Properties of the Cupric Sites in Bovine Superoxide Dismutase Studied by Nuclear-Magnetic-Relaxation Measurements

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Proton nuclear-relaxation rates have been measured as a function of frequency, temperature, pH and cyanide concentration in aqueous solutions of superoxide dismutase from bovine erythrocytes. The results show that, whereas for $\text{pH} \leq 9$ only one water molecule is bound to each Cu^{2+} ion, at higher pH a second co-ordination site for OH^- becomes available; it is proposed that this involves cleavage of the bond between Cu^{2+} and the histidine residue that bridges to Zn^{2+} .

Bovine superoxide dismutase catalyses the dismutation of O_2^- into O_2 and H_2O_2 according to

The catalytic rate for the reaction is close to diffusion-controlled and is independent of pH over the range 5–10 (Rotilio *et al.*, 1972); this suggests that the protons required by the stoichiometry are not derived directly from solvent water. The enzyme is composed of two subunits with identical primary structure, each subunit containing one Zn^{2+} and one Cu^{2+} ion (Fridovich, 1975). Chemical and spectroscopic studies have shown (Fielden *et al.*, 1974) that the copper ions undergo reduction and then re-oxidation during the catalytic cycle: recent studies (Lippard *et al.*, 1977) indicate that Zn^{2+} plays a role in determining the structure at the active site.

The structure of a crystalline form of the enzyme to 0.3 nm resolution has been determined (Richardson *et al.*, 1975) by X-ray diffraction. The results show that the active-site region in each subunit has Cu^{2+} and Zn^{2+} ions separated by a distance of 0.6 nm and linked through a bridging histidine residue. The Zn^{2+} ion is tetrahedrally co-ordinated by three histidine residues (His-61, His-69 and His-78) and Asp-81. His-61 is the bridging ligand to Cu^{2+} to which His-44, His-46 and His-118 are also co-ordinated. The arrangement of the histidine residues around the Cu^{2+} ion is roughly planar, though recent studies (Beem *et al.*, 1977) show that the bridging histidine residue lies slightly out of the plane. Furthermore, one of the co-ordination positions above the plane is unoccupied by a ligand from the protein.

Early proton nuclear-magnetic-relaxation measurements in aqueous solutions of bovine superoxide dismutase (Gaber *et al.*, 1972; Boden *et al.*, 1974) have demonstrated that the Cu^{2+} ions were accessible to solvent water. Gaber *et al.* (1972) interpreted their data as showing that each Cu^{2+} ion had at least one co-ordinated water molecule that exchanges rapidly

(10^6 – 10^8 s^{-1}) with solvent water. Fee & Gaber (1972) demonstrated that the enhancement of water-proton relaxation is eliminated when 2 molar equivalents of cyanide are bound to the enzyme, indicating that there is only one co-ordinated water per Cu^{2+} ion, which occupies the position above the plane of the protein ligands. Terenzi *et al.* (1974) have observed that the proton-relaxation rate increases rapidly with pH value above 9. This behaviour was attributed to deprotonation of the bound water molecules with a concomitant shortening of the Cu^{2+} –H distance. In a corresponding study on aqueous solutions of the copper-containing enzyme benzylamine oxidase, Barker *et al.* (1979) showed that deprotonation of water molecules co-ordinated to Cu^{2+} ion gave a factor-of-2 decrease in the proton-paramagnetic-relaxation rate at pH 8–9. This result implies the absence of any significant change in the Cu^{2+} –H distance and argues against the substantial changes in this distance required by the interpretation proposed by Terenzi *et al.* (1974).

New proton magnetic relaxation measurements on solutions of superoxide dismutase are presented here. The results are interpreted by a model in which an additional co-ordination site for OH^- becomes available at high pH; the H_2O co-ordinated to Cu^{2+} ion at neutral pH is unaffected for $\text{pH} \leq 12.2$. It is proposed that the co-ordination site for OH^- at high pH is a consequence of cleavage of the bond between Cu^{2+} and the bridging histidine residue.

