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Water ^1H Nuclear Magnetic Relaxation Dispersions (NMRD) of Cu_2Zn_2SOD with Some Anions and ^1H NMR Spectra of Cu_2Co_2SOD in the Presence of CN^-

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In order to further understand the interaction of CN^-, N_3^-, NCO^-, NCS^-, and $H_2PO_4^-$ with copper zinc superoxide dismutase (SOD), water ^1H nuclear magnetic relaxation dispersions (NMRD) in the magnetic field range 0.23 mT to 1.17 T have been measured. It is learned that no water is in the vicinity of the copper ion in the case of the cyanide and azide derivatives, whereas there is some, though at a nonbonding distance, in the case of the cyanate derivative. Thiocyanate has an intermediate behavior. In the case of phosphate, which binds to Arg-141, the water ^1H T_1^{-1} values are higher than those for native SOD. The ^1H NMR spectra of Cu_2Co_2SOD in the presence of increasing amounts of cyanide show that the anion is in slow exchange on the NMR time scale. The spectra, however, are similar to those of the other anions, indicating that all of them, except phosphate, bind the metal and displace the same coordinated histidine.

Introduction

It is known that cyanide binds copper(II) in copper zinc superoxide dismutase (SOD)[1,2]. This ligand has the same shape as the natural substrate superoxide and therefore deserves particular attention. Moreover single-crystal EPR studies are available on the cyanide derivative.[3]

It has been also recently shown that azide, cyanate, and thiocyanate bind copper in bovine native and copper cobalt superoxide dismutase,[4-6] i.e. the enzyme in which the native zinc is substituted with cobalt.[7-9] Anion binding causes displacement of a coordinated histidine residue, probably His-44.[5,6] The Cu_2Co_2SOD derivative is well suited to be investigated through ^1H NMR spectroscopy since the protons of all histidines coordinated to both cobalt and copper can be observed. The proton signals assigned to His-44 go toward the diamagnetic position upon anion binding. Azide has been also shown to displace the homologous histidine in human and yeast Cu_2Co_2SOD derivatives.[10]

We have undertaken a similar ^1H NMR research on Cu_2Co_2SOD in the presence of cyanide to ascertain the mode of binding of the latter anion. We have also measured the water ^1H NMRD (nuclear magnetic relaxation dispersion) of solutions containing the native bovine Cu_2Zn_2SOD and various amounts of N_3^-, NCO^-, CN^-, NCS^-, and phosphate, with the aim of checking the interaction of water with copper(II).

Experimental Section

Bovine liver SOD was purchased from Diagnostic Data Inc., Mountain View, CA. Demetalation was obtained as previously described[5] through dialysis against 0.05 M acetate buffer containing 0.01 M EDTA at pH 3.8. The excess of chelating agent was removed through exhaustive dialysis of the apoprotein solution against a 0.05 M acetate buffer containing 0.1 M NaCl at pH 3.8. The latter was then removed by dialysis against acetate buffer 0.05 M at pH 3.8. The spectrum of E_2Co_2SOD (E stands for empty) was fully developed when $CoSO_4$ was gradually added to the apoprotein at pH 5.5 up to a cobalt/protein molar ratio of about 2:1. The Cu_2Co_2SOD derivative was obtained by slowly infusing the required amount of $CuSO_4$ in a dilute solution of E_2Co_2SOD over about 24 h.

The native Cu_2Zn_2SOD solutions were prepared in 0.05 M acetate buffer at pH 5.5.

The ^1H NMRD experiments were performed by using the field cycling relaxometer homebuilt at the IBM laboratories at Yorktown Heights, NY, as previously described.[11] The ^1H NMR spectra were obtained on Bruker CXP 300 and MSL 200 spectrometers by using the modified DEFT[12,13] pulse sequence in order to suppress H_2O and bulk protein signals. T_1 values were estimated by measuring the signal intensity as a function of the time between subsequent pulses of the modified DEFT sequence.[12,13] Irradiation of selected signals for saturation transfer experiments was performed during the τ interval following the 180° pulse and immediately before the 90° observation pulse of the modified DEFT sequence.

