Investigation of Cu_2Co_2SOD and Its Anion Derivatives. ^1H NMR and Electronic Spectra

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Abstract: Bovine erythrocyte superoxide dismutase (SOD) in which the native zinc(II) is substituted by cobalt(II) has been investigated through ^1H NMR spectroscopy. Owing to the magnetic-exchange coupling between cobalt(II) and copper(II), proton signals of the histidine residues coordinated to both cobalt(II) and copper(II) have been observed. The signals are relatively narrow, i.e., of the same quality as those of the copper-deprived cobalt(II) SOD. Assignment of histidine NH signals is obtained through deuterium exchange, and a tentative assignment of the other signals is proposed on the basis of T_1 and T_2 measurements. Anions like N_3^-, NCO^-, and NCS^- cause large variations in the position of the histidine proton signals. The same kind of variations, although to a smaller extent, occur at pH 9, probably due to the binding of the OH^- anion. The spectra have been interpreted in terms of one histidine (possibly His 44) being removed from coordination by the anion ligand. The electronic and CD spectra of Cu_2Co_2SOD and its derivatives as compared to those of native SOD derivatives indicate that the two systems behave in a quite similar fashion.

Bovine erythrocyte superoxide dismutase (SOD hereafter) is an enzyme containing zinc(II) and copper(II) ions, the latter in the site at which the catalytic reaction occurs.[1]

The X-ray structure has definitely shown that zinc(II) is bound to two histidines (His 69 and His 78) through their ND1 nitrogens to an aspartate residue (Asp 81) and to an histidine (His 61) which is also bound to copper(II).[2] The latter is bound to three more histidines (His 44, His 46, and His 118) in a flattened tetrahedral structure and to an apical water molecule. The investigation in solution is mainly based on the electronic absorption spectra and on the EPR signal of copper(II).[3-5] The electronic spectra show some evidence of the interaction of copper(II) with anions like halides,[4,6] CN^-,[3,4] N_3^-,[3,4] NCO^-,[3] and NCS^-.[7]

The EPR spectrum of the native enzyme is extraordinarily rhombic[4] whereas it is pseudoaxial with essentially the same parameters with most of the above anions. The presence or absence of water in the coordination sphere under various conditions has been monitored through water ^1H[7-10] and ^{17}O NMR.[11] Further information have been obtained on the zinc(II)-deprived derivative[12,13] as well as on the phenylglyoxal-modified derivative.[14-16] It would, however, be desirable to have a spectroscopic technique which allowed us to monitor changes in the active cavity and the surrounding of the two metal ions when anions are added to the solution containing the enzyme or when the pH is changed. The NMR technique, which is the most appropriate tool for this kind of investigation, cannot be used on the native enzyme since the signals of nuclei close to copper(II) are too broad to be detected, whereas those of nuclei close to zinc(II) are buried by all the signals of the whole protein. The enzyme derivative in which the native zinc is substituted with cobalt(II) is now well characterized.[3,17-19] Such a derivative has an activity comparable with that of the native enzyme[19,20] and, therefore, is a good model of the latter. The cobalt and copper ions are magnetically coupled, as evidenced by the lack of the copper(II) EPR signal.[19,20] Upon addition of N_3^-, the EPR signal does not reappear, indicating that the anion does not detach the bridging histidinate moiety.[21]

We know that high-spin cobalt(II) can be used to detect isotropically shifted proton signals of groups directly coordinated to the metal ion.[22-26] When even weak magnetic coupling occurs between two metal ions, the electronic relaxation time is unique for the whole system and may be shorter than the shorter one in the absence of magnetic coupling.[27] This gives a chance to detect the proton signals of the groups attached to cobalt(II) and of those attached to copper(II) in the Cu_2Co_2SOD and, therefore, to have a highly sensitive technique to monitor the structure and catalytic properties of the enzyme.

The interaction of anions with Cu_2Co_2SOD has been investigated through ^1H NMR spectroscopy. Electronic and CD spectroscopies have been used to characterize the Cu_2Co_2SOD derivatives and to compare their behavior with that of the native SOD systems. For comparison purposes, the ^1H NMR spectrum

