THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 248, No. 11, Issue of June 10, pp. 3855-3859, 1973
Printed in U.S.A.

Magnetic Circular Dichroism of Cobalt-Copper and Zinc-Copper Bovine Superoxide Dismutase*

(Received for publication, January 4, 1973)

GIUSEPPE ROTILIO, LILIA CALABRESE, AND JOSEPH E. COLEMAN‡

From the Institute of Biological Chemistry, University of Rome, and Center of Molecular Biology, National Research Council, Rome, Italy, and the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510

SUMMARY

The magnetic circular dichroism of the *d-d* transitions of Co(II) when substituted for Zn(II) in the Zn(II)-Cu(II) enzyme bovine superoxide dismutase enzyme is reported. Magnetic circular dichroism of the Co(II) chromophore in the Co(II)-Cu(II) enzyme is typical of tetrahedral Co(II) compounds. The magnetic circular dichroism band pattern is almost identical with the magnetic circular dichroism of the anion complexes of Co(II) carbonic anhydrase, implying similar coordination geometries in the two enzyme Co(II) complexes. The Cu(II) chromophore is only weakly induced by the magnetic field, with induced ellipticity an order of magnitude less than that of the Co(II) chromophore. Reduction of the Co(II)-Cu(II) protein causes minor, but significant, changes in the Co(II) site as measured by magnetic circular dichroism.

Magnetic circular dichroism of the Co(II) chromophore located at the active sites of metalloenzymes has proved to be a valuable probe of structure for two reasons. First, MCD[1] arises from the Zeeman splitting of the energy levels of the *d* orbitals of the metal ion and thus reflects the intrinsic geometry of the coordination complex. In contrast the natural circular dichroism is subject to perturbations produced by the surrounding potential field of the protein. Ellipticity induced in the Co(II) absorption bands by the latter mechanism is not related in any direct way to the underlying coordination geometry. Second, MCD arising from different coordination geometries shows major differences in both magnitude and band pattern and thus can be used to identify the general type of coordination geometry at the Co(II) site.

Although naturally occurring cobalt metalloproteins appear to be largely restricted to the cobalamin-containing enzymes, Co(II) can be substituted for Zn(II) in several zinc-metalloenzymes with retention of full or partial enzymatic activity (1). This laboratory has reported previously on the MCD of the active Co(II) erythrocyte carbonic anhydrases (2). The present work reports an MCD study of the active site of a naturally occurring metalloenzyme, bovine superoxide dismutase, containing both Zn(II) and Cu(II) in the native state, in which the Zn(II) ion has been substituted with Co(II). As isolated from bovine erythrocytes, superoxide dismutase (discovered by Mann and Keilin as erythrocuprien (3)) is a dimer of molecular weight 32,000, each subunit having a molecular weight of 16,300. Each subunit contains 1 Zn(II) and 1 Cu(II) in the oxidized state (4). Recent renewed interest in the protein has arisen from the discovery that it rapidly catalyzes the dismutation of the superoxide radical, O_2^-, to H_2O_2 (4). Exchange of the native Zn(II) ion for Co(II) results in a Co(II)-Cu(II) enzyme which retains full enzymatic activity (5).

EXPERIMENTAL PROCEDURE

Enzymes and Chemicals—Bovine superoxide dismutase and human erythrocyte carbonic anhydrase B were prepared as described previously (4, 6) and stored as lyophilized powders at −20°. Stock solutions of superoxide dismutase were made by dissolving the enzyme in metal-free water at pH 6.0. Stock solutions of carbonic anhydrase were made up in 0.025 M Tris, pH 9.0. Co(II) carbonic anhydrase was prepared by the addition of 1 g atom of Co(II) per mole of protein to the apoenzyme prepared with 1,10-phenanthroline (7). Co(II) superoxide dismutase was prepared by dialysis of the native enzyme against 0.5 % Co(II) contained in acetate buffer, 0.1 M at pH 5.4 for 36 hours at 4°, followed by exhaustive dialysis against metal-free water (5). Under these conditions the native Zn(II) exchanges for Co(II), whereas the native Cu(II) ion does not exchange.[2] Metal-free buffers were prepared as described previously (8). All other chemicals were reagent grade and used without further purification.

* This work was supported by Grant GB-13344 from the National Science Foundation and Grant AM 09070-08 from the National Institutes of Health.

