Superoxide Dismutase-Thiocyanate: A Study of the Binding Sites of Anions on Copper(II) in Superoxide Dismutase

I. Bertini,* C. Luchinat, and A. Scozzafava

Contribution from the Istituto di Chimica Generale ed Inorganica della Facoltà di Farmacia dell'Università di Firenze and Laboratorio per lo Studio dei Composti di Coordinazione del C.N.R., 50121 Firenze, Italy. *Received February 25, 1980*

Abstract: The system bovine superoxide dismutase-thiocyanate has been investigated through ESR, electronic absorption, and ^1H of water and ^{13}C of $N^{13}CS$ NMR spectroscopies. Evidence is reported for a 1:1 adduct in which a water molecule remains bound at the copper ion and the coordination number remains unaltered. Probably a histidine nitrogen is substituted by the anion. On the contrary, azide and cyanide are known to displace the water molecule, but, despite their binding to sites different from that of thiocyanate, the two kinds of anions do not simultaneously bind to the copper ion. For comparison purposes the ^{13}C relaxation rates of the $N^{13}CS$ adduct of a copper-macrocyclic ligand are reported.

Introduction

Superoxide dismutase is a dimeric enzyme that catalyzes the dismutation of the superoxide anion in red blood cells.[1] Each of the two enzyme subunits contains both a copper(II) and a zinc(II) ion which are bridged by a histidyl imidazolate residue.[2-4] It appears well established that the two equivalent copper(II) ions in the dimer are exposed to solvent as well as to solute molecules, while the two zinc(II) ions are not capable of such interactions.[4,5] The copper ion is bound to four histidyl nitrogens and to a water molecule.[2,3]

Detailed water proton relaxation studies at variable magnetic field provided information on the proton-exchange rates as well as on the rotational and electronic correlation times;[6-8] they have also shown that competitive anionic inhibitors such as cyanide, azide, fluoride, and chloride bind to the copper atom by displacing the coordinated water molecule:[9] this suggests the same water, inhibitor, and substrate binding sites, although in some cases the affinity constants obtained through NMR were somewhat lower than those estimated through activity measurements.[9] In the case of the thiocyanate ion previous investigations have shown its ability to affect the ESR spectra of copper(II) superoxide dismutase but not the water proton relaxation of enzyme solutions,[10] while it has been subsequently proved not to affect the catalytic activity.[9]

In order to further characterize the binding sites of anions at the copper(II) site in superoxide dismutase, we have undertaken a deeper study of the interactions between the thiocyanate ion and the enzyme by means of electronic, ESR, and ^1H and ^{13}C NMR spectroscopies. For comparison purposes the complex $(Cu(Me_4(14)aneN_4)NCS)(ClO_4)$ ($Me_4(14)aneN_4$ = 1,4,8,11-tetramethyl-1,4,8,11-tetraazacyclotetradecane) has also been investigated.

Experimental Section

Ninety percent ^{13}C-enriched potassium thiocyanate was purchased from Prochem B.O.C.; all the other chemicals were analytical grade, and freshly bidistilled water was used throughout. $Cu(Me_4(14)aneN_4)(ClO_4)_2$ was prepared as previously reported.[11] The complex $(Cu(Me_4(14)aneN_4)NCS)(ClO_4)$ was prepared from water solutions of potassium thiocyanate and the above compound in a 1:1 molar ratio. Both complexes were checked through elemental analysis. Their molar conductance measured on 10^{-3} M water solutions through a WTW Model LBR/B conductance bridge is 193 and 196 Ω^{-1} cm^2 mol^{-1}, respectively, which is indicative of 1:2 electrolytes.

Bovine erythrocyte superoxide dismutase was obtained as a lyophylized powder from Sigma (Lot 38C-8190, 2900 Activity units/mg according to McCord and Fridovich[1]) and further chromatographed on DEAE Cellulose according to the last step in the purification procedure of McCord and Fridovich.[1] The electronic and ESR spectra were identical with those already reported.[10] In particular the A_{265}/A_{680} ratio, the former absorption being relative to the protein part and the latter to the copper center, was 44, indicating the same degree of purity reached by McCord and Fridovich.[1] Furthermore, the ESR spectrum, even under large sensitivity conditions, showed no evidence of paramagnetic impurities in the protein solution, which could in principle alter the NMR results. Enzyme concentrations were calculated from the intensity of the copper d-d transition (ϵ_{680} = 300 M^{-1} cm^{-1}/dimeric unit[1]). All the experiments were performed in unbuffered solutions at pH around 8: superoxide dismutase is known not to undergo active site ionizations up to pH 10.5.[12]

Spectroscopic, NMR, and ESR measurements were performed by adding aliquots of concentrated thiocyanate solutions by means of an automatic micropipette to enzyme or $Cu(Me_4(14)aneN_4)(ClO_4)_2$ solutions. Final dilutions were not larger than 20% and were taken into account in the calculation.

