mL of 85% aqueous hydrazine hydrate solution (0.054 mol), and 50 mL of methanol was refluxed for 1 h and cooled and 50 mL of water added. The methanol was removed under reduced pressure, and 50 mL of hydrochloric acid (concentrated) was added to the remaining aqueous solution. The mixture was refluxed for 1 h, cooled, and filtered. The filtrate was evaporated to dryness. Water was added, and the solution was extracted with chloroform (3 × 30 mL). The pH was raised to ~7 (10% NaOH), and the solution was extracted with chloroform. The aqueous solution was evaporated, 100 mL of ethanol added, and the solution again evaporated. This process was repeated three times. Finally, ethanol was added and the solution refluxed for 15 min and filtered to remove NaCl. The filtrate was dried ($MgSO_4$), evaporated, and dried in vacuo to yield a glassy yellow hygroscopic solid (9.6 g, 91%): NMR (D_2O, DSS) δ 3.3 (m, 8, $H_2N(CH_2)_2N^+(CH_3)_2(CH_2)_2N(CH_3)_2$), 3.20 (s, 6, $N^+(CH_3)_2$), 2.35 (s, 6, $N(CH_3)_2$). The butyl and hexyl derivatives were prepared in a similar manner, with similar yields. **5**, n = 4: NMR (D_2O, DSS) δ 3.4 (m, 8, $H_2NCH_2(CH_2)_2CH_2N^+(CH_3)_2CH_2CH_2N(CH_3)_2$), 3.15 (s, 6, $N^+(CH_3)_2$), 2.37 (s, 6, $N(CH_3)_2$), 1.83 (m, 4, $H_2NCH_2(CH_2)_2$). **5**, n = 6: NMR (D_2O) δ 3.2 (m, 8, $H_2NCH_2(CH_2)_4CH_2N^+(CH_3)_2CH_2CH_2N(CH_3)_2$), 3.06 (s, 6, $N^+(CH_3)_2$), 2.32 (s, 6, $N(CH_3)_2$), 1.5 (m, 8, $H_2NCH_2(CH_2)_4$).

***N,N,N'*-Trimethyl-*N'*-(2-aminoethyl)ethylenediamine, L_2, by Demethylation of 5, n = 2.** To (2-(dimethylamino)ethyl)(2-aminoethyl)dimethylammonium chloride (**5**, n = 2) (7.0 g, 0.036 mol) in 150 mL of ethanol (100%) was added a solution of 9.5 g (0.072 mol) of sodium thiophenoxide in 20 mL of ethanol and the mixture was stirred for 20 min, filtered, and washed with ethanol. The filtrate was evaporated in vacuo, and 400 mL of 2-butanone (freshly distilled from zinc dust) was added to the residue. The mixture was refluxed under nitrogen for 72 h. Following removal of the solvent, 40 mL of water and 50 mL of chloroform were added, the chloroform layer was removed, and the aqueous layer was extracted three times with chloroform (3 × 30 mL). The chloroform fractions were combined and evaporated. After 40 mL of 10% hydrochloric acid was added to the residue, the solution was repeatedly extracted with ether. The aqueous layer was made basic (pH >11, 10% NaOH), extracted with ether (3 × 30 mL), and then extracted with chloroform. The chloroform extracts were dried (Na_2SO_4) and evaporated to yield a brown oil which was distilled to give 2.4 g (46%) of L_2 as a colorless oil. The properties of this compound were identical with the triamine isolated from the *N,N,N'*-trimethylethylenediamine route. In some cases the oil contained contaminants which could not be removed upon distillation. The oil can be purified by using a silicagel column. The butyl (L_4) and hexyl (L_6) derivatives were prepared in a similar manner to yield 59 and 67% of the alkyl triamines.

Transamination of d-TpC with L_6. The alkyl triamine, L_6 (0.42 g, 0.002 mol), was dissolved in 0.5 mL of water and the pH adjusted to between 8.5 and 9.0 with hydrochloric acid (concentrated). Sodium bisulfite (0.0012 mol) was added and the mixture shaken vigorously until most of the sodium bisulfite was dissolved, adding water if necessary. The pH was carefully adjusted to between 7.0 and 7.2 and the dinucleoside monophosphate (15 mg, 0.027 mmol) dissolved. The final volume of the solution was 1.4 mL. Nitrogen was bubbled through the solution for 1 min. The vial was sealed and incubated at 38–40 °C.