Table I. Ambient Temperature (301 K) Shifts and T_1 Values of the 300-MHz ^1H NMR Signals of the Cu_2Co_2SOD–CN^- Derivative[a]

signal	shift, ppm	T_1, ms	corresponding signals in Cu_2Co_2SOD
a	76.6	1.6	A
b	60.2	4.9	B
c	51.1	2.6	C
d, e	50.1	3.5	D, E
f	47.2	2.6	F
g	37.3	1.8	G, I, J, J′
h	33.7	1.8	M[b]
i	32.1	4.0	H
j	20.4	3.2	N[b]
k	16.8	24.0	*b*, *c*
	9.1		L[b]
l	14.2	2.9	O
m	13.5	15.0	*d*
n	13.0		K
o	−8.7		Q
p	−8.7		R

[a] The labeling is that of Figure 1A,C. For some of the signals the tentative correspondence with the signals of the unligated derivative is shown. [b] Checked through saturation transfer. [c] This signal is related to a signal at 8.3 ppm in the unligated enzyme and therefore does not belong to protons of the coordinated histidines. [d] Signal assigned to a protein residue not interacting with the metal ion. The signal disappears in D_2O.

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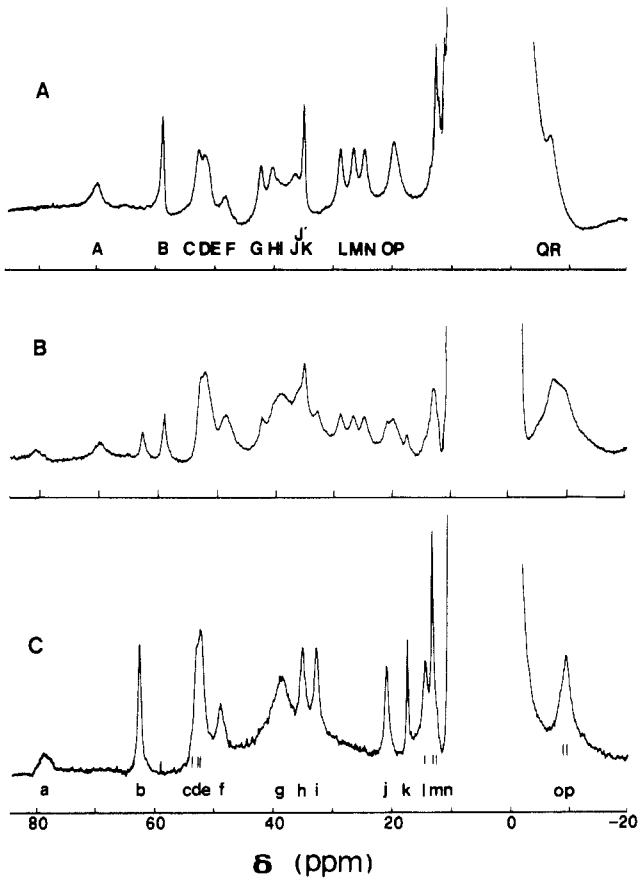

Figure 1. 300-MHz, 275 K ^{1}H NMR spectra: (A) Cu_2Co_2SOD, 0.05 M acetate buffer, pH 5.5; (B) Cu_2Co_2SOD after addition of cyanide in 1:1 molar ratio with respect to the protein; (C) Cu_2Co_2SOD after addition of cyanide in slight excess with respect to the stoichiometric 2:1 ratio.

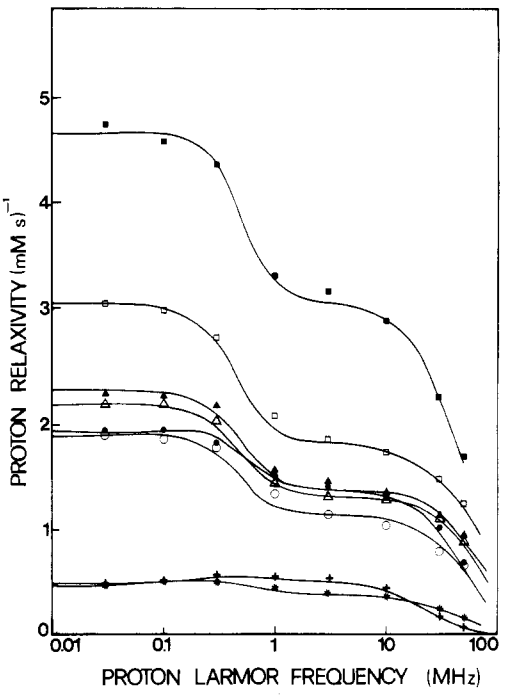

Figure 2. 298 K water proton NMRD profiles of Cu_2Zn_2SOD solutions in 0.05 M acetate buffer, pH 5.5, in the presence of (■) 0.5 M $H_2PO_4^-$, (▲) 0.5 M NCS^-, (△) 1.0 M NCS^-, (○) 2.0 M NCS^-, (●) 0.5 M NCO^-, (+) a slight stoichiometric excess of CN^-, and (*) 0.1 M N_3^-. For comparison purposes the data in the absence of anions (□) are also reported. The solid lines are best fit curves obtained by using the anisotropic A values obtained from EPR data.[4] The best fit parameters are reported in Table II.