The electronic spectra were run on a Cary 17D spectrophotometer in the absorbance range 0-0.1; X-band ESR spectra of frozen solutions were registered on a Bruker 200 TT spectrometer at liquid-nitrogen temperature. The infrared spectra were recorded on a Perkin Elmer 283 spectrometer. The 80-MHz ^1H and 20-MHz ^{13}C NMR spectra were recorded on a Varian CFT 20 spectrometer; T_1 measurements were obtained by the inversion recovery method, while T_2 values were obtained from the line widths at half-peak height through the relation $T_2^{-1} = \pi\Delta\nu$. The estimated accuracy in the reported relaxation values is ±5%.

Results

In order to check whether the thiocyanate ion is able to bind to the copper center in superoxide dismutase in aqueous solutions, we have measured ^{13}C NMR spectra of ^{13}C enriched potassium thiocyanate. Upon addition of even small amounts of superoxide dismutase, the thiocyanate signal experiences a dramatic broadening as well as a noticeable T_1 shortening, indicating the existence of a strong interaction between the anion and the paramagnetic center. However, no isotropic shift could be measured up to the maximal enzyme: NCS^- ratios (1:1000) used in the present work. The relaxation parameters for the thiocyanate ^{13}C nucleus in a typical experiment are shown in Table I; the paramagnetic effect

* To whom correspondence should be addressed the Istituto di Chimica Generale ed Inorganica della Facoltà di Farmacia dell'Università di Firenze.

(1) McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1969**, *244*, 6049-6055.
(2) Richardson, J. S.; Thomas, K. A.; Rubin, B. H.; Richardson, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 1349-1353.
(3) Richardson, J. S.; Thomas, K. A.; Richardson, D. C. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 986-992.
(4) Rotilio, G.; Calabrese, L.; Mondovì, B.; Blumberg, W. E. *J. Biol. Chem.* **1974**, *249*, 3157-3160.
(5) Rigo, A.; Terenzi, M.; Franconi, C.; Mondovì, B.; Calabrese, L.; Rotilio, G. *FEBS Lett.* **1974**, *39*, 154-156.
(6) Gaber, B. P.; Brown, R. D.; Koenig, S. H.; Fee, J. A. *Biochim. Biophys. Acta* **1972**, *271*, 1-5.
(7) Koenig, S. H.; Brown, R. D. *Ann. N.Y. Acad. Sci.* **1973**, *222*, 752-763.
(8) Boden, N.; Holmes, M. C.; Knowles, P. F. *Biochem. J.* **1979**, *177*, 303-309.
(9) Rigo, A.; Stevanato, R.; Viglino, P.; Rotilio, G. *Biochem. Biophys. Res. Commun.* **1977**, *79*, 776-783.
(10) Fee, J. A.; Gaber, B. P. *J. Biol. Chem.* **1972**, *247*, 60-65.
(11) Barefield, E. K.; Wagner, F. *Inorg. Chem.* **1973**, *12*, 2435-2439.
(12) Rotilio, G.; Finazzi Agrò, A.; Calabrese, L.; Bossa, F.; Guerrieri, P.; Mondovì, B. *Biochemistry* **1971**, *10*, 616-621.

0002-7863/80/1502-7349$01.00/0

Table I. ^{13}C Relaxation Parameters for the Thiocyanate Ion in Copper–Enzyme or $Cu(Me_4(14)aneN_4)^{2+}$ Solutions

A[a]			B[b]		
T_1(obsd)	T_1(p)	fT_1(p)	T_1(obsd)	T_1(p)	fT_1(p)
0.29 s	0.30 s	1.0×10^{-4} s	0.60 s	0.65 s	3.7×10^{-3} s
T_2(obsd)	T_2(p)	fT_2(p)	T_2(obsd)	T_2(p)	fT_2(p)
5.1×10^{-3} s	5.8×10^{-3} s	2.0×10^{-6} s	7.1×10^{-3} s	7.9×10^{-3} s	4.5×10^{-5} s

[a] 4.7×10^{-1} M KNCS, 8.0×10^{-5} M dimeric superoxide dismutase. [b] 1.4×10^{-1} M KNCS, 8×10^{-4} M $Cu(Me_4(14)aneN_4)(ClO_4)_2$. f represents the Cu:NCS^- molar ratio.