Ten microliters of solution was removed periodically to monitor the progress of the reaction, using Sephadex gel chromatography or electrophoresis. The reaction was terminated after 8–10 days.

The contents of the vial was rinsed into a small separatory funnel. The pH was adjusted (>11) with 10% sodium hydroxide (2.5 mL) and the mixture then extracted with chloroform (3 × 15 mL) to remove unreacted amine. One molar barium chloride solution (0.0012 mol) was added and the aqueous mixture centrifuged. The aqueous layer was filtered and concentrated to 1–2 mL at 35–40 °C under reduced pressure. The solution was placed on a Sephadex G-10 column (75 × 2.5 cm) and eluted with water. The major ultraviolet absorbing peak was collected, concentrated, and purified by preparative cellulose TLC (solvent B). The major ultraviolet absorbing band (R_f 0.80), dTpC–L_6, was isolated by elution with water. The product was concentrated and lyophilized to yield 0.0186 mmol (68%) of a white solid as judged by ultraviolet spectroscopy using $\lambda_{max}^{pH\ 7.0}$ = 272 nm (ϵ = 21 500). Transaminations using L_2 and L_4 were carried out by similar procedures.

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2. Binding Sites of Anions in Superoxide Dismutase[29]

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Contribution from the Istituto di Chimica Generale ed Inorganica, Facoltà di Farmacia, Università degli Studi di Firenze, and the Istituto per lo Studio della Stereochimica ed Energetica dei Composti di Coordinazione del C. N. R., Firenze, Italy. *Received March 20, 1981*

Abstract: The electronic absorption spectra in the range (10–25) × 10^3 cm^{-1} and the ESR spectra have been carefully remeasured for copper–zinc superoxide dismutase in the presence of increasing amounts of NCO^-, N_3^-, and F^-. The results have been compared with those obtained by multinuclear NMR spectroscopy regarding ^1H of the water solvent, ^{13}C of $N^{13}CO^-$, ^{19}F, and ^{14}N and ^{15}N of NCO^-, NCS^- and N_3^-. A model is proposed, which takes into account also the spectroscopic behavior of CN^- and NCS^-. Within this frame, the ligands bind the enzyme at the copper site in a 1:1 ratio, substituting an equatorial histidine nitrogen. The CN^- derivative is square planar, whereas the NCO^-, NCS^-, and F^- derivatives are five-coordinate, with an apical water molecule. Azide may behave like CN^-, although the possibility of a copper(II)–inihibitor (1:2) is also taken into consideration.

Bovine erithrocyte superoxide dismutase is a dimeric metalloenzyme containing a zinc(II) and a copper(II) ion in each subunit;[1-5] its biological role is to prevent the accumulation of the toxic O_2^- ion in tissues,[6] by catalyzing the reaction

$$2O_2^- + 2H^+ \rightleftarrows H_2O_2 + O_2$$

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The copper(II) ion is essential to the enzymatic activity, being reduced and reoxidized during the catalytic cycle.[7,8] Much is known about the chemical behavior of the apoenzyme with the various metal ions, as well as about the stability constants of the resulting metal derivatives.[9-18] On the contrary, the interaction of the inhibitors with the enzyme is still rather controversial.

The main source of information on inhibitor binding is the copper(II) chromophore, which has been the target of extensive spectroscopic studies. At the beginning of the last decade the electronic and ESR spectra of superoxide dismutase have been reported, together with those of the enzyme adducts with the inhibitors azide and cyanide.[19,20] The superhyperfine splitting of the ESR signals of the latter adduct showed the presence of at least three nitrogen donors in the copper(II) coordination sphere,[20] while the general shape of the ESR and electronic spectra of the pure enzyme could indicate a flattened tetrahedral or a distorted square-pyramidal geometry.[20-22] The cyanide adduct could be safely assigned as square planar[20] on the grounds of the high energy of the d-d transition and of the large $A_{\parallel}$ value observed in the ESR spectrum, while the spectral changes induced by the azide ion were less dramatic.[19-21]

^1H T_1^{-1} measurements on water protons in superoxide dismutase solutions showed the presence of a water molecule in the copper(II) coordination sphere.[19,23,24] Fee and Gaber[19] found that the paramagnetic effect was reduced by addition of cyanide or azide, suggesting displacement of water from the coordination sphere of the metal; the same authors also noted that cyanate and thiocyanate, as well as azide in low concentration, were able to affect the ESR spectra of the enzyme without apparently perturbing the coordinated water molecule. They proposed binding of the above anions to the zinc(II) center, reflected in the copper(II) chromophore by some structural changes of the protein backbone.

