzymatic and spectroscopic properties to those of the native protein (Beem et al., 1974). Metal-substitution studies of bovine superoxide dismutase have shown that cobalt, copper, cadmium, and mercury may be substituted for zinc without affecting the catalytic activity of the enzyme (Valentine & Pantoliano, 1980). The copper binding site displays greater specificity; only silver (Beem et al., 1977) and, under specific conditions, cobalt (Calabrese et al., 1979) have been reported to be incorporated.

A number of studies have attempted to elucidate the relative role of the two active sites in the catalytic activity of the enzyme (Bannister et al., 1974; Calabrese et al., 1980; Fielden et al., 1974; Malinowski & Fridovich, 1979). Initial reports based on pulse radiolytic experiments (Fielden et al., 1974) suggested half-sites reactivity for the enzyme. However, subsequent hybridization studies, in which active and inactive monomers were coupled (Malinowski & Fridovich, 1979), failed to support this hypothesis.

We recently demonstrated that the dissociation constants for both copper and zinc differ between the two subunits in yeast Cu_2,Zn_2SOD (Dunbar et al., 1982). In addition, we have observed apparent subunit asymmetry and interactions between the active sites during metal reconstitution of apoSOD at pH 5.0 (Dunbar et al., 1984). However, in the latter study, cobalt used as a probe to determine the configuration of the zinc binding site could be incorporated into only one of the two subunits. In the current experiments, we have further defined the active site asymmetry by facilitating the incorporation of cobalt into both subunits. In addition, we have attempted to relate structural changes at the active site and interactions between the subunits to the enzymatic activity of the protein.

Materials and Methods

Cu_2,Zn_2SOD, purified from *Saccharomyces cerevisiae*, was provided by Pharmacia (Uppsala, Sweden). ApoSOD was prepared by dialysis of the native enzyme against 25 mM 1,10-phenanthroline, pH 3.2, according to the procedure of Dunbar et al. (1982). Solutions of $ZnSO_4$, $CoSO_4$, and $CuCl_2$ were prepared from spectroscopically pure salts (Johnson-Matthey Co. Ltd., England), and the final concentrations of stock solutions were determined by atomic absorption. Distilled, deionized water was used at all times, and buffers were rendered metal-free by extraction with 0.01% dithizone in CCl_4 according to the procedure of Thiers (1957).

Lyophilized apoSOD was dissolved in buffers of required pH as stated in the text and the initial protein concentration was determined from the absorbance at 280 nm and an absorptivity $E^{1\%}_{280} = 1.3$ (Dunbar et al., 1984). Reconstitution was carried out by addition to the protein of aliquots of 12.5 mM $ZnSO_4$, $CoSO_4$, or $CuCl_2$ over a period of 1–2 h. Initial protein concentration was 10–15 mg·mL^{-1} for spectral studies and 1 mg·mL^{-1} for enzymatic studies.

Visible absorption spectra were recorded on a Cary 219 spectrophotometer, and CD and MCD measurements were performed on a Cary 61 spectropolarimeter equipped with a Varian V4145 superconducting magnet. MCD spectra were recorded at a magnetic field strength of 40 kG and are ex-

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[1] Abbreviations: SOD, superoxide dismutase; CD, circular dichroism; DETPA, diethylenetriaminepentaacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; MCD, magnetic circular dichroism; apoSOD, apo-superoxide dismutase; Cu_2,-Zn_2SOD, native SOD 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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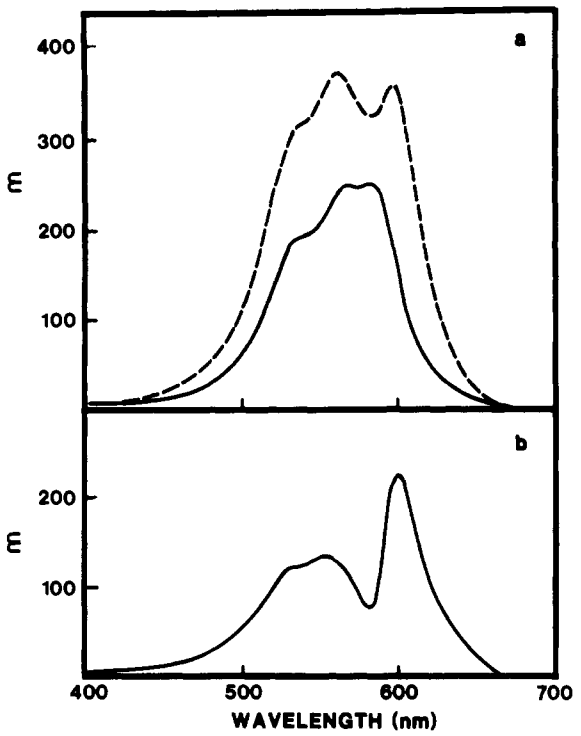