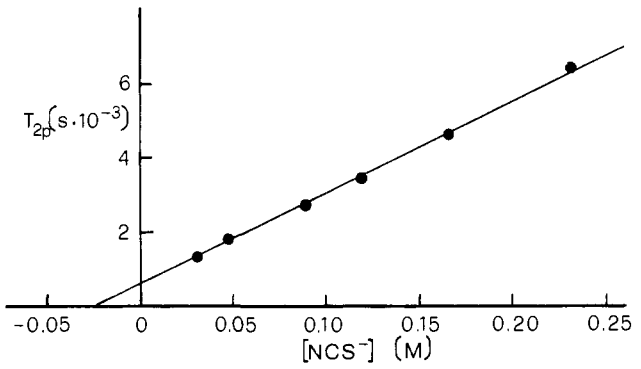

Figure 1. T_2(p) values of the ^{13}C NMR signal of $KN^{13}CS$ in the presence of 3.5×10^{-5} M dimeric superoxide dismutase as a function of thiocyanate concentration.

on T_2 decreases with increasing temperature. From the concentration dependence of the transverse relaxation time a stability constant of 40 ± 10 M^{-1} can be estimated (Figure 1); the experimental points fit satisfactorily to a straight line, indicating a 1:1 interaction.

The ESR spectra of superoxide dismutase at liquid-nitrogen temperature have been measured at thiocyanate concentrations in the range 10^{-2}–2 M. As previously noted[10] the ESR parameters of the copper(II) ion are affected by the presence of thiocyanate (Figure 2), the resulting spectrum showing a decreased anisotropy in the perpendicular part. The changes are almost complete when the anion concentration is 1.4×10^{-1} M, in agreement with the stability constant calculated from the ^{13}C NMR measurements. The ESR parameters for the adduct are (the spectra are assumed to be axial) $g_{\parallel} = 2.27$, $g_{\perp} = 2.06$, $A_{\parallel} = 153 \times 10^{-4}$ cm^{-1}, which compare with $g_{\parallel} = 2.27$, $g_{\perp} = 2.08$, and $A_{\parallel} = 143 \times 10^{-4}$ cm^{-1} for the native enzyme. All the above values are typical of a five-coordinate structure[13] and are different from the values expected for tetragonal four- or six-coordinate chromophores.[14,15] On the other hand a five-coordinate structure of the copper ion in superoxide dismutase has been suggested on the basis of a preliminary X-ray study.[2,3]

The water proton longitudinal relaxation times of superoxide dismutase solutions have been measured as a function of thiocyanate concentration (Figure 3). The ^1H relaxation rate is almost unaffected by the above ion, unless its concentration is raised above 1 M. The electronic spectra of superoxide dismutase in the region 11 000–25 000 cm^{-1} do not show any appreciable variation with the thiocyanate concentration until it approaches 1 M. The absorption maximum at 14 700 cm^{-1} and its extinction coefficient of 150 M^{-1} cm^{-1} for each copper center are again consistent with a five-coordinated chromophore.[14] At high concentrations of thiocyanate the copper(II) d–d absorption is slightly shifted to higher energies, as shown in Figure 4.

In order to further characterize the type of interaction, we carried out NMR experiments in the presence of both thiocyanate and azide ions. In Figure 5 the titration with azide of the water proton relaxation of an enzyme solution is shown in the presence of a fixed amount of thiocyanate; the parallel decrease of the transverse relaxation rate of the thiocyanate ^{13}C is reported as well.

For comparison purposes the thiocyanate adduct of the $Cu(Me_4(14)andN_4)(ClO_4)_2$ complex has been prepared and investigated. The moiety $(M(Me_4(14)aneN_4))^{2+}$ (M = Zn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}) is known to be able to bind ligands like halides, pseudohalides, or solvent molecules in the fifth axial position, giving rise to a square-pyramidal geometry.[11] The metal has been shown to be above the mean N_4 plane, and the sixth position is hindered by the macrocyclic ligand.[16,17] The complex $(Cu(Me_4(14)aneN_4)NCS)(ClO_4)$ shows an electronic absorption at 14.2×10^3 cm^{-1} in the solid state and at 15.6×10^3 cm^{-1} in water solution. The latter value is equal to that shown by the $Cu(Me_4(14)aneN_4)(ClO_4)_2$ complex in water solution, indicating that in both cases the species $(Cu(Me_4(14)aneN_4)H_2O)^{2+}$ is formed. Consistent with this hypothesis conductivity measurements indicate the presence of 1:2 electrolytes in both cases (see Experimental Section). The replacement of the NCS group by the water molecule is consistent also with the observed shift in the electronic absorption band, the absorption occurring at higher energy for the chromophore with the weaker axial ligand. Addition of increasing amounts of KNCS to water solutions of the $Cu(Me_4(14)aneN_4)(ClO_4)_2$ complex allowed us to determine, through electronic spectroscopy, a value of 125 ± 10 for the stability constant of the equilibrium (1), the limit spectrum of the NCS

$$(Cu(Me_4(14)aneN_4)H_2O)^{2+} + NCS^- \rightleftharpoons (Cu(Me_4(14)aneN_4)NCS)^+ + H_2O \quad (1)$$

adduct in solution being equal to that of the solid.