In 1975 the X-ray structure of superoxide dismutase was completed at 3-Å resolution;[25,26] it was apparent that the copper and zinc centers in each subunit were only about 6-Å apart, bridged by an imidazolate ion from His-61. Zinc(II) was found to be tetracoordinated and buried into the protein, while copper(II) is coordinated to three more histidine nitrogens, besides to His 61, in a distorted square-planar geometry. In particular, His 61 is somewhat above the plane identified by the copper atom and the remaining three nitrogens, on the same side which is exposed to the solvent.[27] The coordination sphere is completed by a water molecule.

Although the X-ray structure pointed out the close proximity of the zinc(II) and copper(II) ions, metal substitution experiments have definitely shown that the zinc center is not accessible to solvent or solute molecules;[28] the early proposal of Fee and Gaber of a different anion-binding site has been consequently overlooked.

Recently, we have shown that the thiocyanate ion is capable of binding the copper(II) ion without removing the coordinated water molecule and of maintaining a five-coordinate chromophore.[29]

In an attempt to rationalize the binding properties of anions toward superoxide dismutase, we have reinvestigated the behavior of azide, cyanate, and fluoride ions by means of ESR, electronic spectroscopy, and ^1H, ^{13}C, ^{14}N, ^{15}N, and ^{19}F NMR. For comparison purposes some data are reported on the (1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane)copper(II) perchlorate complex, which in water has a square-pyramidal $CuN_4(H_2O)^{2+}$ chromophore.[30]

Experimental Section

Ninety percent ^{13}C-enriched potassium cyanate and 97% ^{15}N-enriched sodium azide were purchased from Prochem B.O.C.; all the other chemicals were analytical grade. All the solutions were made from freshly bidistilled water. (1,4,8,11-Tetramethyl-1,4,8,11-tetraazacyclotetradecane)copper(II) perchlorate ($(Cu(Me_4(14)aneN_4)(ClO_4)_2)$) was prepared as previously reported[30] and satisfactorily analyzed for C, H, and N.

Bovine erithrocyte superoxide dismutase was obtained as a lyophylized powder from Sigma and purified and checked as previously reported.[29] Enzyme concentrations were calculated from the intensity of the copper d-d transition (ϵ_{680} = 300 M^{-1} cm^{-1}/dimeric unit[6]); all the experiments were performed in unbuffered solutions at a pH of around 8. The pH of each sample was checked by using a microelectrode.

The electronic spectra were recorded on a Cary 17D spectrophotometer in the absorbance range 0-0.1; X-band ESR spectra were recorded on a Bruker 200 TT spectrometer at both room and liquid-nitrogen temperature. The 80-MHz ^1H and 20-MHz ^{13}C NMR spectra were run on a Varian CFT 20 spectrometer; the 4.3-MHz ^{14}N, 6.1-MHz ^{15}N, and 56-MHz ^{19}F NMR spectra were recorded on a Bruker CXP 100 spectrometer equipped with a 1.4-T Varian DA 60 magnet. The measurements were performed at room temperature, unless otherwise specified.

Longitudinal relaxation times, T_1, were measured with the inversion recovery method using an appropriate nonlinear least-squares fitting program; transverse relaxation times, T_2, were obtained from the line width at half-peak height, properly reduced for the line broadening introduced by exponential multiplication of the free induction decay, through the relation $T_2^{-1} = \pi\Delta\nu$.

Affinity constants of some anions were measured through least-squares fitting of both the room-temperature ESR spectra and the water proton relaxation data obtained at various anion concentrations. In the ESR measurements care was taken to keep constant the overall signal intensity throughout the experiment. The affinity constant of fluoride was measured through a similar treatment of the ^{19}F NMR data. In every case the binding constants are well-defined and reproducible under different experimental conditions.