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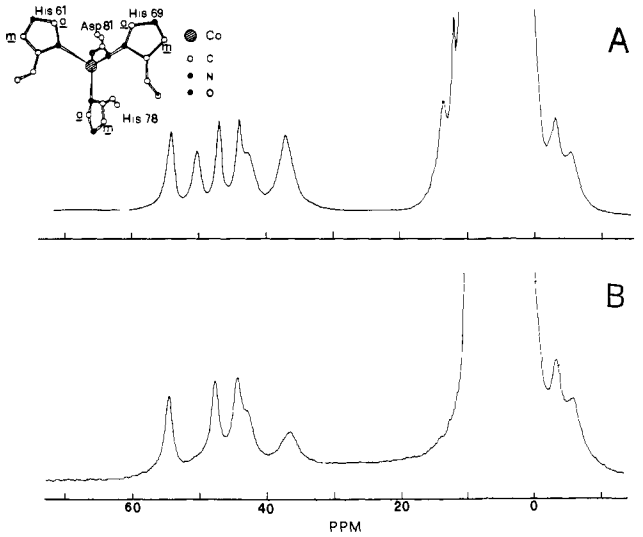

Figure 1. ^1H NMR spectrum (300 MHz) at 30 °C of E_2Co_2SOD in 10 mM acetate buffer at pH 5.5 in H_2O (A) and in D_2O (B).

of the enzyme with cobalt(II) in the place of zinc(II), in the absence of copper(II), is also reported.

Experimental Section

Native SOD was purchased from Diagnostic Data Inc., Mountain View, CA. The A_{280}/A_{680} ratio is 33, as reported in the literature;[28,29] therefore, the enzyme was used without further purification. Demetallation was obtained through dialysis against 0.05 M acetate buffer at pH 3.8, containing 0.01 M EDTA, as described elsewhere.[28] The excess of chelating agent was removed through exhaustive dialysis of the apoprotein solution against a 0.05 M acetate buffer at pH 3.8 containing 0.1 M NaCl. This latter was then removed by dialysis against the same acetate buffer. The solution was then dialyzed against a 0.05 M acetate buffer at pH 5, and $CoSO_4$ was gradually added up to a cobalt/protein ratio 2:1.[20] The spectrum of E_2Co_2SOD fully developed.[20]

Cu_2Co_2SOD was obtained by slowly infusing the required amount of $CuSO_4$ in a dilute solution (10^{-4} M) of E_2Co_2SOD in 24 h.[20] Many independent preparations provided samples with electronic spectra always equal to that reported in the literature and with the same ^1H NMR spectrum. Deuteration was obtained by concentrating the protein through ultrafiltration, diluting the sample with D_2O buffer solutions, and repeating the procedure 3 times.

The CD spectra were obtained on a Jasco J 500C spectrophotometer by using 1 cm path length cells. Ellipticity is expressed as molar ellipticity (θ) with units of deg·cm·dmol^{-1}. The visible spectra were obtained with a Cary 17D.

The ^1H NMR spectra were obtained on a Bruker CXP 300 and, at 60 MHz, with a Bruker CXP console attached to a 1.4-T Varian electromagnet. The spectra were recorded by using the modified DEFT[24,30] pulse sequence in order to suppress H_2O (HDO) and bulk protein signals. The residual signals cover an area of ~20 ppm around TMS. Typical spectra consisted of ~20 000 scans with 16 000 data points over a 50 000-Hz bandwidth; a search for far-shifted signals was performed over a 125 000-Hz bandwidth. Peak shifts were measured from H_2O (HDO) and given relative to TMS to be 4.8 ppm upfield from H_2O. Exponential multiplication of FID to improve the signal-to-noise ratio was such as to introduce a 20-Hz additional line-broadening which was subtracted for line-width measurements. T_1 values of D_2O solutions were measured through the inversion recovery method, while those of H_2O solutions were estimated by measuring the signal intensity as a function of the time between subsequent pulses of the modified DEFT sequence.[24,30] The pulses were adjusted for each sample by measuring the intensity of the FID for the water signal.

The affinity constants of anions for both Cu_2Zn_2SOD and Cu_2Co_2SOD have been determined by measuring the CD spectra at various anion concentrations and constant protein content. The spectral data have then been treated with a best fitting program.

Magnetic susceptibility measurements were performed with the Evans method.[31]