The infrared spectrum of the $Cu(Me_4(14)aneNCS)(ClO_4)$ complex has been recorded in order to determine the coordinated atom of the NCS group. The C–N stretching occurs at 2055 cm^{-1} and the C–S stretching at 760 cm^{-1}; these values are typical for N-bound NCS groups.[18,19] ^{13}C relaxation measurements of 8×10^{-4} M solutions of the copper complex and 1.4×10^{-1} M of $KN^{13}CS$ have shown sizable T_2^{-1} and T_1^{-1} enhancements (see Table I).

Discussion

The large paramagnetic effects observed in the NMR parameters as well as the changes of the ESR spectrum upon addition of the thiocyanate ion to the enzyme solution indicate that there is a strong interaction between the paramagnetic copper center and the above ion. In principle NCS^- could bind the diamagnetic zinc center in superoxide dismutase, affecting only indirectly the ESR spectrum of the copper chromophore. However, in such a case the distance between the carbon atom of the NCS^- ion and the paramagnetic center would be too large to observe any sizable effect on the NMR parameters.

Analysis of the ^{13}C Relaxation Data. From the observed T_1 and T_2 values in paramagnetic solutions (Table I) the paramagnetic contributions T_i(p) were obtained by subtracting the natural diamagnetic contributions (T_i^{-1}(p) = T_1^{-1}(obsd) – T_i^{-1}(dia)). The experimental conditions were chosen in such a way to measure relaxation rates always 1 order of magnitude larger

(13) Bencini, A.; Bertini, I.; Gatteschi, D.; Scozzafava, A. *Inorg. Chem.* **1979**, *17*, 3194–3197.
(14) Hathaway, B. J.; Billing, D. E. *Coord. Chem. Rev.* **1970**, *5*, 143–207.
(15) Bertini, I.; Scozzafava, A. In "Metal Ions in Biological Systems"; Sigel, H., Ed.; Marcel Dekker: New York; Vol. XII, in press.
(16) Alcok, N. W.; Herran, N.; Moore, P. *J. Chem. Soc., Dalton Trans.* **1978**, 1282–1288.
(17) D'Aniello, M. J.; Mocella, M. T.; Wagner, F.; Barefield, E. K.; Paul, I. C. *J. Am. Chem. Soc.* **1975**, *97*, 192–194.
(18) Sabatini, A.; Bertini, I. *Inorg. Chem.* **1965**, *4*, 959–961.
(19) Sabatini, A.; Bertini, I. *Inorg. Chem.* **1965**, *4*, 1665–1667.

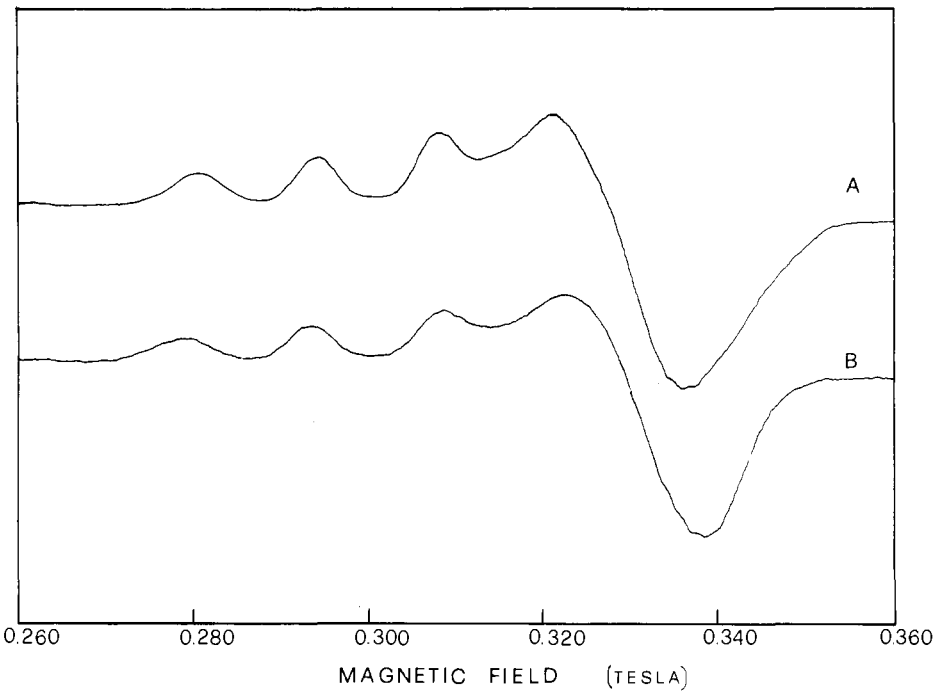

Figure 2. X-Band ESR spectra of water solutions of 8.0×10^{-4} M dimeric superoxide dismutase by itself and in the presence of 1.4×10^{-1} M KNCS (B), at liquid-nitrogen temperature.

than the diamagnetic values; the thiocyanate concentrations were high enough to ensure complete formation of the adduct on the ground of the measured affinity constants for both the enzyme and the model complex. From the data of Table I it appears that the paramagnetic effects on T_2 are much larger than those on T_1. The paramagnetic effects decrease with increasing temperature; although the temperature dependence of the NMR parameters can be field dependent,[6] it appears that the ligand exchange is fast on the NMR time scale[20] at the magnetic field used.

