References

Ambler, R. P. (1967), *Methods Enzymol. 11*, 155.
Bannister, J., Bannister, W., and Wood, E. (1971), *Eur. J. Biochem. 18*, 178.
Boyer, P. D. (1954), *J. Amer. Chem. Soc. 76*, 4331.
Carrico, R. J., and Deutsch, H. F. (1970), *J. Biol. Chem. 245*, 723.
Davis, L. C., Brox, L. W., Gracy, R. W., Ribereau-Gayon, G., and Horecker, B. L. (1970), *Arch. Biochem. Biophys. 140*, 217.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1958), *Anal. Chem. 30*, 350.
Edelhoch, H. (1967), *Biochemistry 6*, 1948.
Gros, C., and Labouesse, B. (1969), *Eur. J. Biochem. 7*, 463.
Hoare, D. G., and Koshland, E. A. (1967), *J. Biol. Chem. 242*, 2447.
Keele, B. B., McCord, J. M., and Fridovich, I. (1971), *J. Biol. Chem. 246*, 2875.
McCord, J. M., and Fridovich, I. (1969), *J. Biol. Chem. 244*, 6049.
Poulik, M. D. (1957), *Nature (London) 188*, 1477.
Rotilio, G., Finazzi Agrò, A., Calabrese, L., Bossa, F., Guerrieri, P., and Mondovi, B. (1971), *Biochemistry 10*, 616.
Rotilio, G., Morpurgo, L., Giovagnoli, C., Calabrese, L., and Mondovi, B. (1972), *Biochemistry 11*, 2187.
Spencer, R. L., and Wold, F. (1969), *Anal. Biochem. 32*, 185.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Trotman, C. N. A., and Greenwood, C. (1971), *Biochem. J. 124*, 25.
Wood, E., Dalgleish, D., and Bannister, W. (1971), *Eur. J. Biochem. 18*, 187.
Woods, K. R., and Wang, K. T. (1967), *Biochim. Biophys. Acta 133*, 369.

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

Giuseppe Rotilio,* Laura Morpurgo, Carlo Giovagnoli, Lilia Calabrese, and Bruno Mondovì

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

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

Materials and Methods

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

† From the Institute of Biological Chemistry, University of Rome, Institute of Applied Biochemistry, University of Rome, and Center of Molecular Biology, National Research Council, Rome, Italy. *Received December 30, 1971.* A preliminary report of this work has been presented at the First La Cura Conference on Copper Proteins, La Cura, Sept 1971. This is the third paper of a series on the metal sites of copper proteins. The second paper has been published (Rotilio *et al.*, 1971).

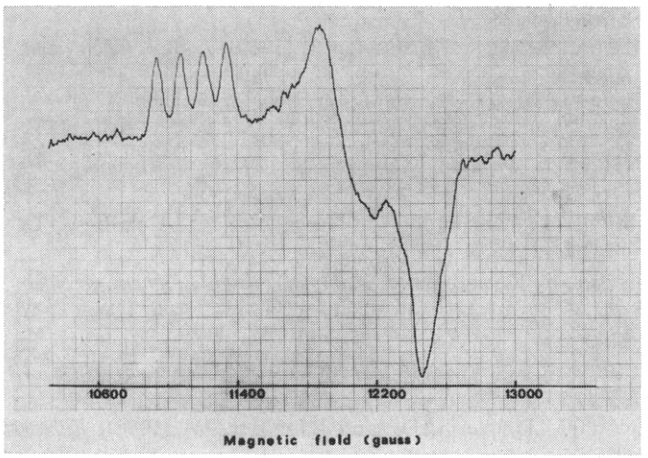

FIGURE 1: Epr spectrum of the bovine enzyme (5%) in water at approximately 35 GHz. Modulation frequency, 100 kHz; modulation amplitude, 10 G; microwave power, 20 mW; temperature, approximately −100°.

Montreal, Canada. ^{63}Cu was obtained from Oak Ridge National Laboratory in the form of CuO; [^{63}Cu]$CuCl_2$ was prepared by dissolving CuO in HCl. The bovine enzyme was purified according to McCord and Fridovich (1969). Copper-free protein was prepared as previously described (Rotilio *et al.*, 1972). The protein concentration was calculated from absorbancy at 680 nm (McCord and Fridovich, 1969). Epr spectra at 9 GHz, optical spectra, and pH measurements were obtained as previously described (Rotilio *et al.*, 1971). Epr spectra at 35 GHz were recorded in a Varian V-4502-14 spectrometer, equipped with a V-K3544 35-GHz conversion kit. Superoxide dismutase activity was tested as described in the previous paper (Rotilio *et al.*, 1972). Programs for simulation of the epr spectra were written in Fortran V using a Univac 1108 computer.