FIGURE 1: (a) Visible absorption spectra of Co_1SOD (—) and Co_2SOD (--). The cobalt derivatives were prepared by cobalt reconstitution of apoSOD in 20 mM HEPES, pH 7.0. (b) Difference spectrum obtained by recording the spectrum of Co_2SOD against Co_1SOD.

pressed as $[\theta]_M$ in units of deg·cm^2·dmol^{-1}·G^{-1}. CD is expressed in units of deg·cm^2·dmol^{-1}.

Reconstituted protein samples were analyzed for metal content after gel filtration on Pharmacia PD-10 columns packed with Sephadex G-25. The columns were rendered metal-free prior to use by washing with 50 mM HCl, followed by distilled H_2O and finally the appropriate metal-free buffer. The protein was eluted with the same buffer employed for the reconstitution of the sample. The metal concentration of the eluted protein was determined on a Perkin-Elmer 603 atomic absorption spectrometer, and the enzyme activity was estimated by the pyrogallol assay described by Marklund & Marklund (1974). The corresponding protein concentration was estimated by quantitative amino acid analyses after hydrolysis of the protein in 6 M HCl for 24 h at 110 °C. The analyses were performed on a Durrum D-500 amino acid analyzer.

Results

Cobalt Reconstitution of ApoSOD. Reconstitution of apoSOD at pH 5.0 results in maximal incorporation of 1 equiv of cobalt per dimer (Dunbar et al., 1984). In contrast, titration of apoSOD at pH 7.0 with 2 equiv of Co(II) yields an enzyme with 1.6 ± 0.12 equiv of the metal per dimer. A stoichiometric incorporation of 2 mol of cobalt per dimer could be achieved by the addition of an approximate 25% excess of the metal ion. The increase in the extinction of the cobalt spectrum during the titration is essentially instantaneous upon the addition of each increment of cobalt and generates absorption spectra for the derivatives containing 1 and 2 equiv of cobalt per dimer as shown in Figure 1a. Co_1SOD displays absorption maxima at 568 and 585 nm with a shoulder at 540 nm, identical with the spectrum obtained for Co_1SOD when the reconstitution of apoSOD is carried out at pH 5.0. Coordination of the second equivalent of cobalt at pH 7.0 is associated with increased resolution of the two major peaks of absorbance with a shift of the 585-nm maximum to 600 nm. The difference spectrum between Co_1SOD and Co_2SOD is shown in Figure 1b and may be considered to correspond to the lower affinity cobalt binding site, assuming that the coordination of the second equivalent of cobalt does not perturb the structure and spectral properties at the higher affinity site.