In the fast exchange limit the $fT_i(p)$ values are given by the Solomon–Bloembergen–Morgan equations (2) and (3).[21,22] The

$$\frac{1}{fT_1(p)} = \frac{1}{T_1(M)} = \underbrace{\frac{2S(S+1)\gamma_I^2 g^2 \beta^2}{15r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right)}_{\text{dipolar term}} + \underbrace{\frac{2S(S+1)A^2}{3\hbar^2}\left(\frac{\tau_e}{1+\omega_S^2\tau_e^2}\right)}_{\text{contact term}} \quad (2)$$

$$\frac{1}{fT_2(p)} = \frac{1}{T_2(M)} = \underbrace{\frac{S(S+1)\gamma_I^2 g^2 \beta^2}{15r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_S^2\tau_c^2} + 4\tau_c\right)}_{\text{dipolar term}} + \underbrace{\frac{S(S+1)A^2}{3\hbar^2}\left(\tau_e + \frac{\tau_e}{1+\omega_S^2\tau_e^2}\right)}_{\text{contact term}} \quad (3)$$

dipolar terms are governed by the correlation time τ_c which in turn is related to the electronic and rotational correlation times

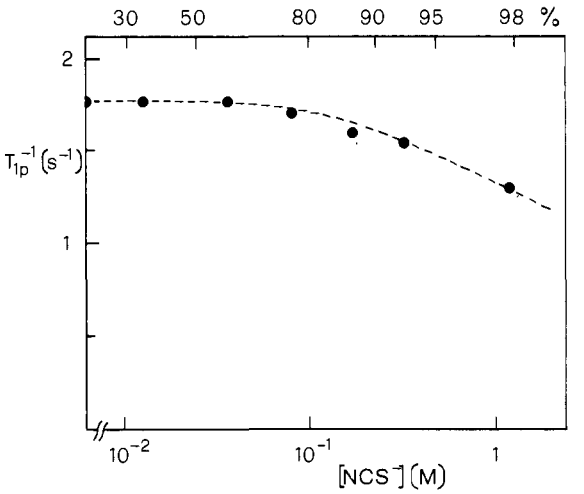

Figure 3. $T_1^{-1}(p)$ values of the ^1H NMR signal of water solutions containing 6.0×10^{-4} M dimeric superoxide dismutase as a function of thiocyanate concentration. The amount of enzyme adduct formed in the same range of concentration is also reported (as calculated from the affinity constant).

(τ_e and τ_r) through eq 4. Therefore τ_c is determined by whichever

$$\tau_c^{-1} = \tau_e^{-1} + \tau_r^{-1}$$

of the two times is smaller. Although often the electronic relaxation time is the important term in copper containing macromolecules[23] while the reverse is true in the case of small copper complexes,[24] Koenig et al. have found[6] for this particular system that τ_e is strongly field dependent at the magnetic field used in the present work and estimated τ_e to be $\simeq 10^{-8}$ s, i.e., of the order of τ_r. A successive paper, however, estimated such value to be $\simeq 10^{-9}$ s.[8] The contact term is governed by the electronic relaxation time, the molecular rotation being uneffective.[23]

As pointed out by Espersen and Martin[25] a large difference between $T_1(M)$ and $T_2(M)$ is not unexpected for copper complexes owing to their long electronic relaxation times ($T_1(e) = \tau_e = 10^{-8}$–$10^{-9}$ s) which cause the contact term to dominate in (3) but

(20) Swift, T. J. "NMR of Paramagnetic Molecules"; La Mar, G. N., Horrocks, W. DeW., Jr., Holm, R. H., Eds.; Academic Press: New York, 1973; pp 53–83.
(21) Solomon, I. *Phys. Rev.* **1955**, *99*, 559–565. Bloembergen, N. *J. Chem. Phys.* **1957**, *27*, 572–573.
(22) Koenig, S. H. *J. Magn. Reson.* **1978**, *31*, 1–10.
(23) Mildvan, A. S.; Engle, L. J. *Methods Enzymol.* **1972**, *26*, 654–682.
(24) Bertini, I. *J. Mol. Struct.* **1978**, *45*, 173–197.
(25) Espersen, W. G.; Martin, R. B. *J. Am. Chem. Soc.* **1976**, *98*, 40–44.