Results

Epr Spectrum of the Bovine Enzyme at 35 GHz. The epr spectrum of the bovine enzyme at 35 GHz is shown in Figure 1. Three distinct g values were determined, that is $g_z = 2.265$; $g_y = 2.108$; $g_x = 2.023$. This demonstrates that the typical line shape observed at 9 GHz (see Figure 3, a) is due to partial resolution of g_x and g_y as already suggested (Rotilio *et al.*, 1971). The hyperfine splitting constant in the z direction was 130 G. A partially resolved pattern of lines was also observed in the g_y and g_x regions; the average splittings between these lines were found to be approximately 40 and 70 G, respectively.

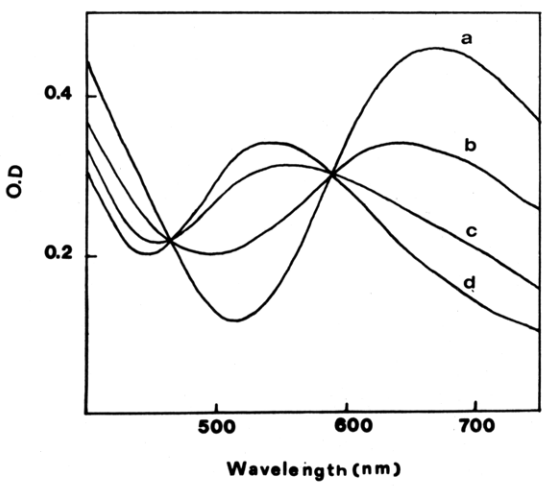

FIGURE 2: Optical spectra between 400 and 700 nm of the bovine enzyme in the presence of increasing amounts of cyanide. Curve a: bovine enzyme (2%) in 0.05 M phosphate buffer, pH 7.2; curve b: NaCN/Cu ratio = 0.5; curve c: NaCN/Cu ratio = 1; curve d: NaCN/Cu ratio = 2.

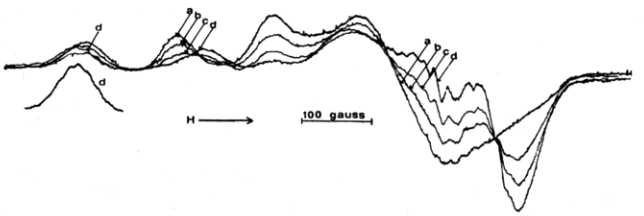

FIGURE 3: Epr spectra at approximately 9 GHz of the bovine enzyme in the presence of increasing amounts of cyanide. The letters indicate the same incubation mixtures as in Figure 2 except for protein concentration. The protein concentration was 1.6%; modulation amplitude, 10 G; microwave power, 20 mW; temperature, −196°. Inset: first hyperfine line of curve d at greater amplification (×4).

Reaction of the Bovine Enzyme with Cyanide. DETERMINATION OF THE AFFINITY CONSTANT AND STOICHIOMETRY. Figure 2 shows the optical spectra of the bovine enzyme in the presence of increasing amounts of cyanide; the broad absorption band at 680 nm decreased and a new band appeared at 550 nm. Two isosbestic points were observed at 590 and 470 nm. A parallel change occurred in the epr spectrum, giving rise to an axial line shape as compared to the rhombic one, characteristic of the native protein (Figure 3).

Two moles of cyanide per mole of protein is required for complete reaction at pH 10.5 (0.05 M carbonate buffer), but higher amounts seem to be necessary when the pH of the buffered solutions is lowered. These data can be rationalized under the hypothesis of independent equilibrium reactions of the two copper sites with one CN^- each, and by taking into account cyanide hydrolysis which becomes very extensive at pH <10, that is by means of the two following equations

$$K = \frac{[CuCN]}{[Cu][CN^-]} \tag{1}$$

$$[CN^-] = [CN^-]_{total} - [CuCN] - [HCN] \tag{2}$$

where [Cu] is the concentration of the free copper sites and [CuCN] that of the sites having bound cyanide. Both can be obtained from visible or epr spectra and from the concentration of the native protein. Introducing the HCN dissociation constant (K_a) the above equations can be rearranged in the form

$$K \Big/ \left(1 + \frac{[H^+]}{K_a}\right) = \frac{[CuCN]}{[Cu]\{[CN^-]_{total} - [CuCN]\}} = K' \text{ (at a given pH)} \tag{3}$$

K' (the "apparent" affinity constant) should increase by one order of magnitude as the pH is increased by one unit, as $[H^+]/K_a \gg 1$, and becomes $\simeq K$ at the higher pH values.