Materials and Methods

All chemicals were of reagent quality and were purchased from either BDH, Poole, Dorset, U.K., or from Hopkin and Williams, Chadwell Heath, Essex, U.K. Sodium azide was further purified as described by Barker *et al.* (1979). Superoxide dismutase was purified from bovine erythrocytes by the method of

McCord & Fridovich (1969) and was judged pure by polyacrylamide-gel electrophoresis, analytical ultracentrifugation and e.s.r. spectroscopy at 35 GHz; further details of the methods used in all these purity determinations are described by Barker *et al.* (1979). Protein concentrations were routinely measured from the absorbance at 680 nm by using an absorption coefficient of $300 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Enzyme activity was assayed as described by Beauchamp & Fridovich (1971).

Samples for nuclear-spin-relaxation measurements were prepared by dialysis of the enzyme against sodium phosphate buffer (50 mM, pH 7.4) for 24 h at 277 K and then vacuum dialysis to decrease the volume of the sample to approx. 0.5 ml. The concentrated solution (protein concentration in the range 0.3–1.0 mM) was transferred to an n.m.r. tube (0.75 cm outer diam.) and deoxygenated by ten cycles of evacuation and then exposure to an Ar atmosphere, after which the tube was sealed.

The ^1H nuclear-spin-relaxation measurements were made at 10, 30 and 60 MHz with a Bruker B-KR 306s pulse spectrometer. The spin-lattice relaxation times, T_1 , were measured by using the standard 90° – τ – 90° pulse sequence, and the transverse relaxation times, T_2 , by the Meiboom–Gill modification of the Carr–Purcell spin-echo experiment. The values of T_2 were independent of pulse spacing over the range 5×10^{-2} – 1×10^{-4} s. The temperature of the sample was controlled by an N_2 gas flow cryostat that was calibrated by using a thermocouple immersed in glycerol in a standard tube. Temperatures are considered accurate to within 1 K.

Results and their Interpretation

1. Spin relaxation in aqueous solutions of the native protein

The proton spin-relaxation measurements in aqueous solutions of the native protein are interpreted by assuming the water molecules undergo fast exchange between bulk solution and the co-ordination spheres of the two Cu^{2+} ions in the protein. The paramagnetic contributions T_{1p}^{-1} and T_{2p}^{-1} due to water in the first co-ordination sphere of the Cu^{2+} ions were obtained from the observed T_1^{-1} and T_2^{-1} by subtracting values of the corresponding rates measured in diamagnetic buffer solutions: contributions from outer-sphere-relaxation effects were estimated and found to be negligible. T_{1p}^{-1} and T_{2p}^{-1} were normalized with respect to the paramagnetic ion concentration by dividing by $[\text{Cu}^{2+}]/55.6$. The resulting values are summarized in Fig. 1.

$(p_m T_{1p})^{-1}$ and $(p_m T_{2p})^{-1}$ have been interpreted by using the Luz–Meiboom (1964) and Swift–Connick (1962) equations, which here reduce to:

$$(p_m T_{1,2p})^{-1} = q/(\tau_m + T_{1,2m}) \quad (1)$$

Fig. 1. Temperature-dependence of the normalized paramagnetic contributions to the water-proton spin-relaxation rates measured in aqueous solutions of superoxide dismutase, pH 7.4

The solid lines were calculated by using eqns. (2) and (3). Symbols: $\blacktriangle$, $1/(p_m T_{1p})$ at 60 MHz; $\blacksquare$, $1/(p_m T_{1p})$ at 30 MHz; $\bullet$, $1/(p_m T_{1p})$ at 10 MHz; $\triangle$, $1/(p_m T_{2p})$ at 60 MHz; $\square$, $1/(p_m T_{2p})$ at 30 MHz; $\circ$, $1/(p_m T_{2p})$ at 10 MHz.