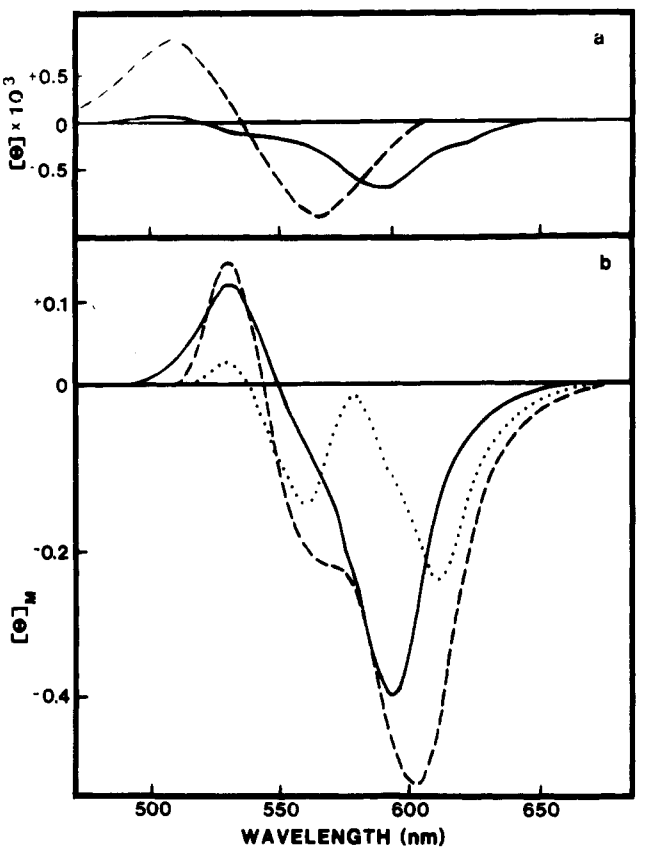

FIGURE 2: (a) Circular dichroic and (b) magnetic circular dichroic spectra of Co_1SOD (—) and Co_2SOD (--) reconstituted in 20 mM HEPES, pH 7.0. The dotted line represents the difference MCD spectrum of Co_2SOD minus Co_1SOD. The MCD spectra were recorded at a magnetic field strength of 40 kG, and the ellipticities are calculated on the basis of subunit molarity (M_r of 15 000).

The cobalt binding sites are also clearly distinguished by their CD and MCD spectra (Figure 2). The natural circular dichroism of Co_1SOD is comprised predominantly of a negative band at 585 nm and a shoulder of negative ellipticity near 540 nm. In contrast, the major negative CD band in Co_2SOD appears near 560 nm while positive bands occur at 520 and 470 nm. The MCD spectrum of Co_1SOD at pH 7.0 (Figure 2b) is typical of cobalt in a tetrahedral-like geometry (Holmquist & Vallee, 1978). Additional optically active bands near 470 and 520 nm appear in the MCD spectrum of Co_2SOD together with a shoulder at 565 nm and a shift of the 590-nm maximum to 600 nm. Subtraction of the spectrum of Co_1SOD yields a resolved spectrum corresponding to the lower affinity binding site, which is consistent with that of model pentacoordinate cobalt complexes (Kaden et al., 1974).

Atomic absorption analyses indicated that the addition of 2 equiv of zinc to Co_2SOD displaces cobalt from the protein. The loss of cobalt occurs slowly, over a period of 24 h, and the concomitant spectral changes are indicative of initial displacement of cobalt from the site of tetrahedral-like[2] ge-

[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.

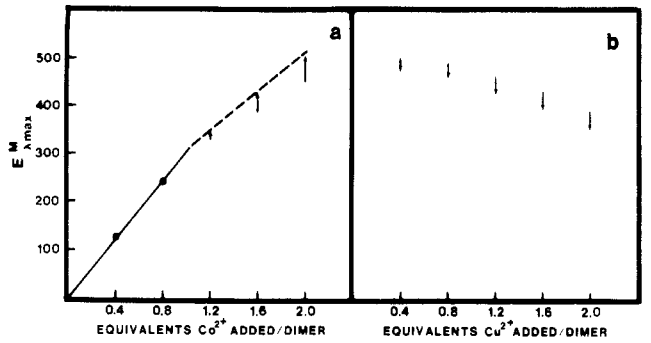

FIGURE 3: (a) Cobalt titration of apoSOD (10 mg·mL^{-1}) in 20 mM sodium phosphate, pH 7.0, and (b) copper titration of Co_2SOD. The extinction corresponds to the peak of maximum absorbance and in (b) is corrected for the absorbance arising from overlap of the copper and cobalt spectra. The arrows indicate the time-dependent changes in the cobalt spectrum upon the addition of copper.

ometry. Copper titration of Co_2SOD at pH 7.0 results in the incorporation of 2 equiv of copper, as indicated by atomic absorption analyses. No time-dependent changes in the cobalt absorption spectrum are apparent upon the coordination of copper to the protein. The resolved cobalt absorption spectrum obtained by measuring the spectrum of Cu_2,Co_2SOD against Cu_2SOD is not significantly different from that of Co_2SOD. The cobalt absorption maxima occur at 540 and 600 nm with extinction coefficients of 364 and 384 M^{-1} cm^{-1}, respectively.