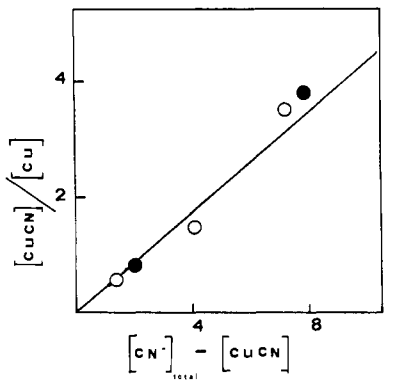

FIGURE 4: The reaction of the bovine enzyme with cyanide. Open circles: values from epr data (experiment of Figure 3). Black circles: values from optical data (experiment of Figure 2).

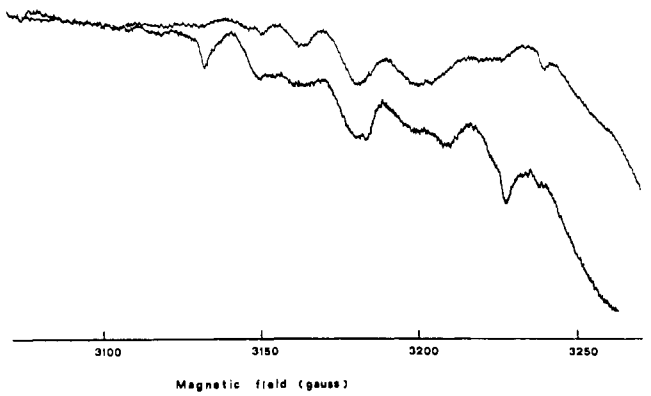

FIGURE 5: Epr spectrum at approximately 9 GHz of the bovine enzyme (2%) in the presence of a fivefold excess of cyanide. The superhyperfine pattern at high field is shown. Upper curve: [^{12}C]-cyanide. Lower curve: [^{13}C]cyanide. Modulation amplitude, 5 G; microwave power, 20 mW; temperature, −170°.

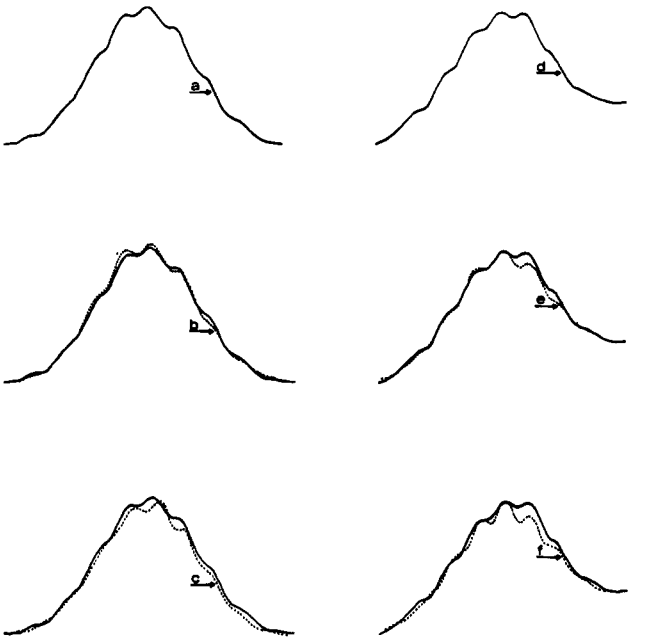

FIGURE 6: Hyperfine line at lowest field of epr spectra at approximately 9 GHz of the bovine enzyme in the presence of cyanide. Continuous lines represent experimental curves, dotted lines the corresponding computed curves superimposed on the experimental spectra. Left: native protein. Right: protein reconstituted with ^{63}Cu from apoprotein. Curve a: bovine enzyme (1.6%) in 0.05 M phosphate buffer, pH 7.2, in the presence of 2×10^{-3} M NaCN; curve b: computed curve to obtain the best fit for the interaction with three nitrogen ligands, assuming a Gaussian line shape and ΔH (line width at half-height) = 0.47 G, A_N (nitrogen hyperfine coupling constant) = 14.1 G, $A_\parallel$ ^{63}Cu = 186 G, and $A_\parallel$ ^{65}Cu = 199 G; curve c: computed curve to obtain the best fit for the interaction with four nitrogen ligands with the same assumption as in b, except for A_N = 12.9 G; curve d: 10^{-2} N NaCN was added to apoprotein (5%) containing 1.5 moles of zinc and 0.5 mole of copper per mole incubated with an excess of ^{63}Cu and then dialyzed; curves e and f: the same as in b and c, taking into account the percentage of the two copper isotopes in the reconstituted sample. Modulation amplitude, 5 G; microwave power, 20 mW; temperature, −160°.