where q is the number of water molecules co-ordinated to each Cu^{2+} ion, τ_m is the mean lifetime of a co-ordinated water molecule and T_{1m} and T_{2m} are the relaxation times of the bound protons. The temperature-dependence observed for both $(p_m T_{1p})^{-1}$ and $(p_m T_{2p})^{-1}$ necessitates $(p_m T_{1,2p})^{-1} = q/T_{1,2m}$. We will assume T_{1m}^{-1} and T_{2m}^{-1} are represented by the Solomon–Bloembergen equations (Solomon, 1955; Bloembergen, 1957):

$$\frac{1}{T_{1m}} = \frac{1}{15} \frac{\mu_{\text{eff}}^2 \gamma_1^2}{r^6} \left(\frac{6\tau_c}{1 + \omega_1^2 \tau_c^2} + \frac{14\tau_c}{1 + \omega_s^2 \tau_c^2} \right) + \frac{1}{3} \frac{S(S+1)A^2}{\hbar^2} \left(\frac{2\tau_e}{1 + \omega_s^2 \tau_e^2} \right) \quad (2)$$

$$\frac{1}{T_{2m}} = \frac{1}{15} \frac{\mu_{\text{eff}}^2 \gamma_1^2}{r^6} \left(4\tau_c + \frac{3\tau_c}{1 + \omega_1^2 \tau_c^2} + \frac{13\tau_c}{1 + \omega_s^2 \tau_c^2} \right) + \frac{1}{3} \frac{S(S+1)A^2}{\hbar^2} \left(\tau_e + \frac{\tau_e}{1 + \omega_s^2 \tau_e^2} \right) \quad (3)$$

ω_1 and ω_s are respectively the Larmor frequencies of the proton and electron, μ_{eff} is the effective electronic magnetic moment of Cu^{2+} , γ_1 is the gyromagnetic

ratio for the proton, r is the ion-proton distance, A is the hyperfine scalar interaction and τ_c and τ_e are the correlation times for respectively the dipolar and scalar interactions. τ_c is given by:

$$\tau_c^{-1} = \tau_r^{-1} + \tau_m^{-1} + \tau_s^{-1} \quad (4)$$

and τ_e by

$$\tau_e^{-1} = \tau_m^{-1} + \tau_s^{-1}$$

where τ_r is the re-orientational correlation time, which together with τ_m , the mean lifetime of a bound water molecule, is assumed to have an Arrhenius temperature-dependence. τ_s may be identified with the electronic spin-lattice-relaxation time in macromolecular solutions (Koenig, 1972). There is, at present, no adequate theory of electron-spin relaxation for a Cu^{2+} ion bound in a macromolecule. In the analysis of the experimental measurements we first attempted to represent the behaviour of τ_s with a model containing contributions from modulation of the anisotropic g tensor and the anisotropic hyperfine coupling interaction (McLachlan, 1964; Luz & Poupko, 1972) as follows:

$$\tau_s^{-1} = (B\omega_s^2 + C) \tau_j / (1 + \omega_s^2 \tau_j) \quad (6)$$

where τ_j is the correlation time for the dynamic process modulating these interactions and B and C are constants whose values are respectively 2×10^{-3} and $4 \times 10^{18} \text{ rad}^2 \cdot \text{s}^{-2}$. An acceptable fit could not, however, be obtained even when the anisotropies in the g tensor and hyperfine interaction were included. Moreover, we also found that eqn. (6) could not account for the frequency-dependence of the more extensive $(p_m T_{1p})^{-1}$ values reported by Gaber *et al.* (1972). Nevertheless, the condition $\tau_s^{-1} \leq \tau_r^{-1}$ must hold because the dynamic process modulating the electron spin interactions is the re-orientational motion of the enzyme molecule; the dynamic Jahn-

Teller effect is not expected to be important for a metal ion in a highly distorted ligand field (Luz & Poupko, 1972). In the analysis of the experimental measurements we were therefore forced to treat τ_s empirically.

Eqns. (2) and (3) were fitted to the $(p_m T_{1p})^{-1}$ and $(p_m T_{2p})^{-1}$ measurements in Fig. 1. The small frequency-dependence of $(p_m T_{1p})^{-1}$ indicated a contribution to τ_c from a frequency-dependent τ_s . It was found necessary to include a scalar contribution to $(p_m T_{2p})^{-1}$, similar to that reported by Luz & Shulman (1965) for Cu^{2+} ions in aqueous solution. The solid lines represent the best fits of the measurements that yielded the values for the various parameters given in Table 1.