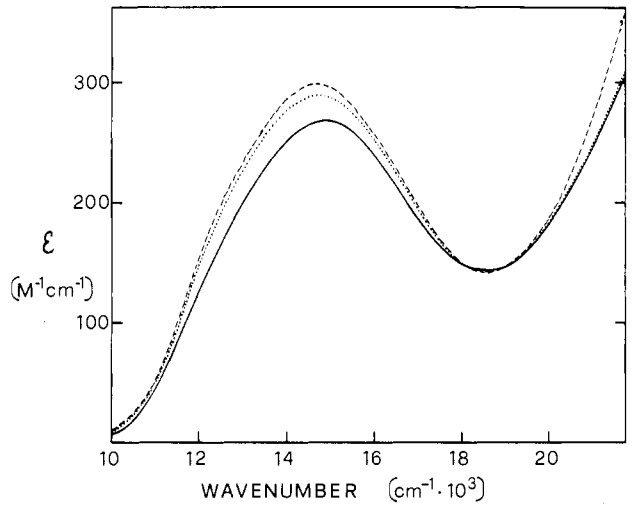

Figure 4. Electronic spectra of water solutions of 8.0×10^{-4} M dimeric superoxide dismutase by itself (---) and in the presence of 0.2 M (···) and 2.0 M KNCS (—).

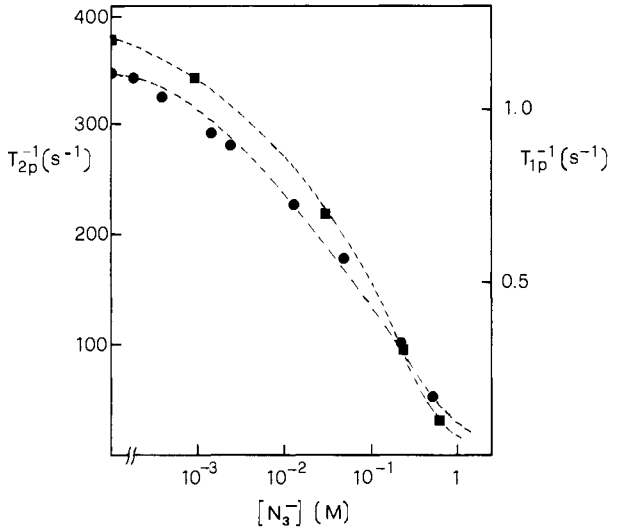

Figure 5. Effect of the addition of NaN_3 on the NMR parameters of superoxide dismutase solutions containing 0.43 M $KN^{13}CS$: T_2^{-1}(p) values of the thiocyanate ^{13}C signal (●, left-hand scale) and T_1^{-1}(p) values of the water ^1H signal (■, right-hand scale) at enzyme concentrations of 1.5×10^{-4} and 4.5×10^{-4} M, respectively, as a function of azide concentration.

not in (2), since $\omega_s^2\tau_e^2 \gg 1$. The ratio between the dipolar terms of eq 3 and 2, respectively, is strongly dependent on τ_c. For τ_c values $\ll 8.10^{-9}$ s, as presumably τ_c is for the model compound, the above ratio is 1.17 while it can be as large as 5.3 for a τ_c value of 2×10^{-8} s corresponding to the previous estimated τ_r limit for the enzyme.[6] Since the T_2^{-1}(M) values for both the enzyme and the model compound (see Table I) are about 2 orders of magnitude larger than the corresponding T_1^{-1}(M), the measured T_2(M) are largely determined by the contact term, implying therefore the existence of the copper–ligand chemical bond. The existence of a contact contribution to the transverse relaxation mechanism was also suggested for the water protons of the enzyme.[8]

In the case of the enzyme–thiocyanate adduct the value of the coupling constant $A/\hbar$ can be reasonably estimated from the measured T_2^{-1}(M) value to be as large as 10^7–10^8 s^{-1} for τ_e values in the usual range 10^{-8}–10^{-9} s.[26] Introducing the above values in the contact term of eq (1) leads to the conclusion that contact contributions to T_1(M) are negligible, and hence the copper–carbon distance can be calculated through the dipolar part of eq (1). Since the τ_c function shows a maximum for $\tau_c = \omega_I^{-1} = 8 \times 10^{-9}$ s, $r = 350$ pm is the maximum value which can be obtained no matter what the uncertainty is on τ_c. The above value is by itself consistent with a coordinated thiocyanate ion; by varying τ_c, we obtain distances even shorter than expected for the usual coordination; however, ligand-centered dipolar relaxation mechanisms are often present on ligand nuclei other than protons, simulating shorter metal–nucleus distances.[27,28]

In the case of the copper macrocycle–thiocyanate adduct both longitudinal and transverse relaxation effects are more than 1 order of magnitude smaller than those in enzyme solutions (Table I).