In Figure 4 [CuCN]/[Cu] is plotted *vs.* ([CN]$_{total}$ − [CuCN]) at pH 7.2 (0.05 M phosphate buffer) (Curtis and Curtis, 1966). A straight line is obtained. The angular coefficient gives $K' = 4.4 \times 10^8$, from which an order of magnitude of 10^5–10^6 can be derived for the affinity constant, using pK_a = 9.36 (Curtis and Curtis, 1966). At pH 8 K' is measurable with some difficulty, but appears to be in the range of 10^4. At pH 10.5 ($K' \simeq K$) the "apparent" affinity constant is so high that the reaction seems to proceed stoichiometrically with one mole of cyanide per copper site.

Complete reversibility is observed by dialysis, but the equilibrium can be partially reversed just by leaving the solution at room temperature for 2–3 hr at the lower pH values. Phosphate concentrations were kept low ($\leq$0.05 M), as phosphate interfered in the cyanide reaction. When the [CN$^-$]/[Cu] ratio was increased above 50, copper was slowly reduced, as monitored by the gradual decrease of the epr signal under these conditions.

Epr Spectrum of Cyanide-Treated Bovine Enzyme. A superhyperfine pattern attributable to copper–nitrogen coupling had already been observed (Rotilio *et al.*, 1971) in the $g_\perp$ region of the epr spectrum of the bovine enzyme after exposure to high concentrations of cyanide. A careful inspection of the spectrum, however, even in the presence of a lower amount of cyanide, clearly reveals the presence of a superhyperfine pattern also in the $g_\parallel$ region on the first (lowest in magnetic fields) copper hyperfine line (Figure 3). To rule out the possibility that such hyperfine structure might be due to the nitrogen atom of cyanide itself epr spectra were recorded in the presence of cyanide containing ^{13}C (spin = 0.5). The superhyperfine splitting in the $g_\perp$ region was modified (Figure 5), as expected for a cyanide–copper interaction *via* the cyanide carbon end. Figure 6 shows in detail the first hyperfine line ($g_\parallel$) of cyanide-treated bovine enzyme. Curve a is just a part of the spectrum of Figure 3d: 8 superhyperfine lines can be detected. Curves b and c are the computed lines for the interaction of copper with 3 and 4 nitrogen atoms, respectively, taking into account the natural abundances and the magnetic moments of the two natural isotopes of copper. Curve d is the first hyperfine line of the epr spectrum of a sample of the bovine enzyme almost completely depleted of copper and then reconstituted with ^{63}Cu. Curves e and f are the computed lines for three and four nitrogen atoms, respectively, to simulate the curve d.

The epr spectrum of the cyanide–protein complex was not modified by raising the pH of the solution until pH 12–13. H_2O_2 did not react with copper in this complex as it does in the native protein (Rotilio *et al.*, 1972).

Effect of Azide and Fluoride on the Epr Spectrum of the Bovine Enzyme. Figures 7 and 8 show the 9 GHz epr and