The value of $2.65 \times 10^{-9} \text{ s}$ for τ_c is obtained directly from the maximum at 284 K in the $(p_m T_{1p})^{-1}$ measurements at 60 MHz. This value is significantly different from that of $2 \times 10^{-8} \text{ s}$ quoted by both Gaber *et al.* (1972) and Terenzi *et al.* (1974); these authors assumed τ_c was determined by τ_r , which they estimated by assuming the Stokes-Einstein hydrodynamic relation. By using the above value for τ_c in the Solomon-Bloembergen equations, we have been able to calculate the ratio q/r^6 . Setting $q = 1$ as shown by the experiments of Fee & Gaber (1972) and assuming equivalent Cu^{2+} ions gives $r = 0.34 \pm 0.01 \text{ nm}$. The larger value of 0.39 nm obtained by Terenzi *et al.* (1974) with $q = 1$ is a consequence of using an incorrect value for τ_c .

2. Effects of pH

Fig. 2 illustrates how $(p_m T_{1p})^{-1}$ varies with pH at 295 K and 30 MHz. The relaxation rate is approximately constant up to pH 9.3 and then it increases rapidly; at pH 12.2 the enzyme undergoes an irreversible transformation and is inactivated. The

Table 1. Quantities derived from the proton-relaxation measurements on superoxide dismutase at 284 K compared with the properties of co-ordinated water or nitrogen ligands in model complexes

Abbreviations used: Cu^{2+} -dien, copper complex with diethylenetriamine; Cu^{2+} -tren, copper complex with 2,2',2''-triiminotriethylamine. For other abbreviations see the text.

Quantity	Superoxide dismutase		Model copper complex		Tetrahydroxy Cu^{2+}
	Site I	Site II	Cu^{2+} -dien (axial ligand)	Cu^{2+} -tren (equatorial ligand)	
τ_m (s)	1.6×10^{-7}	1.0×10^{-5}	$1.0 \times 10^{-8*}$	$0.5 \times 10^{-5\dagger}$	$10^{-5\dagger}$
r (nm)	0.34	0.27	0.237§	0.196	—
q	1	$\frac{1}{2}$	1	1	$\frac{1}{2}$
τ_c (s)	2.7×10^{-9} (at 60 MHz)	2.7×10^{-9} (at 60 MHz)	—	—	—
A/h (Hz)	$(19.5 \pm 1.0) \times 10^5$	—	—	—	—

* Eigen (1963).

† Cayley *et al.* (1976).

‡ Lin *et al.* (1975).

§ Copper-axial nitrogen distance in Cu^{2+} (dien)(penta-acetate) (Seccombe *et al.*, 1975).

|| Copper-NCS distance in Cu^{2+} (tren)(thiocyanate) (Jain & Lingafelter, 1967).

profiles of the frequency-dispersion curves (Fig. 3) for $p_m T_{1p}$ are the same for pH 7.5 and 11.5, showing that τ_c is pH-independent; this conclusion obtains from eqn. (2), which predicts $dT_1/d\omega^2 \propto \tau_c$. The pH-dependence of $(p_m T_{1p})^{-1}$ cannot, therefore, result from a change in τ_c .

Terenzi *et al.* (1974) obtained similar results to those in Fig. 2, but interpreted them in terms of deprotonation of the axially co-ordinated water molecule with a concomitant shortening of the Cu^{2+} -H distance. Assuming that this interpretation is correct and taking $q = \frac{1}{2}$ (OH^- ion) and $\tau_c = 1.80 \times 10^{-9}$ s at 295K and 30MHz, we obtain from $(p_m T_{1p})^{-1}$ at pH 12.2 the value 0.26nm for r . This corresponds to a shortening of the Cu^{2+} -H distance