Methods

Copper(II) is a d^9 ion with a single unpaired electron; its relaxation rate is typically about 10^9 s^{-1}.[22,31] It generally gives rise to well-resolved ESR spectra whose parameters are now well rationalized in terms of their dependence on the geometry of the chromophore.[22,32,33] Its electronic relaxation rates are such as to cause dramatic nuclear relaxation rate enhancements on nuclei close to the metal itself. Such enhancements are due both to dipolar coupling mechanisms which are among the most efficient within 3d metal complexes and to contact contributions due to spin delocalization which are also very effective. Therefore protons of the coordinated water display a quite large T_1^{-1} enhancement with respect to pure water, and the effect is still sizeable under rapid exchange conditions, which decrease the T_1^{-1} value by a

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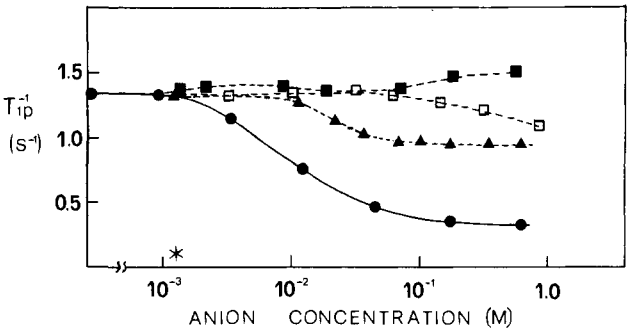

Figure 1. Water proton T_{1p}^{-1} values for 6×10^{-4} M superoxide dismutase solutions as a function of anion concentration: ■, F^-; ●, N_3^-; ▲, NCO^-; □, NCS^-; *, 1:1 CN^-/Cu ratio.

Table I. ESR and Electronic Parameters of Superoxide Dismutase and Its Inhibitor Derivatives

	$g_\parallel$	$g_\perp$[a]	$10^4 A_\parallel$, cm^{-1}	$10^{-3}\nu$, cm^{-1}
pure enzyme	2.26	2.07	143	14.7
NCS^-	2.25	2.06	148	14.7
NCO^-	2.26	2.05	158	15.1
N_3^-	2.24	2.04	157	15.1
F^-	2.26	2.07	143	14.7
CN^-[b]	2.21		188	19.3

[a] The spectra are assumed to be axial. [b] From ref 11 and 19.

factor equal to the molar fraction of the bound water.[24,34,35] When the anion removes the water molecule from the coordination sphere, the T_1^{-1} values of water solutions containing superoxide dismutase decrease in such a way that it is possible to follow the substitution and to determine the affinity constant for that binding site. Also the NMR signals of nuclei of the ligand show dramatic T_1^{-1} and T_2^{-1} enhancements upon binding to the paramagnetic center. In principle, by following the T_1^{-1} or T_2^{-1} enhancements with concentration, it is possible to independently measure the affinity of the anion for the metal ion. This method was successful in the case of the thiocyanate ligand.[29]

The electronic and ESR spectra in solution can also be used to determine the affinity constants of anions.

It should be noted that the affinity constants from activity measurements are somewhat more ambiguous, since they may reflect binding to copper(I) which is formed during the catalytic cycle.[36]

Results

By addition of cyanate to water solutions of superoxide dismutase the ^1H T_1^{-1} values of water are only slightly affected (Figure 1), as previously found,[19] indicating that the ligand does not remove water from the coordination sphere of the paramagnetic ion. Nevertheless, the electronic spectra show an ipsochromic shift,[21] the maximum shifting from 680 to 660 nm, and the ESR spectra, as already observed,[19] become more axial (Figure 2 and Table I).

From a best fitting procedure of the room-temperature ESR spectra the affinity constant of cyanate was estimated to be 42 ± 2 M^{-1}. This value is consistent with the observed variations of the electronic spectra, although the latter were too small for a computer treatment to be attempted. The slight change observed in the ^1H T_1^{-1} values of water also matches the above value (Figure 1).