The two spectrally distinct cobalt binding sites are apparent during reconstitution of apoSOD in either Tris-HCl, Tris–maleate, or HEPES buffer at pH 7.0 but are not detected during analogous titration of apoSOD in the presence of phosphate. Stoichiometric incorporation of cobalt into apoSOD in phosphate, pH 7.0, similarly requires the addition of excess cobalt. The titration curve is shown in Figure 3, and there are significant time-dependent increases in the extinction at the visible absorption maxima during the addition of the second cobalt equivalent. The visible absorption spectrum corresponding to the second cobalt binding site is very similar to that of Co_1SOD, although differences are detected in both the wavelength and relative intensity of the absorption maxima (Figure 4). Nonetheless, the MCD spectra are indicative of tetrahedral-like geometries at each of the cobalt binding sites.

The addition of 2 equiv of zinc to Co_2SOD reconstituted in phosphate results in displacement of the incorporated cobalt in a very slow reaction that requires several days incubation at room temperature to reach completion. Copper titration of Co_2SOD significantly influences the cobalt absorption spectrum. Concomitant with an approximate 30% decrease in the extinction of the cobalt spectral envelope (Figure 3), there is a shift in the absorption maxima to yield the spectrum shown in Figure 5. The spectrum is no longer consistent with two tetrahedral-like cobalt binding sites but rather resembles that of Co_2SOD reconstituted in HEPES and reflects one tetrahedral-like and one pentacoordinate-like cobalt complex.

Regeneration of Enzyme Activity. The ability to restore dismutase activity by the addition of zinc and copper to apoSOD is inversely related to the pH of reconstitution. Consequently, maximal enzyme activity is regenerated at pH 5.0, while reconstitution at pH 7.0 results in a protein with less than 50% of the activity of the native enzyme (Figure 6). Protein samples reconstituted at greater than pH 6.0 also reveal significant time-dependent increases in activity during incubation at room temperature for 24 h. Furthermore, the activities regained at pH > 6.0 are consistently lower when reconstitution is carried out in phosphate buffers in comparison to protein samples reconstituted in MES, HEPES, or Tris at

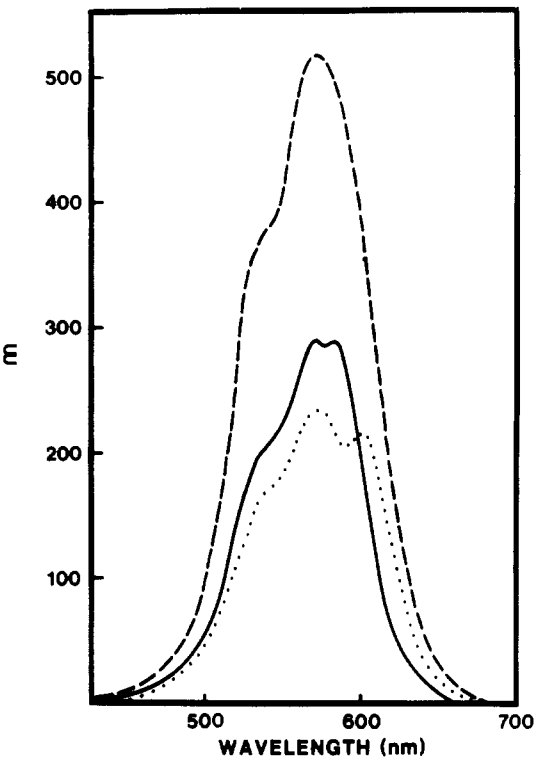

FIGURE 4: Visible absorption spectra corresponding to the titration of apoSOD in 20 mM sodium phosphate, pH 7.0, with 1 (—) and 2 equiv (– –) of cobalt per dimer. Difference spectrum (···) obtained by recording the spectrum of Co_2SOD against Co_1SOD.

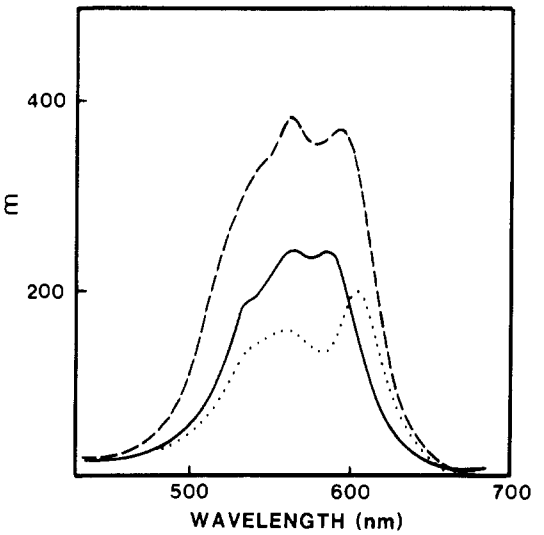

FIGURE 5: Cobalt absorption spectrum of Co_1,Cu_2SOD (—) and Co_2,Cu_2SOD (– –) reconstituted in 20 mM sodium phosphate, pH 7.0, and the spectrum recorded against Cu_2SOD. Spectrum of Co_2,Cu_2SOD recorded against Co_1,Cu_2SOD (···).

the corresponding pH (Figure 6).