‡ To whom reprint requests should be directed at Yale University, New Haven, Connecticut 06510.

[1] The abbreviations used are: MCD, magnetically induced circular dichroism; CD, natural circular dichroism.

[2] The exchange method for making Co(II)-Cu(II) superoxide dismutase (5) results in variable replacement of the Zn(II) with Co(II) depending on the time of dialysis. The extensive treatment required for complete exchange often results in some loss of enzymatic activity. Therefore the best preparations showed 50 to 80% exchange of Co(II) for Zn(II). Variable exchange does not appear to alter the absorption spectrum or circular dichroism of the Co(II) chromophore if corrections are made for the contribution of unexchanged enzyme.

Metal Analysis—Metal determinations were performed by atomic absorption spectroscopy using a Hilger-Watts Atomspek model H 1170.

Absorption Spectra—Spectra were obtained on a Cary 15 recording spectrophotometer.

Circular Dichroism and Magnetic Circular Dichroism—CD and MCD were recorded on a Cary 61 spectropolarimeter equipped with a Varian superconducting solenoid to produce an axial magnetic field at the sample of up to 50 kilogauss. The solenoid can be operated in the normal (or reverse) mode which produces an axial magnetic field parallel (or antiparallel) to the direction of incidence of the light. Often the magnitude of the natural CD is insignificant compared to the MCD; however, when the MCD and CD are of comparable magnitude, the reverse mode may offer advantages over the normal mode depending on the band pattern and sign of the natural CD (see below). Slit widths were programed to maintain constant light intensity and were 1 mm or less. Sample temperature was 25°. CD and MCD are expressed in terms of molecular ellipticity, $[\theta] = 2.303\ (4500/\pi)\ (\epsilon_L - \epsilon_R)$ with units of deg cm² per dmole. For the MCD the exact magnitude of the inducing field is indicated in each figure.

RESULTS

CD and MCD of the Co(II) Chromophore in Superoxide Dismutase—The natural CD of the native Zn(II)-Cu(II)-containing enzyme and the Co(II)-Cu(II)-containing enzyme is shown in Fig. 1. Both proteins have a large negative CD band centered near 800 nm ($\theta \simeq -4 \times 10^3$ deg cm² per dmole). Approximately one-half of this band appears in the region of 700 to 800 nm. The latter negative band is associated with the Cu(II) chromophore and the substitution of Co(II) for Zn(II) has little effect on this band. The native enzyme has a large positive CD band centered at 607 nm ($\theta \simeq 3.9 \times 10^3$ deg cm² per dmole) also associated with the Cu(II) chromophore.

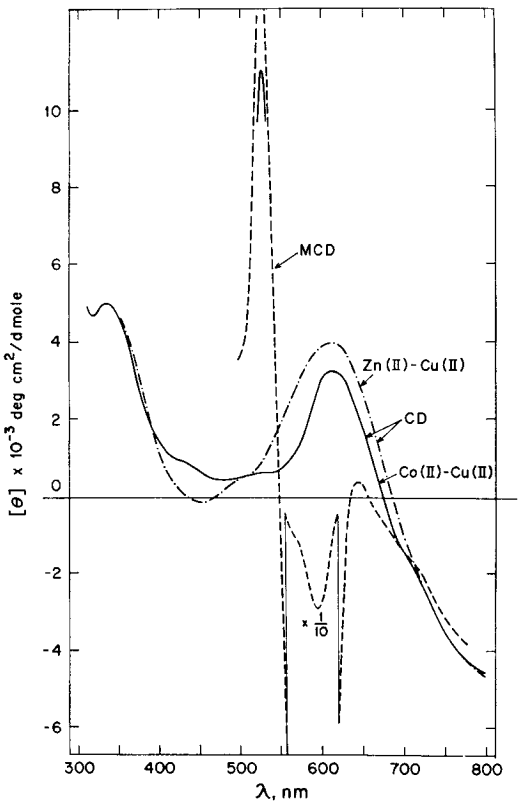

FIG. 1. CD of Zn(II)-Cu(II) (-·-) and Co(II)-Cu(II) (——) bovine superoxide dismutase. MCD (- - -) of Co(II) bovine superoxide dismutase. Conditions: Zn(II)-Cu(II) protein, 3.3×10^{-4} M, Co(II)-Cu(II) protein, 2.0×10^{-4} M, metal-free H_2O, pH 6.0, 25°; field = 43.5 kilogauss. Molar ellipticity for natural CD is expressed per mole of enzyme dimer; for MCD per mole of Co(II) site.