The final proof of binding to the copper center is, however, given by the ^{13}C and ^{14}N NMR spectra. The ^{13}C NMR spectra have been recorded at ligand concentrations ranging from 7×10^{-2} to 5×10^{-1} M, the protein concentration being 9×10^{-5} M. The spectrum of the pure ligand consists of a triplet arising from the coupling with the ^{14}N nucleus; in the presence of superoxide dismutase no isotropic shift is observed, while the triplet collapses in a single signal over the entire concentration range investigated and broadens with decreasing ligand/protein ratio owing to the coupling between the unpaired electron and the resonating nucleus. Variable-temperature measurements at cyanate concentration of 7.7×10^{-2} and 3.2×10^{-1} M have shown that the line width increases with increasing temperature, indicating that the transverse relaxation is dominated by the ligand exchange time, τ_M.[37] The values of $(fT_{1p})^{-1}$ and $(fT_{2p})^{-1}$, where f is the molar fraction of the bound ligand for a solution of 7×10^{-2} M cyanate and 9×10^{-5} M enzyme, are reported in Table II. These values, in absence of measured isotropic shift, are related to T_{1M} and T_{2M}, which are paramagnetic contributions to the nuclear relaxation times of ^{13}C in the bound ligand, through eq 1.[34,35,37]

$$fT_{ip} = T_{iM} + \tau_M \quad (1)$$

In the present case $(fT_{2p})^{-1}$ represents the lower limit for T_{2M}^{-1}, since τ_M is the dominant term in 1. The large value of $(fT_{2p})^{-1}$ is by itself strongly indicative of substantial binding already at 7×10^{-2} M cyanate concentration, although an estimate of the

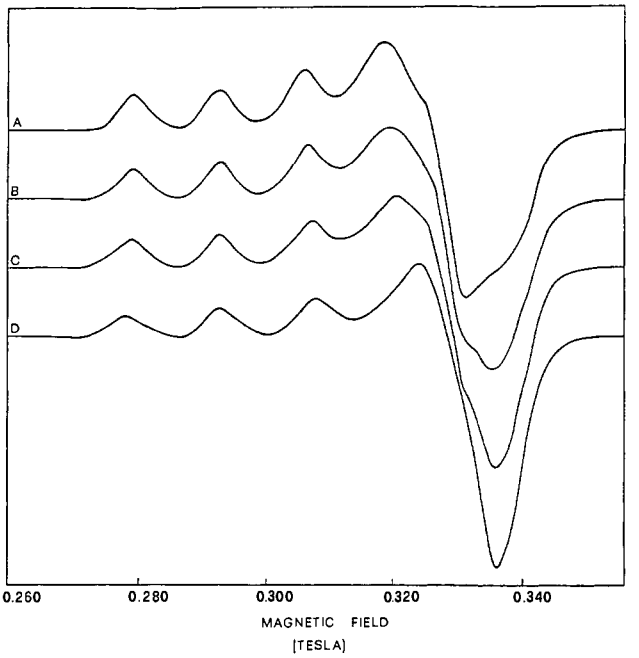

Figure 2. Room-temperature ESR spectra of superoxide dismutase solutions, [SOD] = 1.0×10^{-3} M, in the presence of increasing amounts of NCO^- (spectra A–D, 0, 8.3×10^{-3}, 2.5×10^{-2}, 2.9×10^{-1} M, respectively).

Table II. Nuclear Relaxation Parameters for Anions Bound to Copper(II) in Superoxide Dismutase (SOD) and $Cu(Me_4(14)aneN_4)(ClO_4)_2$ (TMC)

	SOD		TMC	
ligand	$(fT_{1p})^{-1}$, s^{-1}	$(fT_{2p})^{-1}$, s^{-1}	$(fT_{1p})^{-1}$, s^{-1}	$(fT_{2p})^{-1}$, s^{-1}
NCS^- (^{13}C)	1.0×10^4	5.0×10^5	2.7×10^2	2.2×10^4
(^{14}N)		1.6×10^7		
NCO^- (^{13}C)	5.3×10^3	5.6×10^5	2.8×10^2	6.9×10^4
(^{14}N)		5.0×10^6		
N_3^- (terminal ^{15}N)		1.1×10^7		7.3×10^4
(terminal ^{14}N)		7.0×10^6		
(central ^{14}N)		2.1×10^5		
F^- (^{19}F)	5.2×10^6	2.1×10^7	2.5×10^4	6.7×10^5