Partial reconstitution of apoSOD with zinc and copper yields the enzyme activities shown in Table I. (The activity of Cu_1SOD and Cu_2SOD samples could not be determined adequately due to the decreased stability of the copper–protein complexes in the zinc-free enzyme. As a result, there was a loss of copper from the protein in the presence of the DEPTA-containing assay buffer.) The derivative obtained by titration of apoSOD with 1 equiv each of zinc and copper per dimer exhibits approximately 50% of the native enzyme activity, regardless of the pH of reconstitution. The incorporation of the second equivalent of copper to yield Cu_2,Zn_1SOD fully restores the native dismutase activity to the samples recon-

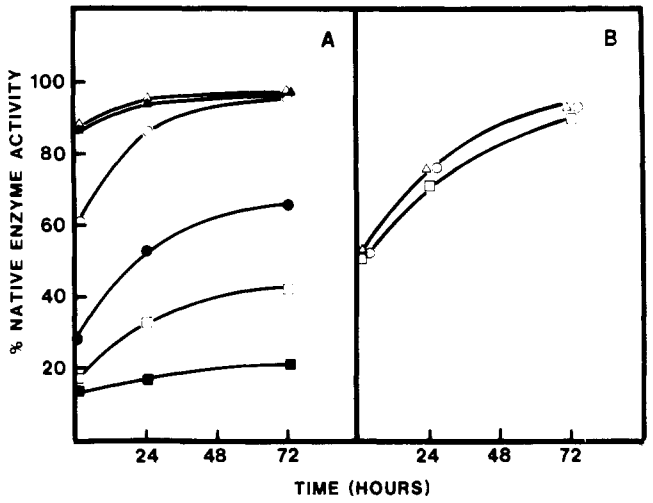

FIGURE 6: Regeneration of enzyme activity following reconstitution of yeast apoSOD. (A) The enzyme (1 mg·mL^{-1}) was reconstituted initially with 2 equiv of zinc followed by 2 equiv of copper, and the enzyme activity was measured at the indicated times. Reconstitution was carried out in 10 mM sodium acetate, pH 5.0 (△), 10 mM sodium phosphate, pH 5.0 (▲), 10 mM MES, pH 6.0 (○), 10 mM sodium phosphate, pH 6.0 (●), 10 mM HEPES, pH 7.0 (□), and 10 mM sodium phosphate, pH 7.0 (■). (B) Activity of Cu_2,Co_2SOD reconstituted in 10 mM sodium acetate, pH 5.0 (△), 10 mM MES, pH 6.0 (○), and 10 mM sodium phosphate, pH 7.0 (□). Native enzyme activity was 7500 units·mg^{-1}.

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

	enzyme activity (units·mg^{-1})[a]		
sample	acetate, pH 5.0	HEPES, pH 7.0	phosphate, pH 7.0
Cu_1,Zn_1SOD	3800	3780	3773
Cu_1,Zn_2SOD	3650	1766	1154
Cu_2,Zn_1SOD	7186	6735	4718
Cu_2,Zn_2SOD	7300	2859	1500
Cu_2,Zn_2SOD[b]	7450	7036	6554

[a] Enzyme activities of the reconstituted copper and zinc derivatives of SOD. The apoSOD was initially reconstituted with 1 or 2 equiv of zinc/mol dimer, and copper was subsequently added. The enzyme activities were measured 24 h after the reconstitution. The native enzyme activity was 7500 units·mg^{-1}. [b] Reconstitution of the apoSOD with copper prior to the addition of zinc.

stituted in acetate and HEPES, suggesting that copper coordinated in each of the binding sites contributes equally to the activity of the protein. The enzyme activity regained is considerably reduced, however, when reconstitution of Cu_2,Zn_1SOD is carried out in the presence of phosphate.