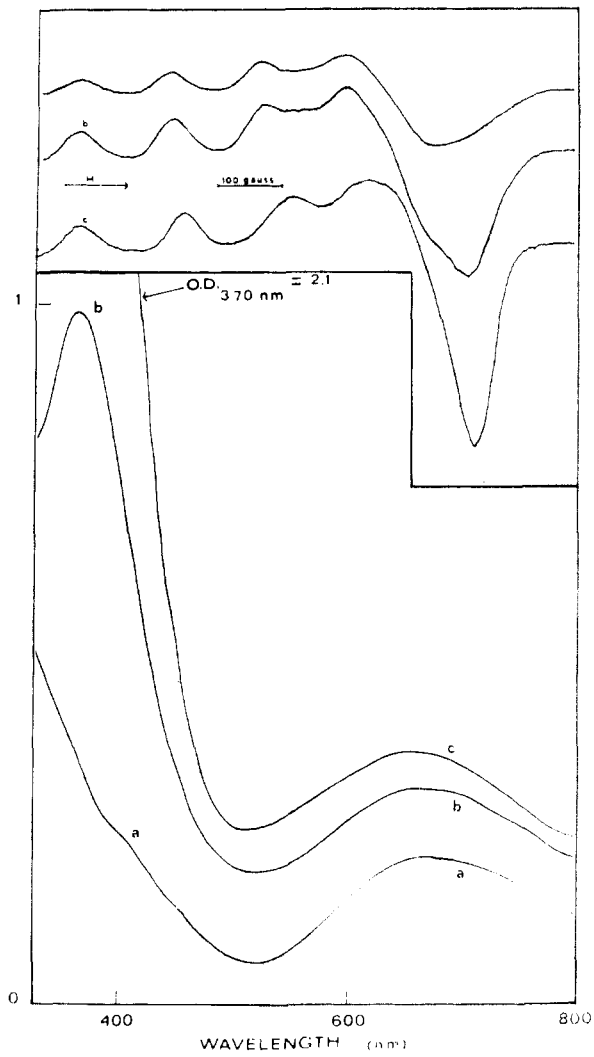

FIGURE 7: The reaction of the bovine enzyme with increasing amounts of N_3^-. Bovine enzyme (1.6% in water, curve a) was reacted with 10^{-3} M (ligand/Cu ratio = 1; curve b), and 1.5×10^{-2} M (ligand/Cu ratio = 15, curve c) sodium azide. Lower part of the figure: optical absorption spectra. Upper inset: epr spectra at approximately 9 GHz (modulation amplitude, 10 G; microwave power, 20 mW; temperature, −150°).

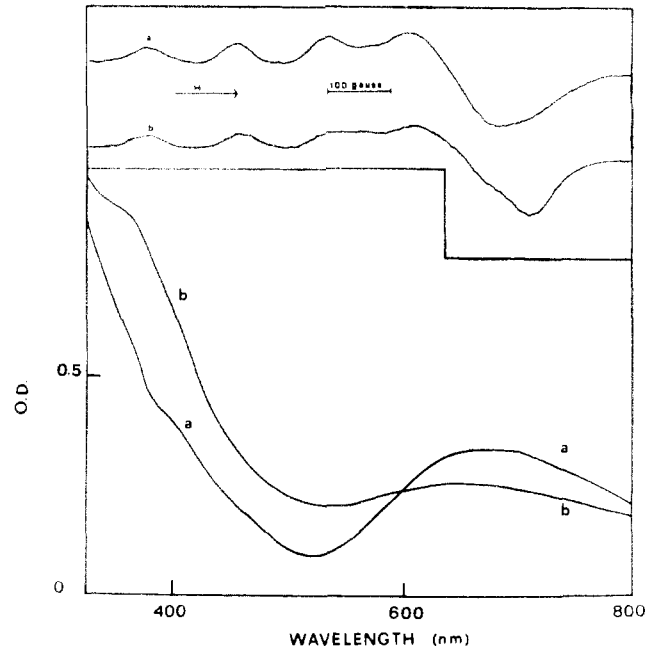

FIGURE 8: The reaction of the bovine enzyme with F^-. The protein (1.6% in water, curve a) was reacted with 1.5×10^{-2} M sodium fluoride (ligand/Cu ratio = 15, curve b). Optical absorption spectra in the lower part of the figure; epr spectra at the same settings as in Figure 7 in the upper inset.

optical spectra of the bovine enzyme in the presence of N_3^- and F^-. The epr spectrum was modified by both anions and patterns attributable to nitrogen hyperfine structure were observed in the $g_\perp$ region of the spectra either in the N_3^- or F^- case. Addition of increasing amounts of N_3^- brought about a shift of the 680-nm band to shorter wavelengths and the appearance of an intense absorption at 370 nm. Similar optical changes were produced by F^-, though a much larger excess was required. All these spectral modifications were reversed by dialysis.

Figure 9 shows the 35-GHz epr spectra of the bovine enzyme in the presence of excess CN^-, N_3^-, and F^-. They clearly indicate that the rhombicity of the untreated protein (Figure 9, d) is removed by anion binding.