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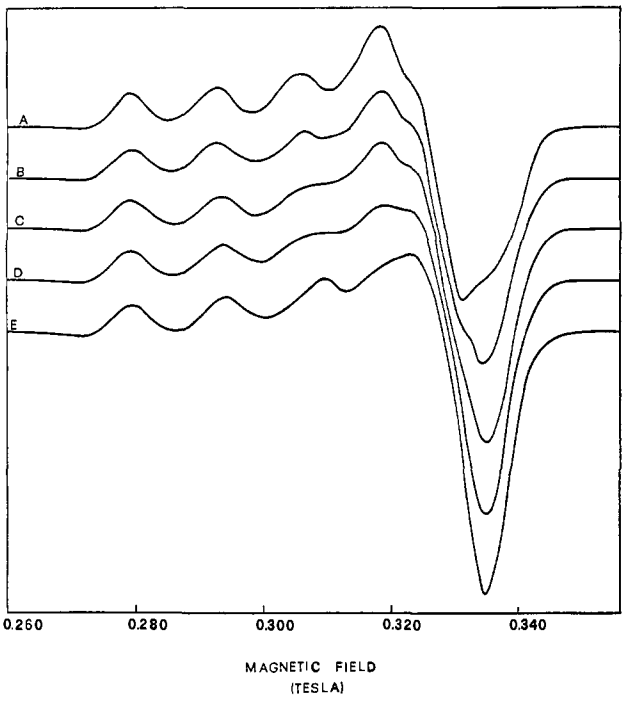

Figure 3. Room-temperature ESR spectra of superoxide dismutase solutions, [SOD] = 1.0×10^{-3} M, in the presence of increasing amounts of N_3^- (spectra A–E, 0, 1.9×10^{-3}, 4.4×10^{-3}, 1.9×10^{-2}, 3.4×10^{-2} M, respectively).

affinity constant through the concentration dependence of the line width was prevented by the slow exchange rate observed.

The ^{14}N NMR spectrum has been recorded for a solution containing 1 M cyanate and 10^{-5} M superoxide dismutase. Again, no isotropic shift occurs, whereas the signal is sensibly broadened, the corresponding $(fT_{2p})^{-1}$ value being reported in Table II.

The azide ion causes a variation in the room-temperature ESR spectra of the metalloprotein similar to that caused by the cyanate ion (Figure 3 and Table I) and similar to that reported at liquid-nitrogen temperature.[19,20] Computer treatment of the experimental data yields an affinity constant of 242 ± 8 M^{-1}. As in the case of cyanate, the small variation observed in the visible electronic spectra upon addition of increasing amounts of azide (Table I) does not allow us to independently estimate the affinity constant. However, addition of azide produces an intense charge-transfer band[19,20] at 375 nm (ϵ = 2500 M^{-1} cm^{-1}) from which an affinity constant of 130 ± 10 M^{-1} is estimated. The ^1H T_1^{-1} values of water are strongly decreased upon addition of azide[19] (Figure 1), indicating that water is displaced from the coordination sphere. From the latter data the affinity constant is estimated to be 90 ± 10 M^{-1}.

^{14}N NMR spectra at 1 M concentrations show two signals with no isotropic shift but with different line broadening with respect to the pure ligand, the broadening being about 10 times larger for the terminal N. The $(fT_{2p})^{-1}$ value for the central atom (Table II) is of the same order of magnitude of that of ^{13}C of NCS^-[29] and NCO^-.

The ^{14}N $(fT_{2p})^{-1}$ value compares well with those of NCS^- and NCO^- derivatives. A ^{15}N-enriched azide sample was also investigated at 1 M concentration, the ratio between ^{14}N and ^{15}N line broadening being of 0.6. The ratio between the γ_I^2 values of the two nuclei is 0.5.

The fluoride ion is known to be a weak inhibitor of the enzyme[36] and has also been successfully proposed by Rotilio et al. as a test for detecting the presence of superoxide dismutase in biological preparations.[38] The test consists of measuring ^{19}F T_1^{-1} enhancements, which are very sensitive to even small amounts of enzyme.[39] Indeed, we have repeated the ^{19}F T_1 and T_2 measurements in the fluoride concentration range 10^{-2}–1 M and the affinity constant has been found to be 3 ± 0.5 M^{-1}, as already reported.[39] On the other hand, the ^1H T_1^{-1} values are unaffected up to 1 M F^- concentration (Figure 1), substantially in agreement with the data reported by Rotilio et al.[36] The electronic and ESR spectra do not allow us to detect the binding of the fluoride ion (Table I), the large ^{19}F T_{ip}^{-1} enhancements being the sole evidence of strong copper–fluoride coupling. It is interesting to observe that a 1 M F^- solution binds 75% of the protein whereas the ^1H T_1^{-1} values are almost unaffected.