Fig. 2. Paramagnetic contributions to the water-proton spin-lattice-relaxation rates in aqueous solutions of superoxide dismutase as a function of pH at 30 MHz and 295 K

Fig. 3. Paramagnetic contributions to the water spin-lattice-relaxation rates in aqueous solutions of superoxide dismutase at pH 7.5 (●) and pH 11.5 (▲) plotted as a function of the square of the resonance frequency

by 0.08 nm, which seems unreasonably large (Kennard *et al.*, 1972). The behaviour observed for another copper-containing protein, benzylamine oxidase (Barker *et al.*, 1979), where $(p_m T_{1p})^{-1}$ undergoes a stoichiometric decrease by a factor of 2 at pH 8–9 due to deprotonation of water molecules co-ordinated to the copper, is also inconsistent with the hypothesis of Terenzi *et al.* (1974). An alternative explanation for the pH-dependence of $(p_m T_{1p})^{-1}$ considered here is that at high pH OH^- displaces one of the histidine ligands from the Cu^{2+} , whereas the water molecule bound at lower pH remains unaffected.

Designating the water co-ordinated at pH 9.3 as I and the OH^- site at pH 9.3 as II, eqn. (1) becomes:

$$(p_m T_{1p})^{-1} = q^I/(\tau_m^I + T_{1m}^I) + q^{II}/(\tau_m^{II} + T_{1m}^{II}) \quad (7)$$

Assuming that pH has little effect on site I:

$$(p_m T_{1p})^{-1} - (p_m T_{1p})^{-1}_{\text{pH } 9.3} = (p_m T_{1p}^{II})^{-1} = q^{II}/(\tau_m^{II} + T_{1m}^{II})$$

In Fig. 4 the value for $(p_m T_{1p}^{II})^{-1}$ at pH 12.2 is plotted against reciprocal temperature; at this pH

Fig. 4. Temperature-dependence of the paramagnetic contributions to the water-proton spin-lattice-relaxation rates measured at 30 MHz in aqueous solutions of superoxide dismutase

▲, pH 9.3; ●, pH 12.2; ○, difference between the curves at pH values 9.3 and 12.2.

site II is assumed to be fully open. The curve has a maximum at 305 K corresponding to $\tau_m^{\text{II}} = T_{1p}^{\text{II}}$ and yields the value 7×10^{-6} s for $\tau_m^{\text{II}}/q^{\text{II}}$; setting $q^{\text{II}} = \frac{1}{2}$ (OH^- ion) gives a value of 1.4×10^{-5} s for τ_m^{II} . By using 1.40×10^{-9} s for τ_c at 305 K, we obtain from $T_{1p}^{\text{II}}/q^{\text{II}}$ a value for r of 0.27 nm; in this calculation it has been assumed that τ_c is the same for both sites since τ_c is dominated by τ_r .

Fig. 5 demonstrates that there is a linear dependence of $(p_m T_{1p})^{-1}$ on $[\text{H}^+]^{-1}$. This behaviour is consistent with the proposed model and yields a value of 53 M^{-1} for the equilibrium constant of the above ligand-displacement reaction.

3. Binding of cyanide

Fee & Gaber (1972) have shown that the addition of two molar equivalents of CN^- to the enzyme at pH 7.4 decreases $(p_m T_{1p})^{-1}$ to near zero and concluded that one water molecule is bound to each Cu^{2+} ion. From their measurements, we have calculated a value of 0.02 mM for the dissociation constant, K_D , of the Cu^{2+} - CN^- complex. Similar experiments with CN^- have been carried out at pH 12.2; the results are summarized in Fig. 6 and show that, as at pH 7.4, $(p_m T_{1p})^{-1} \rightarrow 0$ as $[\text{CN}^-] \rightarrow \infty$, which indicates that CN^- displaces all exchangeable H_2O and OH^- ligands. However, the binding of CN^- at pH 12.2 is weaker and this prevents determination of the binding stoichiometry. We have attempted to fit these measurements assuming sequential binding of CN^- at the H_2O and OH^- co-ordination sites. The fit is represented by the solid line in Fig. 6 giving values for the two dissociation constants of 0.20 mM and 1.4 mM. This provides further support for our proposal of a new OH^- -binding site available at high pH. It should be noted, however, that a reasonable fit to the measurements can also be obtained by assuming