Identical activities are displayed by Cu_2,Zn_1SOD and Cu_2,Zn_2SOD reconstituted at pH 5.0, implying that zinc has no effect on the rate of catalysis. However, comparison of the Cu_2,Zn_1SOD and Cu_2,Zn_2SOD derivatives reconstituted at higher pH reveals that incorporation of zinc into two sites at pH 7.0 is associated with much lower enzyme activities than when only one zinc site is occupied. The capacity of zinc to modify the activity of the enzyme is observed only upon coordination of zinc to the protein prior to the addition of copper. The activity of Cu_2,Zn_2SOD reconstituted initially with copper is not significantly different from that of the native enzyme (Table I).

The pH-dependent restoration of enzyme activity observed during reconstitution of apoSOD with zinc and copper is not apparent when cobalt is substituted for zinc during the reconstitution (Figure 6). Although the dismutase activity of the copper–cobalt derivatives is relatively low immediately following the addition of the metals, subsequent incubation at room temperature yields activities approaching that of native Cu_2,Zn_2SOD.

Discussion

The most frequent representation of the configuration of the metal binding sites in Cu_2,Zn_2SOD is that of a distorted tetrahedron for zinc, while copper is proposed to be coordinated in an uneven tetrahedral distortion from square-planar ligand geometry (Richardson et al., 1975; Tainer et al., 1983). In addition, the structures of the two subunits are generally considered to be identical. However, we have recently demonstrated asymmetry in the active site structures in yeast Cu_2,Zn_2SOD (Dunbar et al., 1982). Reconstitution of apoSOD with cobalt at pH 5.0 results in the coordination of cobalt into one of the two native zinc binding sites to display spectra consistent with a tetrahedral-like geometry, while the configuration of the second zinc site precludes the incorporation of cobalt (Dunbar et al., 1984). In the current experiments, reconstitution of yeast apoSOD at pH 7.0 facilitated the incorporation of cobalt into both subunits and consequently enabled visualization of the coordination geometry at each of the zinc binding sites.

The visible absorption, CD, and MCD spectra of the high-affinity cobalt binding site at pH 7.0 are identical with those previously observed for Co_1SOD reconstituted at pH 5.0. The conformation of this site therefore appears to be pH independent within the range $5 \leq pH \leq 7$. In contrast, incorporation of cobalt into the second zinc binding site not only is a function of pH but also yields spectral properties consistent with a pentacoordinate complex. The subsequent incorporation of 2 equiv of copper into Co_2SOD has no apparent effect on the cobalt spectra, and therefore, it seems unlikely that the pentacoordinate-like complex reflects cobalt incorporation into one of the native copper binding sites.

The asymmetry in the structure of the cobalt binding sites is not apparent when apoSOD is reconstituted at pH 7.0 in the presence of phosphate. Under these conditions, the incorporated cobalt appears to reside in two sites of tetrahedral-like geometry. The high-affinity tetrahedral-like site is spectrally indistinguishable from that observed when reconstitution is carried out in the absence of phosphate. Although subtle differences are detected in the visible absorption spectra ascribed to the high- and low-affinity cobalt binding sites, the similar MCD spectra suggest that the observed variations in the absorption spectra may simply arise from the degree of distortion in the ligand geometry. However, the subsequent addition of copper to Co_2SOD in phosphate induces significant changes in the spectra corresponding to the lower affinity cobalt binding site. These spectral changes are consistent with a transition from a site of tetrahedral geometry to an apparent pentacoordinate-like complex. Although it is possible that the coordination of copper could influence the configuration of cobalt bound in the adjacent site, an alternative and more likely explanation is that in the presence of phosphate the second equivalent of cobalt is initially incorporated into one of the native copper binding sites. The observed shift in the coordination geometry would then be consistent with the displacement of cobalt by copper and subsequent coordination of cobalt into the pentacoordinate-like zinc site. In accordance with this proposal, cobalt reconstitution of Cu_2SOD results in incorporation of cobalt into a site of tetrahedral-like geometry and subsequent coordination of the second cobalt equivalent into a site resembling that of a pentacoordinate-like complex. The appearance of a copper binding site of tetra-

hedral-like geometry is also compatible with recent EXAFS analyses of the active site structure of yeast Cu_2,Zn_2SOD (R. Bauer et al., personal communication) and X-ray structure analysis (Tainer et al., 1983).