Discussion

The epr spectrum at 35 GHz of the bovine enzyme confirms the presence of a rhombic distortion in the copper ligand field which was already suggested on the basis of the spectra at lower frequency (Rotilio *et al.*, 1971). The three g values we have calculated are very close to those obtained by Vänngård (1971)[1] and indicate a high degree of deviation from axial symmetry. This is the fourth case reported of well-defined rhombic symmetry for a copper protein, after stellacyanin (Peisach *et al.*, 1967; Malmström *et al.*, 1970), laccase from *Polyporus versicolor* (Malmström *et al.*, 1968), and dopamine β-hydroxylase (Blumberg *et al.*, 1965). However, the bovine enzyme shows the highest degree of rhombicity. This distortion can be explained by a constraint which is exerted on the ligand atoms of the bovine enzyme copper by the structure of the protein. This is probably also responsible for the optical properties of the protein, which has an extinction coefficient at 680 nm significantly higher than that of low molecular weight complexes of copper. Moreover the energy of the transitions as well as the multiplicity of the CD bands are not the usual ones for a square planar copper complex (Rotilio *et al.*, 1971). The data we have obtained from the reaction of the bovine enzyme copper with some anionic ligands permit a deeper insight into the nature of this distortion.

Our results clearly indicate that cyanide reacts with copper in the ratio of 1 mole of ligand/g-atom of copper. The binding of this molecule of cyanide brings about a drastic modification of the symmetry of the copper site, which becomes more axial, as shown by the optical and epr spectra. The modifications of the $g_\perp$ region of the epr spectrum of the bovine enzyme in the presence of $[^{13}C]CN^-$ (Figure 5) support the view that cyanide binds to copper through its carbon end (Shriver, 1966; Taylor *et al.*, 1970). The appearance of a ^{13}C splitting shows that cyanide coordinates as a planar ligand. For a greatly distorted octahedron, such an interaction with an apical ligand is unlikely (Falk *et al.*, 1970).

[1] Quoted by Fee and Gaber.

Coordination of cyanide *via* its carbon end means that the superhyperfine pattern with spacing of about 15 G present in both the $g_{\parallel}$ and $g_{\perp}$ regions has to be entirely assigned to nitrogen atoms from the protein. In fact, under these conditions, the coupling constant of the cyanide nitrogen would be much smaller than that observed (Hayes, 1967).

In the $g_{\parallel}$ region the number of superhyperfine lines can be related unequivocally to the number of nitrogen ligands, since no overlap from copper hyperfine structure occurs in this region (Rotilio *et al.*, 1971). Eight lines were detected (Figure 6, a) on the first copper hyperfine line in the epr spectrum of the cyanide-treated protein. This even number of superhyperfine lines can only be explained by assuming an overlapping of the contributions from both the natural isotopes of copper, since odd numbers of lines are expected for the interaction of the copper nucleus with magnetically equivalent nitrogen atoms (nuclear spin = 1).[2] Taking into account this fact, we have simulated the first hyperfine line for the interaction with three and four nitrogen nuclei. By superimposing the simulated spectra on the experimental ones, it is easy to see that, keeping the height of the hyperfine line constant, four nitrogen atoms, for every set of nitrogen isotropic coupling constant and line width, always give bands significantly different from the natural ones. On the other hand, the simulated curve for three nitrogens perfectly overlaps the experimental hyperfine line (Figure 6,b). The same result is obtained by comparing the spectrum of ^{63}Cu reconstituted protein (Figure 6,d) with the computed curves for three and four nitrogen atoms (curves e and f). The equivalence of the three nitrogen atoms must only be considered as *magnetic equivalence* and does not imply that the three nitrogens have all the same chemical nature: it is well known that different types of N atoms in a cupric complex can give rise to the same superhyperfine splitting (Falk *et al.*, 1967; Bryce, 1966).

Similar results have been obtained in the presence of N_3^- and F^-, even though the affinity of these ligands is lower than that of CN^-. Direct evidence for binding of these anions to the copper could not be obtained from epr spectra as with CN^-. F^- can give rise to ^{19}F hyperfine splitting. Unfortunately, the low concentration of the modified form (Figure 8) did not allow a detailed analysis of its spectrum. However, all the three anions produced species with axial symmetry and superhyperfine pattern in the epr spectrum and a shift of the visible absorption band to shorter wavelengths. Thus, binding of N_3^- and F^- at the copper site can reasonably be suggested. This view is supported by the appearance, in both cases, of an optical absorption band below 400 nm, which may be assigned to a specific charge transfer from the anion to the cupric copper (Kasper, 1968; Morpurgo and Williams, 1968). With N_3^- this band started to appear already at the 1:1 ratio with respect to copper. This and the subsequent increases on further addition of N_3^- were paralleled by the modifications of the epr spectrum (Figure 7). Since similar changes, that is a shift toward axial symmetry with appearance of a superhyperfine pattern, were observed by raising the pH of the bovine enzyme solutions to 10–11, it can be suggested that they might be due to coordination of OH^- in the plane itself. The CN^- complex is stable even at pH values as high as 12–13 and this may result from competition with OH^- at the same binding position. Also H_2O_2 is without effect on the cyanide-treated protein, thus supporting the hypothesis that the disappearance of the copper signal after addition of H_2O_2 in the absence of oxygen (Rotilio *et al.*, 1972) is mediated by coordination of the peroxide.