Substitution of Co(II) for Zn(II) decreases the magnitude of the CD in the region 500 to 650 nm and produces a small positive band near 450. The majority of these changes appear to be due to the superposition of optically active *d-d* transitions of the Co(II) ion. These bands are negative as shown by the CD difference spectrum in Fig. 2 calculated by subtracting the CD of the native Zn(II)-Cu(II) protein from that of the Co(II)-Cu(II) protein.

The small long wave length bands centered near 650 nm are on the low energy edge of the Co(II) absorption spectrum (see below), and, if they are due to the Co(II) chromophore, they must arise from relatively weak transitions. That they are optically active Co(II) transitions and not associated with Co(II)-induced changes in the Cu(II) chromophore is suggested by the CD of the reduced Co(II)-Cu(I) protein (Fig. 2). The reduced Cu(I) protein has no contribution from the copper chromophore in the visible region and thus the CD in the visible region is entirely due to the Co(II) chromophore. There remains a CD band in the 650-nm region of the reduced protein which is now positive rather than negative as observed in the difference CD of the two oxidized enzymes (Fig. 2). Both the Zn(II)-Cu(II) and the Co(II)-Cu(II) proteins show positive natural CD of the same magnitude associated with the copper charge-transfer band at 340 nm (9).

The effect on the circular dichroism of applying a magnetic field of 43.5 kilogauss to the Co(II)-Cu(II) enzyme is pictured on the same scale in Fig. 1. The magnetic field induces negative MCD bands at 588 and 565 nm and a positive band at 530 nm. These bands are an order of magnitude larger than the natural CD. This set of bands can be almost entirely attributed to the Co(II) chromophore (see below).

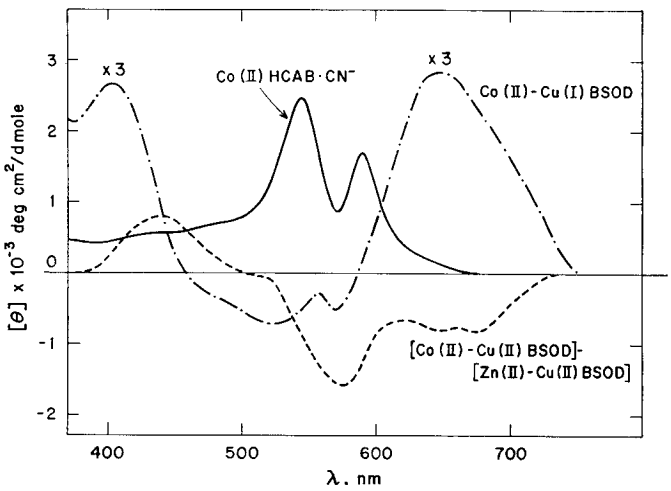

FIG. 2. Natural CD of the 1:1 CN^- complex of Co(II) human carbonic anhydrase B (——) (*Co(II) HCAB·CN⁻*) compared to the natural CD bands associated with the Co(II) chromophore in Co(II)-Cu(II) bovine superoxide dismutase (*BSOD*). The latter CD was determined by difference CD, CD of the Co(II)-Cu(II) protein minus the CD of the Zn(II)-Cu(II) protein (- - -); CD of the reduced Co(II)-Cu(I) protein (-·-). Conditions for superoxide dismutase are as in Fig. 1. Concentration of the carbonic anhydrase-cyanide complex was 1×10^{-3} M Tris, pH 9.0. For superoxide dismutase molar ellipticity is expressed per mole of enzyme dimer.