While a smaller effect on T_1^{-1} of the copper macrocycle complex with respect to the enzyme adduct is accounted for by the smaller τ_c in the former complex (10^{-10} s compared to 10^{-8}–10^{-9} s),[26] the difference in T_2^{-1} probably is not attributable to differences in τ_e, as the ESR spectra of the macrocycle derivative and of the enzyme adducts show signals with comparable line width. Probably the differences in T_2^{-1} values arise from different structural situations which influence the hyperfine coupling constant and consequently the contact term of eq (2). Indeed, it is known that the NCS^- ion can bind through either nitrogen or sulfur[29] and in each case with different M–donor–C angles. The difference in coordination sites, e.g., axial or equatorial, may also give rise to different contact contributions.[30,31] For example, if the same electronic correlation time τ_e for the enzyme and the model complex is assumed, a value of the hyperfine coupling constant $A/\hbar$ 4 times larger in the enzyme adduct than in the model complex is able to account for the difference in T_2^{-1}. A larger contact interaction between carbon and copper centers in the enzyme with respect to the model adduct is the final proof of direct chemical bond between thiocyanate and copper(II) in superoxide dismutase.

Structural Information on the Thiocyanate Adduct. Water proton relaxation measurements have shown that inhibitors like cyanide, azide, or chloride bind the copper atoms of superoxide dismutase by displacing the coordinated water molecule.[9] On the contrary, the thiocyanate ion has almost no effect on the ^1H relaxation rate in the presence of superoxide dismutase, unless its concentration is raised up to 1 M. From the data reported in Figure 2 it appears that the paramagnetic effect on the water proton is decreased only 10% at thiocyanate concentrations for which the Cu–NCS adduct is 90% formed as judged from the measured affinity constants. The decrease observed at high concentrations may be due to additional weak binding of thiocyanate to the water coordination position. Although the electronic spectra are substantially insensitive to thiocyanate binding, the ESR spectra do reflect changes in the electronic parameters of the copper chromophore (Figure 4).

From the above data the following considerations may be made: (i) the thiocyanate ion binds the copper atom of superoxide dismutase; (ii) at variance with other anions which possess inhibitory properties,[9] thiocyanate does not remove the coordinated water molecule upon binding; (iii) the thiocyanate adduct should have a coordination polyhedron similar to that of the noninhibited enzyme since the electronic parameters of the two species are similar. X-ray data[2,3] have shown that the copper atom is in a distorted square-pyramidal geometry, with four imidazole ligands in the plane and the water molecule in the axial position; the sixth coordination position trans to the water molecule is apparently pointing toward the inner part of the protein and is not accessible to solute molecules. Therefore it is tentatively suggested that thiocyanate removes one of the imidazole donors of the protein without causing large variations in the coordination geometry of

(26) Bertini, I. In "ESR and NMR of Paramagnetic Species in Biological and Related Systems"; Bertini, I., Drago, R. S. Eds.; NATO-ASI; D. Reidel Publishing Co. Dordrecht, 1980; pp 201–223.

(27) Doddrell, D. M.; Healy, P. C.; Rendall, M. R. *J. Magn. Reson.* **1978**, *29*, 163–166.

(28) Bertini, I.; Luchinat, C.; Scozzafava; A. *Inorg. Nucl. Chem. Lett.* **1979**, *15*, 89–91.

(29) Burmeister, J. L. In "The Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives"; Newman, A. A. Ed.; Academic Press: London, 1975; pp 68–130.

(30) Wayland, B. B.; Wisniewski, M. D. *Chem. Commun.* **1971**, 1025–1026.

(31) Wayland, B. B.; Kapur, V. K. *Inorg. Chem.* **1974**, *13*, 2517–2520.

the copper chromophore. The substitution of an imidazole nitrogen with that of NCS^- might only negligibly influence the energy of the d-d transitions; the decreased rhombicity observed in the ESR spectrum of the adduct with respect to the spectrum of the native enzyme would only suggest a more regular square-pyramidal geometry in the adduct. The proposed equatorial coordination in the copper enzyme is consistent with the larger contact effect observed with respect to the model system, in which NCS^- occupies an axial position.

Finally there are evidences of additional binding of thiocyanate, at concentrations equal or larger than 1 M, to the water coordination position; however, in such concentrated solutions the results may be altered by the occurrence of conformational changes in the protein.

Influence of Thiocyanate on Inhibitor Binding. In order to check the possibility for superoxide dismutase to accommodate two different anions in the coordination sphere of copper, we carried out NMR experiments in the presence of both thiocyanate and azide ions. The data in Figure 5 indicate that N_3^- displaces both the water molecule and the thiocyanate ion at the same time; there is no evidence of mixed ligand adducts, since the two titration curves in Figure 5 overlap all over the azide/enzyme concentration ratios. From the present experiments the apparent affinity constant of azide for the enzyme-thiocyanate adduct has been estimated to be $15 \pm 5\ M^{-1}$. These values are considerably smaller than the affinity values of N_3^- for the free enzyme.[9,10] This is consistent with a binding competition between the two anions. An analogous titration of the $N^{13}CS$ relaxation with cyanide shows a similar reduction in the cyanide inhibitor affinity constant[9] ($\sim 2 \times 10^3\ M^{-1}$), again indicating competition between cyanide and thiocyanate. Although the latter ion occupies a different binding site, steric effects as well as a decrease in the net positive charge on the metal may account for its detatchment upon addition of inhibitors.