Table III. 80-MHz ^1H T_{1p}^{-1} Values for 1×10^{-3} M Solutions of Copper-Substituted Proteins

protein	T_{1p}^{-1}, s^{-1}	protein	T_{1p}^{-1}, s^{-1}
thermolysin[a]	5.8	transferrin[c,d]	1.5
carbonic anhydrase[b]	4.5	superoxide dismutase[d]	1.0
carboxypeptidase[a]	2.1		

[a] Reference 41. [b] Reference 42. [c] Reference 43. [d] 1×10^{-3} M copper sites.

All the above ligands have been tested against the macrocyclic complex $(Cu(Me_4(14)aneN_4)(ClO_4)_2$. In aqueous solution and in absence of coordinating counterions, the compound interacts with a water molecule in the axial position, the metal ion being above the mean N_4 plane of the macrocyclic ligand.[30] Although the extent of this axial interaction cannot be quantified, coordinating anions are known to give rise to well-defined five-coordinated square-pyramidal chromophores with the anion in the apical position.[30,40]

The affinity constants for the investigated anions determined through spectrophometric measurements range from 500 M^{-1} for azide to about 2 M^{-1} for the fluoride ion.[40] The ^{13}C NMR of cyanate, the ^{15}N NMR of azide, and the ^{19}F NMR of fluoride show line broadening in the presence of the macrocyclic compound which is diagnostic of a copper anion interaction.

The $(fT_{ip})^{-1}$ values, together with those obtained for the thiocyanate ion, are reported in Table II along with the analogous data obtained for the anion–enzyme interactions. In every case, the $(fT_{2p})^{-1}$ values are always larger than the corresponding $(fT_{1p})^{-1}$ values, indicating relevant contact contributions in both the model and enzyme adducts.[29]

The presence of contact contribution on T_{2p}^{-1} is the final proof of direct anion coordination to copper(II), and the larger contact effect operative in the enzyme with respect to the model complex can be accounted for by an equatorial rather than axial coordination of anions in superoxide dismutase. Consistently, the electronic absorption maxima in the enzyme derivatives (Table I) are shifted to higher energy (CN^-, N_3^-, NCO^-) or at most are unchanged (NCS^- and F^-), while in the model complex they invariably decrease[40] according to the well-known effect of axial coordination. As discussed elsewhere,[34,35] T_{1p}^{-1} is smaller for the small model molecule than for the macromolecule since the former experiences shorter rotational correlation times which affect the dipolar coupling contribution to the longitudinal relaxation mechanism.

Discussion

From the above data it is apparent that NCO^-, analogous to NCS^-,[29] binds copper(II) without substantially affecting the water relaxation. Contemporarily the overall shape of the EPR spectra changes from rhombic to almost axial, $A_\parallel$ increases, and the electronic absorption shifts to higher energy. All of these observations are consistent with a strengthening of the basal ligand

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field. Since water remains coordinated, the overall coordination number may be five and the geometry square pyramidal. In light of the X-ray structure, it may be suggested that cyanate and thiocyanate replace one of the histidine ligands, partially releasing the strained coordination imposed by the protein and leaving the water molecule in the axial position. The ^1H T_1^{-1} values of solutions containing the enzyme either in pure form or with added NCO^- and NCS^- also deserve some comments since the absolute values of the T_{1p}^{-1} enhancement are relatively small as compared to those of other copper proteins in which a water molecule is bound to the paramagnetic center (Table III). Since the electronic relaxation rates in copper(II) complexes are rather insensitive to the donor set,[44] this may indicate that the copper–oxygen bond in superoxide dismutase is somewhat longer than the usual coordination bond,[24] as generally observed for axial ligands.[45]

The fluoride ion again binds the copper ion without affecting the ^1H T_1^{-1} values, no major effect being detectable in the ESR and electronic spectra. Probably the binding fashion of the ion is similar to that of NCO^- and NCS^-. Since the EPR and the electronic spectra are not appreciably affected by the fluoride ion, it should be concluded that the ligand field produced by the ion is close to that of the substituted histidine. On the other hand histidine is expected to produce much larger ligand field perturbations than the fluoride ion; therefore, it may be tentatively proposed that the histidine in this case is weakly bound, probably as a result of some strain imposed by the protein part.