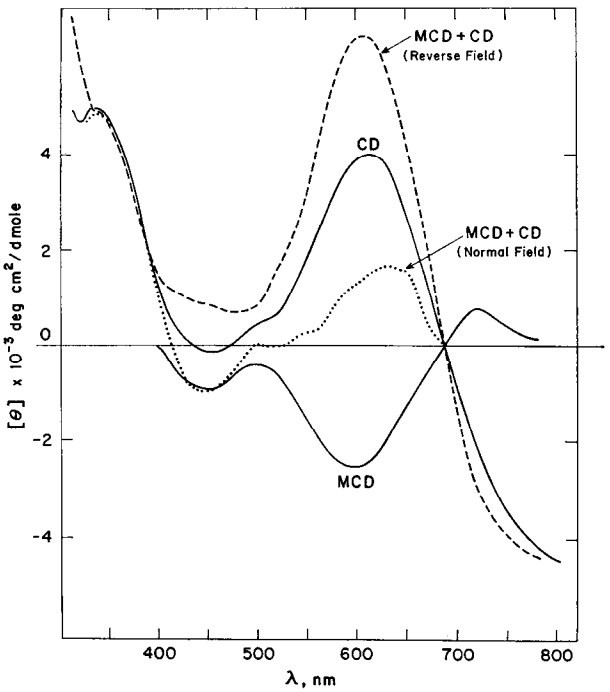

FIG. 3. MCD and CD of Zn(II)-Cu(II) bovine superoxide dismutase. Natural CD (——); MCD + CD, normal field (· · · ·); MCD + CD, reverse field (– – –); Calculated MCD under a normal field (——) = CD − (MCD + CD, reverse field). Conditions: 3.3×10^{-4} M enzyme, metal-free H_2O, pH 6.0, 25°; field = 43.5 kilogauss. Molar ellipticity for both MCD and CD is expressed per mole of enzyme dimer.

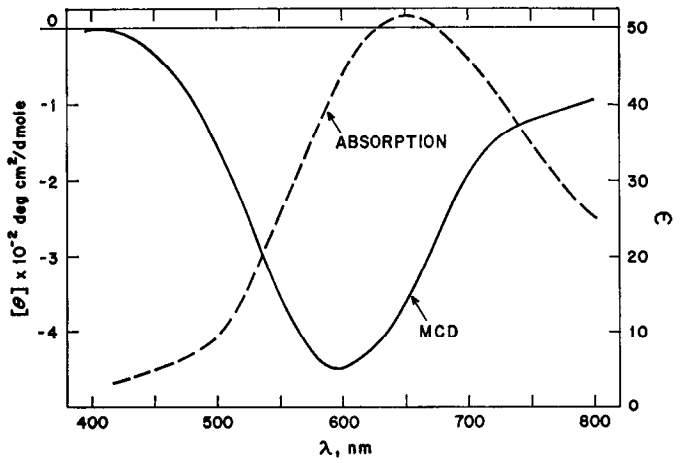

FIG. 4. MCD (——) and absorption spectrum (– – –) of the 2:1 complex of tris(hydroxymethyl)aminomethane (Tris) with Cu(II); Tris, 0.05 M, Cu(II) 0.02 M, pH 8.0, 25°; field = 43.5 kilogauss.

MCD of Cu(II) Chromophore—Since the Cu(II) chromophore is the only one contributing to the visible absorption of the native protein, the MCD contribution from Cu(II) can be assessed by a determination of the MCD of the native protein. The results are shown in Fig. 3 which compares the natural CD to the total circular dichroism observed under a normal field of 43.5 kilogauss and a reverse field of 43.5 kilogauss. These spectra show that the MCD induced in the Cu(II) chromophore is small compared to that induced in the Co(II) chromophore (Fig. 1) and is about equal in magnitude to the natural CD. Since CD and MCD are additive, the true MCD must be determined by subtracting the natural CD contribution. Since the MCD associated with the major Cu(II) absorption band near