Results

^{1}H NMR Spectra of Cu_2Co_2SOD in the Presence of CN^-. When CN^- is increasingly added to the Cu_2Co_2SOD derivative, the ^{1}H NMR signals (Figure 1A) decrease in intensity and simultaneously another set of signals appears. In Figure 1B the spectrum of the system containing a protein:CN^- 1:1 ratio is reported. The spectrum becomes rather complicated owing to the large number of signals, although a tentative assignment is proposed. Finally, in an essentially quantitative fashion, the spectrum is obtained with only the signals of the second species (Figure 1C). The spectra of Figure 1 have been recorded at 275 K; when the ^{1}H NMR spectrum of the solution in Figure 1B is recorded at room temperature, all the signals broaden sizably. This behavior is typical of slow exchange conditions, i.e. of CN^- exchanging slowly from its metal binding site. An upper limit for the exchange rate can be set at about 10^4 s^{-1}. This is consistent with the high affinity constant of the anion.[1] The assignment of the ^{1}H NMR spectrum of the CN^- derivative is performed by comparison with that of the azide derivative, through deuteriation experiments, and by T_1 measurements. Signals M and N of the unligated enzyme (Figure 1A) have been shown through saturation transfer to correspond to signals h and j of Figure 1C, respectively. Signal L is related to a signal at 9.1 ppm in the cyanide derivative. In particular signals K, L, and O, which identify a single histidine (His-44),[6] are essentially inside the diamagnetic region. The same probably holds for signal P, which is not observed in the cyanide adduct.

The signal shifts of the cyanide derivative at room temperature are reported in Table I together with the corresponding T_1 values. The latter values are related to the distance from the paramagnetic center and are sensitive to changes of the electron spin delocalization onto the histidine ring.[6] Essentially the same structures for the two anion adducts can be proposed, owing to their similarity with those of the N_3^- derivative.

Comparison of these results with previous data[5,6] indicate that CN^- and N_3^- or NCO^- and NCS^- bind copper(II) in the same fashion, despite their different steric constraints and despite the different electronic spectra of native SOD with the two sets of ligands.

Water ^{1}H NMRD of Anion Derivatives of Native SOD. The water ^{1}H NMRD profiles have been measured from 0.23 mT to 1.17 T, corresponding to 0.01–50 MHz proton Larmor frequencies for CN^-, N_3^-, NCO^-, NCS^-, and phosphate at pH 5.5 (Figure 2). In the case of CN^-, N_3^-, and NCO^- saturation of the binding site occurs[5] under the experimental conditions of Figure 2 according to the affinity constants (K_{aff} = 138 ± 4 M^{-1} and 51 ± 1 M^{-1} for N_3^- and NCO^-, respectively[5]). The experimental T_1^{-1} values have been corrected for the diamagnetic contribution corresponding to the water proton T_1^{-1} values for the reduced protein in the same concentration. In such a way the paramagnetic contribution to T_1^{-1}, T_{1p}^{-1}, has been calculated and transformed into the fully paramagnetic contribution, T_{1M}^{-1}, which is equal to T_{1p}^{-1} divided by the molar fraction of the bound water. The results are reported normalized to 1 mM concentration of copper in the protein (Figure 2). The relaxivity values thus provide an indication of whether there are exchangeable protons feeling the paramagnetic center at a shorter or larger distance with respect to the noninhibited SOD.[14]

In the latter derivative a water molecule is semicoordinated to copper,[15,16] and the copper–oxygen distance is estimated through NMRD to be around 2.4 Å.[16] The data have been treated with a best-fitting procedure based on a previously derived equation,[16,17]

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Table II. Best Fit Parameters for the Water Proton NMRD Data on Solutions of Native SOD and of Its Inhibitor Derivatives