Calabrese et al. (1979) have reported a novel cobalt binding site in bovine Cu_2,Zn_2SOD. The incorporation of cobalt into this site was phosphate dependent and was proposed to occur subsequent to the incorporation of cobalt into the two native zinc binding sites. However, the visible absorption spectrum arising from the "anomalous" cobalt coordination was identical with that displayed in the current experiments during titration of Co_1SOD to Co_2SOD. Since we have observed that the addition of excess cobalt is necessary to achieve the stoichiometric incorporation of 2 equiv of Co(II) per dimer and that the incorporation of the second cobalt equivalent is time dependent, it appears likely that the cobalt binding detected by Calabrese and co-workers represents the same Co_2SOD species we have observed in which cobalt is proposed to reside in one native zinc and one native copper binding sites. These comparable observations in the titration behavior of yeast and bovine enzymes may be taken as a further reflection of the similarity in the active site structure of Cu_2,Zn_2SOD isolated from the two species (Cass et al., 1978; Hill et al., 1978).

The pH-dependent incorporation of cobalt into the lower affinity pentacoordinate-like binding site presumably reflects protein conformational changes at the active site to provide a more favorable configuration for cobalt binding. The lack of incorporation of cobalt into the same site at pH 5.0 may arise from greater distortion and the inability of cobalt to conform to the constraints of the ligand geometry at the lower pH. Although the incorporation of zinc did not exhibit a comparable pH dependence, the proposed conformational changes at the lower affinity site at pH 7.0 may also underlie the observed pH-dependent regeneration of enzyme activity during reconstitution of apoSOD with copper and zinc (vide infra).

The addition of zinc to Co_2SOD consistently resulted in the displacement of cobalt from the site of tetrahedral-like geometry. The preferential incorporation of zinc into one active site implies that the association constant for zinc differs between the two subunits. Similar conclusions were drawn from the rate of dissociation of zinc from the native enzyme (Dunbar et al., 1982). Extrapolation of the structure of the zinc binding sites from the spectral properties of the incorporated cobalt would then suggest a high-affinity zinc binding site of tetrahedral-like geometry in one subunit and a lower affinity pentacoordinate-like zinc complex in the second subunit.

Asymmetry between the active site structures is observed during metal titration of apoSOD. Subunit interactions occur during reconstitution, and it has been suggested that the asymmetry may be induced by the coordination of metals into one active site (Dunbar et al., 1984). Whether metal coordination into both active sites induces subsequent subunit interactions leading to restoration of equivalent active site structures is at present unknown. However, analysis of the metal binding sites in native yeast Cu_2,Zn_2SOD by EXAFS spectroscopy is consistent with asymmetry.

The relationship between the active site asymmetry and the catalytic mechanism of the enzyme remains to be elucidated. It has been shown previously that copper is essential for catalysis, undergoing alternate oxidation and reduction during the dismutation reaction (Fielden et al., 1973; Klug-Roth et al., 1973). Nonetheless, there appears to be a potential role for zinc in modifying the activity of the enzyme, which, in the current experiments, paralleled the proposed pH-dependent conformational changes at the lower affinity zinc binding site. The ability of zinc to modify the enzyme activity of the protein presumably arises, in part, from its capacity to direct the conformation around the neighboring copper binding site (Dunbar et al., 1982). However, the specific activity of both subunits is apparently decreased by the incorporation of zinc into the lower affinity site at pH 7.0, suggesting that there is also an interaction between the active sites. The pH and zinc dependencies of the regeneration of enzyme activity are not apparent when copper is coordinated to apoSOD prior to the addition of zinc. Furthermore, the incorporation of cobalt into the pentacoordinate-like binding site at pH 7.0 has no effect on the capacity to restore enzyme activity upon the addition of copper. It is possible, then, that the incorporation of zinc at pH 7.0 may induce structural constraints at the active site that are not evident if copper is coordinated first, while conceivably cobalt may be more weakly bound than zinc and unable to induce similar protein structural changes. Decreased flexibility of the zinc binding site at pH 7.0 has also been suggested for bovine Cu_2,Zn_2SOD (Pantoliano et al., 1982).