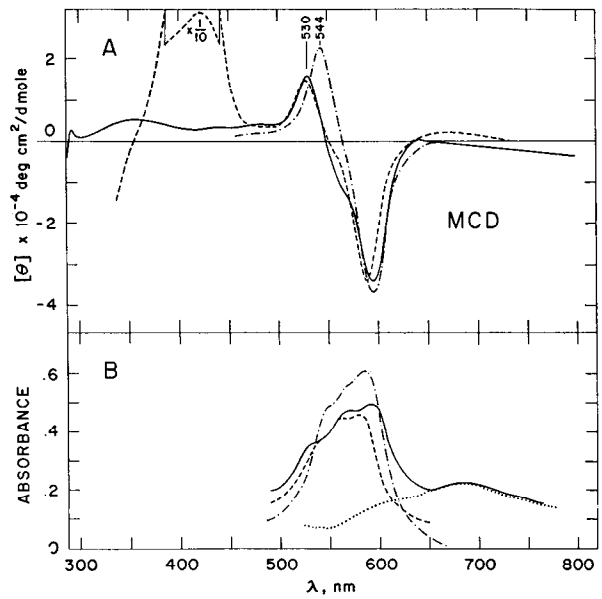

FIG. 5. MCD (*A*) and absorption spectra (*B*) of Co(II)-Cu(II) bovine superoxide dismutase compared to Co(II) human carbonic anhydrase B·CN⁻. –·–, Co(II) human carbonic anhydrase B·CN⁻; ——, Co(II)-Cu(II) bovine superoxide dismutase; – – –, Co(II)-Cu(I) bovine superoxide dismutase; · · · ·, absorption spectrum of native Cu(II)-Zn(II) bovine superoxide dismutase. Conditions: bovine superoxide dismutase, 4×10^{-4} M; metal-free H_2O, pH 6.0, 25°; Co(II) human carbonic anhydrase B·CN⁻, 1×10^{-3} M; 0.025 M Tris, pH 9.0, 25°; field for superoxide dismutase = 43.5 kilogauss; field for cyanide complex of Co(II) carbonic anhydrase = 47.5 kilogauss. Molar ellipticity is expressed per mole of Co(II) site.

600 nm is largely negative, the effect of the normal field is to reduce the observed CD to small values (Fig. 3). Thus better experimental results were obtained with a reverse field which produces a positive deflection for the major MCD band and thus a larger total circular dichroism. The MCD contribution at a normal field was then calculated by the difference, CD − (MCD + CD, reverse field). The resulting MCD curve is plotted in Fig. 3 and consists of three bands, a small negative band at 450 nm, a larger negative band at 600 nm, and a small positive band at ~720 nm. Maximum $[\theta]$ for the MCD is -2.5×10^3 deg cm² per dmole at 600 nm.

The magnitude is small compared to the Co(II) chromophore, but rather similar to a number of Cu(II) models we have examined. One of them, the Cu(II) complex of tris (hydroxymethyl)aminomethane is shown in Fig. 4. Titration data and formation curves for this complex suggest the usual four coordinate Cu(II) complex, presumably square-planar or distorted octahedral (10). A relatively small broad negative MCD band is induced in this symmetrical copper complex in the region of the unresolved broad absorption band of the Cu(II) *d-d* transitions. The charge transfer band at ~340 nm in Zn(II)-Cu(II)-superoxide dismutase does not show significant magnetically induced circular dichroism (Fig. 3).

MCD of Co(II)-Cu(II) Superoxide Dismutase, Comparison with MCD of Reduced Protein and Anion Form of Co(II) Carbonic Anhydrase—The MCD of the Co(II)-Cu(II) enzyme is plotted in Fig. 5A on a much reduced scale. The natural CD makes a negligible contribution to the MCD as indicated by the very small deflection from 650 to 800 nm. The major feature is a large negative MCD band at 595 nm ($[\theta] = -3.4 \times 10^4$ deg cm² per dmole), another negative band at ~565 nm ($[\theta] \simeq -1.1 \times 10^4$ deg cm² per dmole), and a positive band at 530 nm ($[\theta]$ =

TABLE I

Wave lengths, molar extinction coefficients, and magnetically induced molar ellipticity of principle visible bands of Co(II)-Cu(II), Co(II)-Cu(I) bovine superoxide dismutase, and Co(II) human carbonic anhydrase B·CN⁻

Enzyme absorption bands (wave length, nm)	Molar extinction coefficient[a]	MCD[b] (molar ellipticity × 10^{-4})
	M^{-1} cm^{-1}	deg cm^2/dmole
A. Co(II)-Cu(II) superoxide dismutase		
680 615 (Cu(II) bands (see D))		
595	600	−3.9
565	~570	−1.3
530	~420	+1.7
B. Co(II)-Cu(I) superoxide dismutase		
588	~560	−3.9
560	~555	−0.5
530	~410	+1.5
C. Co(II) human carbonic anhydrase B·CN⁻		
585	~650	−3.7
565	~540	~−1.2
544	~510	+1.6[c]
D. Zn(II)-Cu(II) superoxide dismutase		
680	~250	+0.10
615	~170	−0.29

[a] Molar extinction coefficients and molar ellipticity for Co(II) bands are expressed per cobalt site, and for Cu(II) bands are expressed per mole of enzyme dimer.