Fig. 5. Spin-lattice-relaxation rate from water molecules bound at site II plotted as a function of $1/[\text{H}^+]$

Fig. 6. Attenuation of the water-proton spin-lattice-relaxation rate T_{1p}/T_{1p}^* (where the asterisks denote the presence of added salt) as a function of $1/[\text{KCN}]$ at pH 12.2. The broken line represents the best fit to single-site binding ($K_D = 0.7 \text{ mM}$) by using the expression:

$$K_D = [\text{CN}^-]_{\text{free}}\{[\text{Cu}]_{\text{total}} - [\text{CN}^-]_{\text{bound}}\}/[\text{CN}^-]_{\text{bound}}$$

where

$$T_{1p}/T_{1p}^* = \frac{[\text{CN}^-]_{\text{bound}}(\varepsilon_b - 1)}{[\text{Cu}]_{\text{total}}} + 1$$

with ε_b being the value of T_{1p}/T_{1p}^* at infinite $[\text{KCN}]$; the value of ε_b has here been taken to be zero. The solid line represents the best fit to sequential binding at two sites ($K_{D1} = 0.20 \text{ mM}$, $K_{D2} = 1.4 \text{ mM}$) by using the following expressions:

$$K_{D1} = [\text{CN}^-]_{\text{free}}\{[\text{Cu}]_{\text{total}} - [\text{CN}^-]_{\text{bound}}^{\text{I}}\}/[\text{CN}^-]_{\text{bound}}^{\text{I}}$$

$$K_{D2} = [\text{CN}^-]_{\text{free}}\{[\text{CN}^-]_{\text{bound}}^{\text{I}} - [\text{CN}^-]_{\text{bound}}^{\text{II}}\}/[\text{CN}^-]_{\text{bound}}^{\text{II}}$$

where

$$T_{1p}/T_{1p}^* = \frac{[\text{CN}^-]_{\text{bound}}^{\text{I}}(\varepsilon_b^{\text{I}} - 1) - \varepsilon_b^{\text{I}}[\text{CN}^-]_{\text{bound}}^{\text{II}}}{[\text{Cu}]_{\text{total}}} + 1$$

ε^{I} is the value of T_{1p}/T_{1p}^* for site I at infinite cyanide concentration and $[\text{CN}^-]_{\text{bound}}^{\text{I}}$ denotes cyanide bound at sites I and II respectively.

a single dissociation constant of 0.7 mM (see broken line in Fig. 6). The cyanide-binding data at high pH should not be regarded, therefore, as strong evidence for the model.

Discussion

The results presented are consistent with the hypothesis that at higher pH there is a co-ordination site (termed site II) for OH^- ions additional to that for H_2O (termed site I). The properties of these two sites are compared in Table 1 with those for H_2O

co-ordinated to Cu^{2+} in well-characterized model complexes. It can be seen that the values of τ_m and r for site I are consistent with those for axial water, whereas the values for site II correspond to those for equatorial H_2O or OH^- . The pK obtained for site I is ≥ 12.2 , which is higher than the value calculated for axial water in the copper-triethylenetriamine complex (Paoletti *et al.*, 1973). This high pK value might be significant for the role of copper-co-ordinated water in the catalytic mechanism of the enzyme (Khan, 1977).