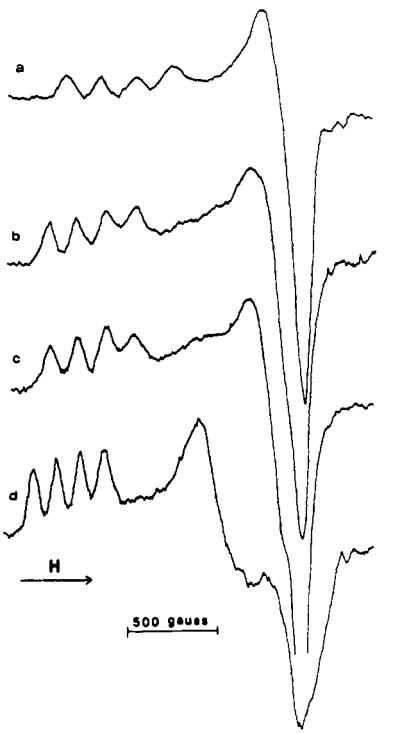

FIGURE 9: Epr spectra at approximately 35 GHz of the bovine enzyme in the presence of different anions. To different samples of a protein solution (5% in water, curve d) sodium cyanide (curve a), sodium azide (curve b), and sodium fluoride (curve c) were added in a ligand/Cu ratio $\simeq$ 30. Instrumental settings as in Figure 1.

If the rhombic symmetry of the bovine enzyme copper can be thought of as a situation resulting from the binding of copper to three nitrogen ligands (strong ligands) and to a fourth weaker ligand, probably not nitrogeneous, which might be water itself, it appears fairly reasonable that anions whose affinity to copper is comparable to that of the strong ligands can replace the fourth one, yielding a complex nearly square planar. Suitable low molecular weight models for such a site may be the Cu(II) complexes with a tetramine macrocyclic ligand described by Cabbiness and Margerum (1969). They exist in two isomeric forms, a red one, with maximal absorbancy at 520 nm (ϵ 140 M^{-1} cm^{-1}), and a blue one, with maximum at 650 nm (ϵ 193 M^{-1} cm^{-1}). The red complex is that with four nitrogen atoms in the square-planar positions while the blue one, which is only found at acid pH values, is believed to have the macrocycle in a folded form. This means three nitrogen atoms in the plane, and it is striking that in this situation these low molecular weight complexes have optical properties similar to those of the bovine enzyme.

Among copper proteins, ceruloplasmin (Kasper, 1968; Curzon, 1966, 1967; Curzon and Speyer, 1968; Andréasson and Vänngård, 1970) and laccase (Malmström *et al.*, 1970; Malkin *et al.*, 1968) have been shown to coordinate anions at the copper site and especially for the type 2 Cu(II) of lac-

[2] The possibility that this pattern of lines may arise from superimposing lines of nonequivalent nitrogen atoms has been excluded on the basis of computer simulations. Moreover, if some of these atoms were nonequivalent, a splitting of approximately 28 gauss would be required to obtain the observed number of lines and such a high value has never been reported for nitrogen hyperfine splitting in copper complexes.

case (Malmström *et al.*, 1970) a great deal of evidence for this has been accumulated. The reaction of laccase with cyanide was extensively studied (Malkin *et al.*, 1968) and shows indeed many similarities to that of the bovine enzyme. It may then not be too farfetched to suggest also a similar functional disposition for both laccase type 2 Cu(II) and copper of the bovine enzyme. In the latter case it is tempting to postulate that this anion binding position is the very site of attachment of the proposed substrate, the superoxide anion radical. The reaction of the bovine enzyme copper with H_2O_2—which is a product of the dismutase reaction—may be taken as a *prima facie* evidence that investigations of this aspect might be revealing.