[b] Normalized to a field of 50,000 gauss.

[c] In a previous reference (2), this band was inadvertently reported as occurring at 570 rather than 544 nm.

+ 1.5 × 10^4 deg cm^2 per dmole). These large MCD bands correspond to the absorption bands of the Co(II) ion in the enzyme at 595 nm, 565 nm, and 530 nm (Fig. 5*B*) (5). Absorption by the Cu(II) chromophore makes only a small contribution to the absorption spectrum in this region as shown by the absorption spectrum of the native enzyme (Fig. 5*B*). The Cu(II) chromophore makes little contribution to the MCD as revealed by reducing the copper with ferrocyanide. This reduction removes the copper absorption and causes small but significant changes in the MCD (Fig. 5*A*). The magnitude remains almost the same, but the low wave length MCD band moves 7 nm to the blue. The large MCD peak at 425 nm is due to the induction of MCD in the transitions of the ferricyanide produced on reduction of the protein.

Metal-binding anions form stable mixed complexes with Co(II) carbonic anhydrase. The MCD of the cyanide complex of human Co(II) carbonic anhydrase B is shown in Fig. 5*A*. Band pattern and position are almost identical with the MCD bands of the Co(II) superoxide dismutase except for the high energy positive band which occurs 14 nm further to the red. The absorption spectrum of the anion form of Co(II) carbonic anhydrase is also almost identical with that of Co(II) superoxide dismutase. The absorption band positions, the MCD magnitudes normalized to 50,000 gauss, and the extinction coefficients of the absorption bands are all compared in Table I. An extensive analysis of the MCD of Co(II) carbonic anhydrase has been made previously and the important features are summarized in the discussion (2).

The natural CD of the Co(II) complex in the Co(II) carbonic anhydrase CN^- complex was compared to the natural CD of the Co(II) complex of superoxide dismutase in Fig. 2.

DISCUSSION

The MCD of Co(II) complexes appears to differentiate very clearly between the two most frequent types of coordination geometry for Co(II), octahedral and tetrahedral (2). At comparable inducing fields, octahedral Co(II) complexes have low induced MCD at the wave lengths of the visible transition, whereas tetrahedral Co(II) complexes show very large MCD corresponding to the absorption band in the visible region arising from the $^4A_2(F) \rightarrow {}^4T_1(P)$ transition which is expected to be split into four bands by spin-orbital coupling (2). The theory for the MCD of tetrahedral Co(II) complexes has received considerable attention (2, 11, 12). The band pattern for the visible MCD of tetrahedral Co(II) (large negative MCD bands associated with the two transitions to the low energy $^4T_1(P)$ levels and smaller positive MCD associated with the transitions to the higher energy $^4T_1(P)$ levels) have been well identified (2, 11, 12). The MCD of the CN^- complex of Co(II) carbonic anhydrase (Fig. 5*A*) has been identified with the tetrahedral band pattern (2).[3] The MCD of most Co(II) carbonic anhydrase anion complexes appears compatible with nearly regular tetrahedral geometry (2). The sensitivity of the MCD band pattern to distortions from the two regular geometries is not clear as yet, although rather different MCD from those pictured in Fig. 5*A* are observed for Co(II) complexes known to have significant deviations from strictly tetrahedral geometry, *e.g.* the uncomplexed alkaline form of Co(II) carbonic anhydrase (2).

The Co(II)-substituted Zn(II) site of superoxide dismutase appears to have a coordination geometry which conforms almost exactly to that of the cyanide complex of Co(II) carbonic anhydrase (Fig. 5*A*). This similarity is suggested by the absorption spectra of the Co(II) form of the superoxide dismutase (Fig. 5*B*) and even more clearly demonstrated by the similarity of the MCD spectra (Fig. 5*A*). The small shifts in the position of the absorption maxima and the postion of the MCD bands upon reduction of the copper are not explainable upon the basis of removal of the Cu(II) absorption and CD bands. The 7-nm shift in position of the low energy band must reflect a small change in the Co(II) coordination complex, coupled to the reduction of Cu(II). This is not a major change in geometry, since no significant change in MCD band pattern occurs, but could be a change in the spin-orbital coupling such that the band splitting of the $^4A_2(F) \rightarrow {}^4T_1(P)$ transition alters. This may suggest that the Co(II) and Cu(II) sites in each monomer are rather close to each other as does electron spin resonance data which show interaction between the electron spins at the two centers (13). Conformational changes propagated through the protein accompanying reduction are also a possibility. Such changes are also suggested by the alteration in the natural CD of the Co(II) bands between the oxidized and reduced proteins (Fig. 2). These changes in natural CD may reflect alterations in the external protein potential field upon reduction and do not necessarily imply changes in the inner coordination sphere of Co(II) (2).