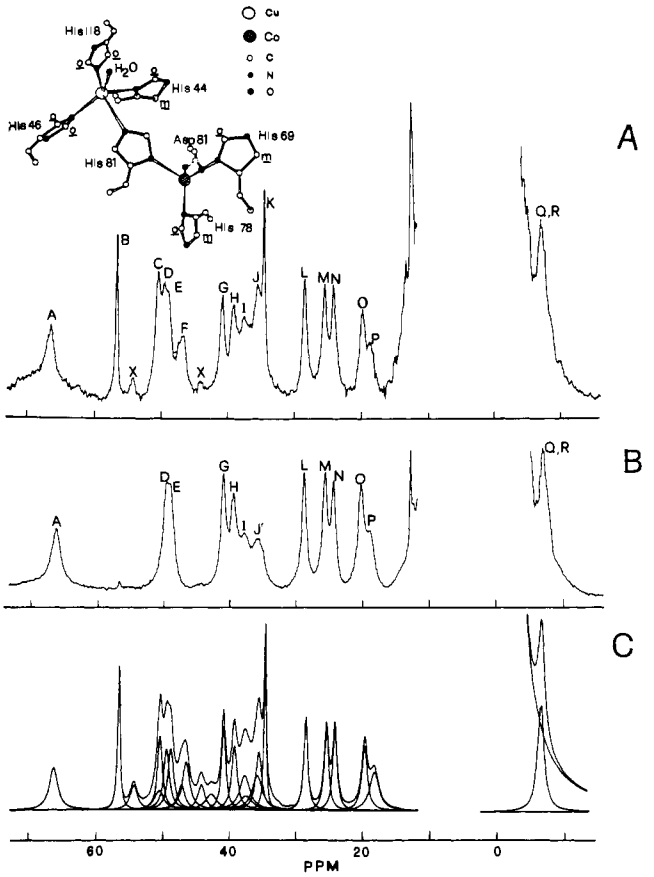

Figure 2. ^1H NMR spectrum (300 MHz) at 30 °C of Cu_2Co_2SOD in 10 mM acetate buffer at pH 5.5 in H_2O (A) and in D_2O (B). (C) Computer simulation of the spectrum reported in (A) through Lorentzian lines of variable line width (see Table I) and a residual contribution from E_2Co_2SOD (signals X in A)).

Results and Discussion

^1H NMR Spectra of E_2Co_2SOD. The ^1H NMR spectrum of E_2Co_2SOD consists of at least six signals between 35 and 55 ppm downfield from TMS and two more signals upfield (Figure 1). Cobalt(II) is bound to three histidines through ND1 (see scheme of Figure 1); therefore, three NH and three CH signals, which we refer to as metalike (m–H hereafter), are expected, experiencing similar line widths and isotropic shifts.[22-25] Three more signals due to ortholike CH protons (o–H hereafter) are expected to be broader, again downfield.

The spectrum in D_2O shows three signals at 54, 47, and 44 ppm downfield from TMS which may be assigned to m-H because they are relatively sharper. The broader signals at 37 and 43 ppm downfield, the latter appearing as a shoulder, can be assigned as o-H. A third o-H is probably very broad either below the signals between 40 and 50 ppm or beyond detection. The two signals at 37 and 50 ppm, which are present only in the spectra recorded in H_2O, are assigned to NH. It is possible that the third NH is not observed owing to fast proton exchange with bulk water (see the spectra of Cu_2Co_2SOD at pH 8 for analogous observations). The assigned signals are in the region typical of corresponding histidine protons bound to tetrahedral cobalt(II) chromophores[22-24] and of model cobalt(II) imidazole complexes.[22] The signals upfield could be consistent with β-CH_2's attached to ortho positions of the histidine rings: a downfield shift on an ortho position may cause an upfield shift on the attached CH_2 protons if the unpaired spin density on the ring is predominantly π in origin. Again, this is found in several other systems.[22-24] The shape and line widths are consistent with such an assignment. The CH_2 protons of the coordinated Asp, although never detected in cobalt(II) proteins, are expected to be shifted downfield.[32]

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Table I. NMR Parameters of the Signals of Cu_2Co_2SOD at 300 MHz and 30 °C

signal[a]	chem shift (ppm from TMS)	line width,[b] Hz	T_1,[c] ms	proposed assign
A	66.2	430	1.1	o-H(Co), o-H(Cu) (His 61)
B	56.5	115	4.1	NH(Cu)
C	50.3	240	*d*	NH(Cu)
D	49.4	295	3.1	m-H(Co)
E	48.8	295	3.1	m-H(Co)
F	46.7	380	*d*	NH(Co)
G	40.6	210	2.8	o-H(Cu)
H	39.0	280	1.7	o-H(Cu)
I	37.4	530	1.4	o-H(Co)
J′	35.6	530	1.6	o-H(Co)
J	35.4	310	*d*	NH(Co)
K	34.5	105	4.5	NH(Cu)
L	28.4	190	4.2	m-H(Cu)
M	25.3	220	2.5	o-H(Cu)
N	24.1	220	2.5	o-H(Cu)
O	19.6	280	2.4	o-H(Cu)
P	18.7	480	1.2	o-H(Cu), m-H(Co) (His 61)
Q	−6.2	330	2.2	β-CH_2
R	−6.2	330	2.2	β-CH_2

[a] The signals are labeled according to Figure 2. [b] Obtained from the spectral simulation in Figure 2. [c] The estimated error on T_1 measurements is ±0.1 ms except for NH protons. For the latter, the measurements were performed through the modified DEFT pulse sequence and the error was ±0.2 ms. [d] Not measured because the signal is within a complex envelope.