Azide 10^{-2} M is reported to be capable of protecting the enzyme against inactivation by H_2O_2, its inhibiting action being effective only at higher concentrations.[32] It had been suggested that N_3^- at concentrations smaller than 10^{-2} M replaces a histidine ligand giving rise to a still active CuN_4OH_2 chromophore; the present data do not further clarify the N_3^- behavior.

Conclusions

Anionic ligands like cyanide, azide, and halides bind superoxide dismutase in a 1:1 ratio by displacing the bound water molecule. They are also capable of inhibiting the enzymatic activity. The thiocyanate ion does not displace the water molecule and does not inhibit the catalytic activity; nevertheless it does bind the copper ion without any major change in the coordination polyhedron, possibly through displacement of a bound histidine. The moiety CuN_4OH_2, which is present in both the native and the NCS^--ligated enzymes, is apparently essential for the catalytic mechanism independently of the type of nitrogen.

Two binding sites at the copper ion in the enzyme are therefore evidenced, the choice of any of the two depending on the nature of the inhibitor.

Acknowledgment. Thanks are expressed to Professor Luigi Sacconi for providing NMR facilities. This research is part of a CNR-NSF joint program, contribution No. 8000316.

(32) Hodgson, E. K.; Fridovich, I. *Biochemistry* **1975**, *14*, 5294-5299.

Conformational Characteristics of Rigid Cyclic Nucleotides. 3. The Solution Conformation of β-Lyxonucleoside Cyclic 2′,5′- and 3′,5′-Monophosphates and of α-Arabinonucleoside Cyclic 2′,5′-Monophosphates. Implications for Evaluation of the Solution Properties of Nucleoside Analogues[1]

Malcolm MacCoss,* Clinton F. Ainsworth, Gregory Leo,[2a] Fouad S. Ezra,[2b] and Steven S. Danyluk

Contribution from the Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439. *Received February 8, 1980*

Abstract: A detailed ^1H 220-MHz NMR study has been made of 1-(β-D-lyxofuranosyl)uracil cyclic 3′,5′-monophosphate (**1a**, 3′,5′-cLUMP), 1-(β-D-lyxofuranosyl)-5,6-dihydrouracil cyclic 3′,5′-monophosphate (**1b**, 3′,5′-cLDHUMP), 1-(β-D-lyxofuranosyl)uracil cyclic 2′,5′-monophosphate (**2a**, 2′,5′-cLUMP), 1-(β-D-lyxofuranosyl)-5,6-dihydrouracil cyclic 2′,5′-monophosphate (**2b**, 2′,5′-cLDHUMP), and 9-(α-D-arabinofuranosyl)adenine cyclic 2′,5′-monophosphate (**3**, α-2′,5′-cAAMP) in D_2O solution. Conformational analyses showed the cyclic 2′,5′-phosphates (**2a,b** and **3**) to exhibit sugar conformations in the range of ^2E whereas the cyclic 3′,5′-phosphates (**1a,b**) showed a preference for the ^3E to ^3_4T conformation. The unusually large $^3J_{PH}$ coupling of ~31 Hz which had previously been observed for $J_{5'P}$ in 9-(β-D-arabinofuranosyl)adenine cyclic 2′,5′-monophosphate (**2c**, 2′,5′-cAAMP) and for 1-(β-D-arabinofuranosyl)cytosine cyclic 2′,5′-monophosphate (**2d**, 2′,5′-cACMP) was again apparent in **2a,b** but not in **3** even though the sugar rings are in the same conformation in all five compounds. This difference is attributed to a steric interaction between the β-oriented base and the cyclic 2′,5′-phosphate ring in **2a-d**, which is not present in **3** where the base is oriented α; this allows the phosphate ring to take up a less-strained conformation to that in **2a-d**. The cyclic phosphate rings of the compounds described in this study fix the sugar rings into a particular conformation, precluding the $^2E \rightleftharpoons {}^3E$ conformer equilibrium usually found in acyclic mononucleotides. In the cases of **1a** and **2a**, this permits an accurate evaluation of the solution properties of the parent lyxonucleoside and the conformations of lyxouridine and lyxoadenosine are shown to be different from a $^3E/^3_4T \rightleftharpoons {}^2_1T/^2_3T$ equilibrium predicted earlier for lyxouridine. Furthermore, a detailed NMR examination has been made of the **2a** → **1a** isomerization in aqueous solution, and a mechanistic rationale is proposed, based on the conformations of the sugar and phosphate rings in **1a** and **2a**.

The conformational properties of flexible nucleoside and nucleotide derivatives have been shown to be best represented in solution as a dynamic equilibrium between various conformers.[3,4] For example, the sugar conformation in most ribo- and deoxy-

0002-7863/80/1502-7353$01.00/0