Work with Co(II) carbonic anhydrases has shown that the sign and magnitude of the natural CD bands are controlled pre-

[3] The MCD spectrum of tetrahedral Co(II) has been identified as arising predominantly from C terms due to the Zeeman splitting of the ground state (2, 11, 12). This assignment has been confirmed for Co(II) carbonic anhydrase·CN^- by the temperature dependence of the MCD spectrum (2). Since C terms give rise to single-signed ellipticity terms corresponding to the absorption bands, the C term assignment explains the correspondence between the absorption bands and the maxima and minima of the MCD spectra in the case of the Co(II) enzyme spectra (Fig. 5).

dominantly by the dissymmetry of the external protein potential field, either from electrostatic perturbations or coupled transitions (2). On the other hand, the MCD is due only to the Zeeman splitting of the intrinsic energy levels of the *d* orbitals and hence much more accurately reflects the geometry of the coordination complex. Hence, the MCD spectra of all analogous carbonic anhydrase complexes look the same, whereas their natural CD spectra do not. The same is true of the comparison of the natural CD bands of the Co(II) site in superoxide dismutase to that in the CN^- complex of Co(II) carbonic anhydrase shown in Fig. 2. The signs of the natural CD bands are opposite even though the MCD (Fig. 5*A*) shows them to have almost identical geometries. The natural CD bands of the Co(II) chromophore of superoxide dismutase also changes radically upon reduction of the Cu(II) (Fig. 2). This change is clearly not a major change in geometry of the complex (Fig. 5).

The great similarity of the Co(II) binding sites in the Co(II) carbonic anhydrase-anion complex and the uncomplexed Co(II) superoxide dismutase is an intriguing finding. Since the Co(II) forms of both enzymes are active (5, 14), it is perhaps not too speculative to suggest that the Zn(II) sites in the anion complexes of carbonic anhydrase and the active form of superoxide dismutase are very similar if not identical. Both enzymes are found in relatively high concentration among the few proteins retained in any significant concentration by the mature mammalian erythrocyte. The dismutase contains two subunits, each about half of the molecular weight of the monomer of carbonic anhydrase containing one Zn(II) or Co(II) active site. One possibility might be that the subunit of superoxide dismutase is in some manner evolutionarily related to the carbonic anhydrase monomer. Such a relationship would require of course that the gene size had either halved or doubled depending on which protein evolved first and that it either acquired or lost the Cu(II) site, assuming that the Zn(II) site corresponds in both proteins, as the MCD suggests (Fig. 5). Further structural information on this point would be of interest.

Since it is the anion form of Co(II) carbonic anhydrase whose coordination geometry corresponds to the Co(II) site of superoxide dismutase, the site, if it is related to that of carbonic anhydrase, must therefore have evolved to one like the anion complex of Co(II) carbonic anhydrase. All evidence indicates the latter to be a four coordinate, nearly regular tetrahedron, with three protein ligands. The anion occupies the fourth ligand position which is occupied by a solvent molecule in the unliganded enzyme (1, 14). In superoxide dismutase this fourth position may be occupied by a fourth protein ligand forcing a more regular tetrahedral geometry. The Co(II) in superoxide dismutase does not appear accessible to anions in contrast to both Zn(II) and Co(II) in the unliganded carbonic anhydrase. Likewise the absorption spectrum of Co(II) superoxide dismutase is insensitive to pH between 6 and 11 (13), similar to the cyanide complex of Co(II) carbonic anhydrase, but unlike the unliganded form of Co(II) carbonic anhydrase, where the marked changes in absorption spectra appear to reflect changes at the solvent-occupied coordination site.