The cyanide ion is the strongest ligand among the known inhibitors of superoxide dismutase, binding in a 1:1 ratio with respect to copper; it reduces almost completely the ^1H T_1^{-1} enhancement whereas $A_\parallel$ increases to 188×10^{-4} cm^{-1} and the electronic absorption band shifts to 19.3×10^{-3} cm^{-1}.[20] These electronic properties are extremely diagnostic of square-planar copper(II) complexes.[22,32] In light of the present proposal for the binding mode of NCO^-, NCS^-, and F^-, the behavior of cyanide may be framed in a unified picture: cyanide binds in the equatorial position removing a histidine nitrogen and giving rise to a planar complex. The reduction of the ^1H T_1^{-1} values in this case can be a consequence of the formation of a square-planar complex with a very strong in-plane ligand field, in which the water has been pushed away rather than directly substituted by the ligand.

It has been observed that the ^1H T_1^{-1} values increase when the pH of superoxide dismutase solutions is raised above 10,[46,47] the ESR spectra becoming more axial.[20] The proposal of Boden et al.[47] of OH^- addition in the basal plane in place of a histidine nitrogen, without displacement of the axial water molecule, nicely fits in the present general anion-binding scheme. (See Note Added in Proof.)

Whereas the behavior of azide is analogous to that of cyanide with respect to displacement of water from the coordination sphere, the changes in the electronic and ESR spectra and in particular the resulting $A_\parallel$ values are similar to those displayed by the NCO^- derivative.

The behavior of azide could be therefore similar to that of cyanide, provided that the resulting in-plane ligand field is weaker than that produced by the latter ion but strong enough to prevent axial coordination of water. However, the affinity constants obtained from the various spectroscopic techniques differ from each other outside the experimental uncertainty. This may indicate a more complex type of interaction between the enzyme and azide. For example, a 2:1 interaction with different stepwise affinity constants could account for this discrepancy. One of the affinity constants should be equal to 90 M^{-1} as found from water proton relaxation measurements while the value of 242 M^{-1} found from the ESR spectra would be the lower limit for the other constant. Indeed, the possibility of a 2:1 azide–protein subunit interaction has already been considered.[19,48] Fee and Gaber from the ESR spectra had proposed that at low N_3^- concentration a 1:1 adduct is formed.[19] The fitting of the experimental spectra, however, does not provide a definite conclusion. It is unfortunate that none of the spectroscopic techniques used in this research are capable of safely discriminating between a single affinity constant and two stepwise affinity constants. In the hypothesis of a 2:1 azide–copper(II) derivative, five-coordination could be retained with a variety of possible stereochemistries as the active cavity has been shown to be rather flexible.[27] The intermediate 1:1 derivative would be analogous to the NCO^- and NCS^- derivatives.

From the present research no indications can be obtained on which histidine is replaced upon coordination. There is a report of the bridging His-61 not being replaced upon coordination of the anions.[14] However, independent of which histidine is replaced, the cavity appears flexible enough[27] to allow the chromophore to attain a more symmetric configuration than that of the unsubstituted enzyme.

A final comment is due on the possible coordination site of the O_2^- substrate; since NCS^- is reported not to display inhibitory capabilities, this may indicate that the site of attack of the superoxide ion is the site of water. In the case of cyanide, which is reported to be a competitive inhibitor,[36] the affinity of ligands for the axial position is drastically reduced, owing to the resulting square-planar complex; neither water nor O_2^- are able to significantly interact with the copper center. Therefore a competitive behavior can be simulated even if the O_2^--binding site is different from that of cyanide. In the hypothesis of azide binding copper in a 2:1 ratio, the intermediate 1:1 adduct would still be active and protected against inactivation by H_2O_2.[48]

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Note Added in Proof. The results of a recent investigation through ^{17}O NMR of $H_2{}^{17}O$ solutions of superoxide dismutase up to pH 11.5 are quite consistent with equatorial binding of the OH^- ion.[49]

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