	G,[a] pm^{-6}	r,[b] Å	τ_c,[c] s	θ,[d] deg
Cu Zn SOD	1.2×10^{-15}	3.4	2.4×10^{-9}	12
+ 0.5 M phosphate	1.5×10^{-15}	3.3	3.2×10^{-9}	36
+ 0.5 M cyanate	6.0×10^{-16}	3.9	3.2×10^{-9}	32
+ 0.5 M thiocyanate	9.1×10^{-16}	3.6	2.3×10^{-9}	19
+ 1 M thiocyanate	8.5×10^{-16}	3.6	2.4×10^{-9}	20
+ 2 M thiocyanate	5.6×10^{-16}	3.9	3.1×10^{-9}	13
+ 0.1 M azide	1.5×10^{-16}	4.8	3.9×10^{-9}	37
+ 9 mM cyanide	1.1×10^{-16}	5.2	7.7×10^{-9}	53

[a] A G value of 4×10^{-15} pm^{-6} corresponds to a regularly coordinated water molecule. [b] Calculated by assuming a single water molecule coordinated to the copper ion with two equidistant protons. [c] τ_c is the correlation time which for copper proteins corresponds to the electronic relaxation time. [d] θ is the angle between the average metal–proton vector and the z axis of the A tensor, assumed to be axial.[16]

which takes into account the dipolar coupling between the water protons and the S manifold, whose splitting due to coupling with the magnetic moment of the copper nucleus is also taken into account.[16] Such treatment provides an estimate of the correlation time for the magnetic coupling between the proton nucleus and the unpaired electron (assumed dipolar in origin[18]) and of a geometrical factor $G = \sum_i 1/r_i^6$, where the sum is over the number of exchangeable protons and r_i is the distance of the ith proton from the paramagnetic center (Table II). Owing to the strong covariancy between the two parameters, the absolute figures may have a large percent error. Nevertheless the data can provide useful information.

The electronic relaxation times are in the range 10^{-8}–10^{-9} s, as usually found in copper(II) systems. In the case of CN^- and N_3^- the G value is so low that a single water molecule would have a Cu–H distance of 4 Å. This is consistent with a previous observation by Fee and Gaber.[19] In the case of NCO^- there is a closer water molecule, as previously noted.[4] Again, the water molecule is outside the first coordination sphere, but in a significantly different position with respect to CN^- and N_3^-. In the case of NCS^- the problem is more complicated owing to the low affinity constant and to the large chaotropic effect of the anion. The affinity constant from electronic spectroscopy is 4 M^{-1} [5] whereas it is significantly larger if measured through ^{13}C T_2^{-1} NMR spectroscopy (40 M^{-1}).[20] In the presence of 0.5, 1.0, and 2.0 M NCS^- the water ^1H T_{1M}^{-1} values decrease from the starting values down to those of NCO^-. It seems, however, that the largest effects occur between 0 and 0.5 M of thiocyanate.

Phosphate was first reported to be an inhibitor of the enzyme;[21] then it was proposed that its action is essentially due to ionic strength effects.[22] Later phosphate was proposed to bind Arg-141, which is present in the active cavity, but not the metal ion. The Cu–P distance is estimated through ^{31}P NMR measurements to be about 5 Å.[23] Surprisingly, phosphate dramatically increases the water ^1H relaxivity relative to that of the pure protein, whereas the spectroscopic properties of the copper chromophore are little affected if not at all.[23] It is possible that the acidic phosphate protons interact with the metal-coordinated water and with another water molecule, with the result of increasing the overall number of exchangeable protons feeling the paramagnetic center.

Concluding Remarks

This research shows that CN^-, N_3^-, NCO^-, and NCS^- all behave in the same way as far as the site of approach to copper is concerned and cause the removal of the same coordinated histidine. This seems to happen irrespective of the steric constraint on the anions and of their affinity for the protein. The resulting chromophore is of tetragonal type,[1] and we suggest it is four coordinated since (i) the detached histidine does not feel the paramagnetic ion anymore and (ii) water is also removed from coordination, although its presence in the cavity varies from anion to anion. This may be relevant as far as the redox mechanism of the inhibited enzyme is concerned.

Phosphate was shown to bind in a different fashion since (i) the ^1H NMR spectra of Cu_2Co_2SOD are insensitive to phosphate concentration[23] and (ii) ^{31}P NMR measurements indicate a P–Cu distance of ca. 5 Å.[23] Water ^1H NMRD data indicate that the number of protons feeling the copper ion increases. A possible picture is that phosphate binds Arg-141 as suggested and increases the number of water molecules near the metal ions.

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Registry No. SOD, 9054-89-1; CN^-, 57-12-5; N_3^-, 14343-69-2; NCO^-, 661-20-1; NCS^-, 302-04-5; PO_4^{2-}, 14265-44-2.

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