Acknowledgments

The authors wish to thank Mr. Vincenzo Peresempio for technical assistance. They also thank Dr. W. E. Blumberg for helpful discussion and suggestions.

References

Andréasson, L. E., and Vänngård, T. (1970), *Biochim. Biophys. Acta 200*, 247.
Blumberg, W. E., Goldstein, M., Lauber, E., and Peisach, J. (1965), *Biochim. Biophys. Acta 99*, 187.
Bryce, G. F. (1966), *J. Phys. Chem. 70*, 3459.
Cabbiness, D. K., and Margerum, D. W. (1969), *J. Amer. Chem. Soc. 91*, 6540.
Curtis, N. F., and Curtis, Y. (1966), *Aust. J. Chem. 19*, 609.
Curzon, G. (1966), *Biochem. J. 100*, 295.
Curzon, G. (1967), *Biochem. J. 103*, 289.
Curzon, G., and Speyer, B. E. (1968), *Biochem. J. 109*, 25.
Falk, K. E., Freeman, H. C., Jansson, T., Malmström, B. G., and Vänngård, T. (1967), *J. Amer. Chem. Soc. 89*, 6071.
Falk, K. E., Ivanova, E., Ross, B., and Vänngård, T. (1970), *Inorg. Chem. 9*, 556 (1970).
Hayes, R. G. (1967), *J. Chem. Phys. 47*, 1692.
Kasper, C. B. (1968), *J. Biol. Chem. 243*, 3218.
Malkin, R., Malmström, B. G., and Vänngård, T. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 1*, 50.
Malmström, B. G., Reinhammar, B., and Vänngård, T. (1968), *Biochim. Biophys. Acta 156*, 67.
Malmström, B. G., Reinhammar, B., and Vänngård, T. (1970), *Biochim. Biophys. Acta 205*, 48.
McCord, J. M., and Fridovich, J. (1969), *J. Biol. Chem. 244*, 6049.
Morpurgo, G., and Williams, R. J. P. (1968), *in* Physiology and Biochemistry of Haemocyanins, Ghiretti, F., Ed., New York, N. Y., Academic Press, p 113.
Peisach, J., Levine, W. G., and Blumberg, W. E. (1967), *J. Biol. Chem. 242*, 2847.
Rotilio, G., Calabrese, L., Bossa, F., Barra, D., Finazzi Agrò, A., and Mondovi, B. (1972), *Biochemistry 11*, 2182.
Rotilio, G., Finazzi Agrò, A., Calabrese, L., Bossa, F., Guerrieri, P., and Mondovì, B. (1971), *Biochemistry 10*, 616.
Shriver, O. F. (1966), *in* Structure and Bonding, Vol. 1, Berlin, Springer-Verlag, p 32.
Taylor, J. S., Mushak, P., and Coleman, J. E. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1410.
Vänngård, T. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1409.

Interpretation of Protein Titration Curves. Application to Lysozyme†

Charles Tanford* and Robert Roxby‡

ABSTRACT: A computer method has been devised for the calculation of hydrogen ion titration curves of proteins according to the theory of Tanford and Kirkwood. The theory is moderately successful in accounting for the experimental titration curve of lysozyme, but only if one of the parameters of the theory is assigned a value that differs from the value expected for it on the basis of previous studies. It is also shown that some titratable groups on lysozyme are significantly affected by nonelectrostatic interactions that are not theoretically predictable, so that *ad hoc* pK assignments have to be made for them. One other difficulty arises from the likelihood that some titratable groups of a protein molecule will have different locations in solution and in the crystalline state, so that calculations based on the structure of the crystalline protein will be subject to error. The overall conclusion is that although the major perturbation of the acidic and basic groups of proteins arises from electrostatic interactions between charged sites, the accurate prediction of pK values of individual groups is not feasible.

It is well known that the titration curves of native proteins differ substantially from the sums of the unperturbed titrations of the constituent acidic and basic groups. It has been commonly assumed that the dominant factor responsible for the difference arises from electrostatic interactions between the titratable groups when they are in their charged

† From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. *Received December 17, 1971.* Part of a Ph.D. Thesis submitted by R. R. to the Graduate School of Duke University. Supported by research grants from the National Science Foundation and the National Institutes of Health.

* Research Career awardee, National Institutes of Health.
‡ Supported by a National Aeronautics and Space Administration Predoctoral Fellowship and a U. S. Public Health Service training grant award. Present address: Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Ore. 97331.