The regeneration of enzyme activity by reconstitution of apoSOD with copper and zinc and the configuration of the metal binding sites during cobalt titration of apoSOD are phosphate dependent at pH 7.0 Phosphate has also been shown to influence the rate of tritium incorporation into the reduced Cu_1,Zn_2SOD (Dunbar et al., 1980), and different perturbed angular correlation spectra are obtained for Cu_1,Cd_2SOD in the presence and absence of phosphate (R. Bauer, personal communication). The mechanism by which phosphate influences the active site is as yet unknown, although phosphate may interact with Arg-143 (corresponding to Arg-141 in the sequence of the bovine enzyme), which lies in close proximity to the active site copper (Richardson et al., 1975), a residue suggested to be essential for enzyme activity (Borders & Johansen, 1980). In this regard, it has also been suggested that the imidazolate bridge in Cu_2,Cu_2SOD is cleaved in the presence of phosphate (Strothkamp & Lippard, 1981).

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

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Escherichia coli Phosphoenolpyruvate-Dependent Phosphotransferase System. Functional Asymmetry in Enzyme I Subunits Demonstrated by Reaction with 3-Bromopyruvate†

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ABSTRACT: In the bacterial phosphoenolpyruvate-dependent sugar transport systems, enzyme I (E_I) is responsible for the initial reaction step which is the transfer of the phosphoryl group from phosphoenolpyruvate to a cytoplasmic phosphocarrier protein (HPr). The inactivation of enzyme I by the substrate analogue 3-bromopyruvate has been investigated. Incubation of enzyme I with only micromolar concentrations of this reagent results in complete and irreversible loss of enzymatic activity within a few minutes. Other alkylation reagents such as 2-iodoacetate, 3-bromopropionate, or 5-bromovalerate are far less effective inhibitors of enzyme I, suggesting that the inactivation by 3-bromopyruvate is brought about by the alkylation of one or more essential residues at the active site. Phosphoenolpyruvate and pyruvate, or phosphoenolpyruvate and oxalate, when added together, protect against inactivation by bromopyruvate. Experiments with bromo[2-^{14}C]pyruvate showed that one residue per enzyme I dimer is first alkylated without causing any loss of enzymatic activity. Alkylation of a second residue causes complete inactivation. Both alkylated residues are cysteines. The observations in this report together with published data on the phosphorylation of E_I suggest that the subunits of the E_I dimer modulate one another's activity during the turnover of the enzyme.

Enzyme I, a component of the bacterial phosphoenolpyruvate (PEP)[1]-dependent phosphotransferase system, catalyzes the transfer of a phosphoryl group from PEP to a phosphocarrier protein (HPr). This is the first step in a series that ultimately leads to the phosphorylation and concomitant transport of hexoses and hexitols into the bacterial cell [for recent reviews, see Hays (1978) and Robillard (1982)]. The enzyme I catalyzed reaction proceeds via a phosphoenzyme I intermediate (Stein et al., 1974; Waygood & Steeves, 1980; Saier et al., 1980; Hoving et al., 1981; Weigel et al., 1982). Even though the activity of enzyme I requires a dimeric form of the enzyme, only one phosphoryl group is bound per dimer (Hoving et al., 1981; Misset & Robillard, 1982). These two observations are consistent with negative cooperative interactions or a flip-flop mechanism.

3-Bromopyruvate has been used successfully for active-site labeling of a large number of PEP- and pyruvate-utilizing enzymes (Meloche, 1965, 1967; Barnett et al., 1971; Berghauser et al., 1981; Hudson et al., 1975; Chang & Hsu, 1977; Yoshida & Wood, 1978; Kameshita et al., 1979; Yon & Suelter, 1979). Saier et al. (1980) have reported inactivation of enzyme I by 3-bromopyruvate. This paper presents studies using 3-bromo[2-^{14}C]pyruvate to specify the number of amino acid residues involved in the inactivation of enzyme I by this substrate analogue and to identify these residues. The object of these studies was to determine whether the asymmetry found in the phosphorylation sites also occurs at the level of the catalytically active base(s).

Materials and Methods

Chemicals and Enzymes. [1-^{14}C]PEP (monocyclohexylammonium salt, sp act. 12 μCi/μmol) and [2-^{14}C]pyruvate (sodium salt, sp act. 15.8 μCi/μmol) were purchased from Amersham. Nonradioactive pyruvate (sodium salt), PEP (monocyclohexylammonium salt), 3-bromopyruvic acid, 3-

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[1] Abbreviations: *S*-CHE-cysteine, *S*-(carboxyhydroxyethyl)cysteine; PEP, phosphoenolpyruvate; PTS, phosphotransferase system; DTT, dithiothreitol; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; Ox, oxalate; Pyr, pyruvate; BrPyr, 3-bromopyruvate.

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