The magnitudes of the binding constants for N_3^- and CN^- at pH 7.4 (Fee & Gaber, 1972) of 100M^{-1} and $5 \times 10^4\text{M}$ respectively are not, however, in keeping with the axial nature of water molecules at site I. Displacement of H_2O co-ordinated axially to Cu^{2+} is extremely difficult; thus, for NH_3 binding to $\text{Cu}(\text{H}_2\text{O})_6^{2+}$, the values of $\log K$ are positive for the equatorial water molecules, but negative for the axial water molecules (Cotton & Wilkinson, 1966). In fact, the association constant for N_3^- binding to the enzyme (10^2M^{-1}) is similar to the value of $2.3 \times 10^2\text{M}^{-1}$ for N_3^- binding at the equatorial positions of $\text{Cu}(\text{H}_2\text{O})_6^{2+}$ (Saini & Ostacoli, 1958). Thus the N_3^- and CN^- binding data suggest, unexpectedly, that a Jahn-Teller rearrangement has occurred where the initial attack of the ligand at the axial position is followed by a rapid rearrangement that leaves the ligand at an equatorial position (Moss *et al.*, 1973). Such a rearrangement would require ligand fluxionality at the Cu^{2+} site of superoxide dismutase, which again might well be an important feature of the catalytic mechanism. It is interesting to speculate that binding of N_3^- or CN^- might result in the bridging histidine residue being moved to a position where it is co-planar with the other histidine residues co-ordinated to the Cu^{2+} (Beem *et al.*, 1977). This could explain the change in the e.s.r. spectrum from rhombic to axial symmetry on binding N_3^- or CN^- (Rotilio *et al.*, 1972), and the structural change might also explain the mode of action of these inhibitors. It should be noted that our proposal on the ligand fluxionality of the active site in the enzyme is not in conflict with structural information from X-ray diffraction (Richardson *et al.*, 1975) or high-resolution n.m.r. data (Cass *et al.*, 1977).

Our interpretation of the relaxation measurements at high pH is quite different from that of Terenzi *et al.* (1974), who proposed that increase in pH causes deprotonation of a single axially bound water molecule. Terenzi *et al.* (1974) did consider the possibility that an increase in the relaxation rate at high pH arose from an increase in the number of bound water molecules, though they rejected this explanation on the grounds that c.d. and electron-spin-relaxation studies indicated no major structural change up to a pH of 12. The 35 GHz e.s.r. studies by Rotilio *et al.* (1972) do, however, show that a structural change

(from rhombic to axial symmetry) at the Cu^{2+} site occurs with increase in pH. Thus the e.s.r. evidence supports our proposal that the opening up of a new co-ordination site for OH^- as the pH is raised involves a structural change at the Cu^{2+} , which we suggest to be cleavage of the bond between Cu^{2+} and the bridging histidine residue. Cleavage of this bond would offer an explanation for the observations by Rotilio *et al.* (1972) that the catalytic activity of superoxide dismutase falls off rapidly at $\text{pH} > 10$. The equatorial nature of site II implied by our results suggests that OH^- rather than H_2O must be co-ordinated, since the pK for equatorial H_2O in model Cu^{2+} complexes is approx. 9 (Brookes & Pettit, 1975). The values for the dissociation constants (K_D) of the enzyme-cyanide complex at pH 12.2 are consistent with equatorial co-ordination. It is interesting that both the K_D values (0.2 and 1.4 mM) imply equatorial co-ordination, whereas it might have been anticipated that one of these values would have been characteristic of axial co-ordination; this result must again be a consequence of Jahn-Teller rearrangements.

Finally, we compare the results obtained in the present paper for superoxide dismutase with those obtained for benzylamine oxidase (Barker *et al.*, 1979). The behaviour of the Cu^{2+} sites in these two enzymes is clearly distinct. First, both axially and equatorially co-ordinated $\text{H}_2\text{O}/\text{OH}^-$ can be displaced by azide and cyanide in superoxide dismutase, whereas for benzylamine oxidase only the equatorially co-ordinated water molecule can be displaced. This behaviour is consistent with the proposal that the Cu^{2+} sites in superoxide dismutase are fluxional, whereas in benzylamine oxidase the axial and equatorial disposition of water ligands is rigidly maintained. Secondly, the pK of the axial water molecule in superoxide dismutase appears to be anomalously high (≥ 12.2) relative to model Cu^{2+} complexes, whereas in benzylamine oxidase the pK of the axial water molecule is low (approx. 8.5). These different properties must relate to the catalytic role of Cu^{2+} in the two enzymes.

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