Unfortunately our MCD studies of Cu(II) complexes and Cu(II) proteins have thus far not revealed any features of the MCD spectra which would be helpful in distinguishing different types of Cu(II) complexes. Most Cu(II) complexes appear to show relatively weak magnetically induced CD (Figs. 3 and 4). At room temperature the MCD spectrum of Cu(II) does not resolve the three *d-d* transitions expected in the visible region for Cu(II) complexes any better than the absorption spectrum (Figs. 4 and 5). The major MCD, however, appears to be induced in the highest energy band, usually assigned to the $d_{x^2-y^2} \rightarrow d_{xy,\,yz}$ transition (15), and does not correspond to the maximum of the absorption spectrum. The same characteristic is observed for the MCD (Fig. 3) and absorption spectra (Fig. 5*B*) of the Cu(II) in superoxide dismutase. The MCD band of the Cu(II) chromophore is similar to that reported previously for the native bovine superoxide dismutase by Weser *et al.* (16). The MCD of the Cu(II) in the superoxide dismutase is somewhat larger than that of the typical model complex even when corrected for the two Cu(II) sites per molecule (Fig. 3). This may reflect the rhombic distortion indicated by the ESR of the enzyme (17). Further studies of other distorted Cu(II) sites will reveal if there is any systematic variation.

The function of the Zn(II) or Co(II) at the second metal site in each monomer of superoxide dismutase is not clear as yet. Extensive electron spin resonance studies of the Cu(II) site have indicated that the copper site consists of three magnetically equivalent nitrogen nuclei from the protein with a fourth coordination site available to the solvent or to metal-binding anions from solution (17). The geometry appears to have axial symmetry in the anion complexes, but with significant rhombic distortion in the unliganded enzyme (17).

There is no evidence that the Co(II) site is readily accessible to solvent or anions, not incompatible with the observation of the similarity between the Co(II) site and that of the CN^- complex of Co(II) carbonic anhydrase (Fig. 5). It appears likely that interactions with O_2^- or with the product H_2O_2 take place near the copper ion (13, 17). The additional Zn(II) or Co(II) ion may play a structural role, perhaps influencing the chemistry of the copper site either by direct proximity or via conformational changes in the protein.

REFERENCES

1. Coleman, J. E. (1971) *Progr. Bioorg. Chem.* **1**, 159–344
2. Coleman, J. E., and Coleman, R. V. (1972) *J. Biol. Chem.* **247**, 4718–4728
3. Mann, T., and Keilin, D. (1939) *Proc. Roy. Soc. London Biol. Sci.* **126**, 3 3
4. McCord, J. M., and Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6056–6063
5. Calabrese, L., Rotilio, G., and Mondovi, B. (1972) *Biochim. Biophys. Acta* **263**, 827–829
6. Armstrong, J. M., Myers, D. V., Verpoorte, J. A., and Edsall, J. T. (1966) *J. Biol. Chem.* **241**, 5137–5149
7. Rickli, E. E., and Edsall, J. T. (1962) *J. Biol. Chem.* **237**, PC 258–260
8. Harris, M. I., and Coleman, J. E. (1968) *J. Biol. Chem.* **243**, 5063–6073
9. Rotilio, G., Finazzi-Agro, A., Calabrese, L., Bossa, F., Guerrieri, P., and Mondovi, B. (1971) *Biochemistry* **10**, 616–621
10. Hall, J. L., Swisher, J. A., Brannon, D. G., and Liden, T. M. (1962) *Inorg. Chem.* **1**, 409
11. Denning, R. G., and Spencer, J. A. (1969) *Faraday Soc. Symp.* **3**, 84
12. Bird, B. D., Briat, B., Day, P., and Rivoal, J. C. (1969) *Faraday Soc. Symp.* **3**, 70
13. Rotilio, G. (1973) *Biochem. Soc. Symp.* **1**, 24–26
14. Coleman, J. E. (1967) *J. Biol. Chem.* **242**, 5212–5219
15. Taylor, J. S., and Coleman, J. E. (1971) *J. Biol. Chem.* **246**, 7058–7067
16. Weser, U., Bunnenberg, E., Cammack, R., Djerassi, C., Flohe, L., Thomas, G., and Voelter, W. (1971) *Biochim. Biophys. Acta* **243**, 203–213
17. Rotilio, G., Morpurgo, L., Giovagnoli, C., Calabrese, L., and Mondovi, B. (1972) *Biochemistry* **11**, 2187–2192