^{1}H NMR Spectra of Cu_2Co_2SOD. The ^{1}H NMR spectra of Cu_2Co_2SOD show numerous, well-shaped, well-spread, and relatively narrow signals between 70 ppm downfield and 10 ppm upfield from H_2O (Figure 2A). The line width of the signals is the same or smaller than that of the E_2Co_2SOD signals (cf. Figures 1 and 2). This shows that the magnetic coupling is relatively large so that both ions have the same electronic relaxation rates which are equal or shorter than those of cobalt(II).

The expected signals of histidines coordinated to either metal ions are 17, whereas the observed signals are consistent with 19 protons. Their assignment is rather complex since at first glance it is not even possible to distinguish between the signals of protons of the cobalt(II) ligands from those of the copper(II) ligands. The upfield signal of intensity 2, which splits in the case of the anion derivatives (see later), is reminescent of the signal observed in the case of E_2Co_2SOD and assigned to β-CH_2 protons of histidines coordinated to cobalt(II). The spectrum in D_2O (Figure 2B) of the same derivative allows us to assign three NH protons out of a total of five. These are the signals labeled B, J, and K, which disappear in D_2O. The spectra in D_2O reveal the presence, under signal J, of a much broader signal J′ which, in aqueous solutions, is not apparent. The existence of the two overlapping J and J′ signals is confirmed also by the spectral simulation (see Figure 2C). Upon standing, signal C decreases in intensity; it can also be assigned to an NH proton of another histidine. If copper(II) is added to a D_2O solution of E_2Co_2SOD, also signals C and F are missing. This is evidence that they are also due to NH of histidines, which are less accessible to solvent in the bimetallic derivative. Both signals are broader than signals B and K. We can now assume that the signals of copper(II) ligands are intrinsically sharper than the corresponding signals of cobalt(II) ligands since every nuclear relaxation mechanism depends on $S(S+1)$ of each atom provided that the electronic relaxation time for the entire system is unique owing to magnetic coupling. This is true if protons of copper(II) ligands mainly feel the electrons on this ion which will be largely described by $S = {}^1/_2$; the same holds for protons of cobalt(II) ligands which will be coupled with electrons in a state largely described by $S = {}^3/_2$. Under these assumptions, also the T_1 values should be longer for protons feeling the copper(II) center than those feeling the cobalt(II) center if the metal–proton distance is the same. From these considerations, it follows that signals B, K, and possibly C belong to histidines coordinated to copper(II) (see Table I), whereas signals J and F belong to histidines coordinated to cobalt(II). A further tentative assignment of the resonances is proposed later.

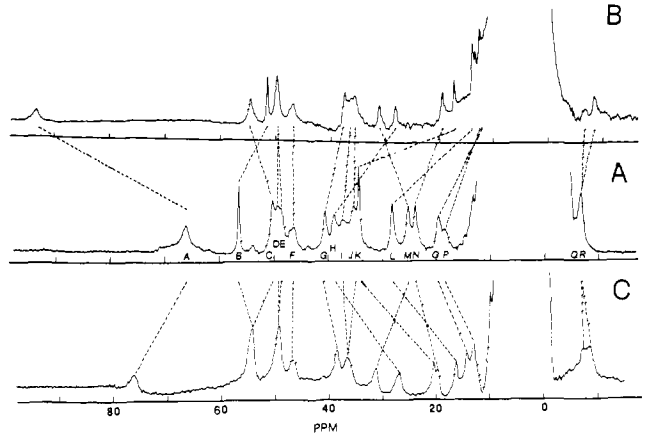

Figure 3. ^{1}H NMR spectra (300 MHz) at 30 °C of Cu_2Co_2 SOD in 10 mM acetate buffer at pH 5.5 (A) and in presence of saturating amounts of N_3^- (B) and NCO^- (C). The correspondence between signals in the unligated enzyme and in the anion adducts is indicated by the dashed lines.

^{1}H NMR Spectra of Cu_2Co_2SOD in the Presence of N_3^-, NCO^-, and NCS^-. The anions N_3^-, NCO^-, and NCS^- interact with copper(II) in the native[4,7] and cobalt-substituted[19b] SOD. The ^{1}H NMR spectra have been recorded at increasing amounts of the anions. The final spectra obtained with N_3 and NCO^- are reported in Figure 3. It appears that two signals, K and L, move dramatically toward the diamagnetic position upon anion binding, and their line width decreases; also signal O moves toward the diamagnetic position with comparable slope. The three signals, one of which has been assigned to a NH of a histidine coordinated to copper(II), clearly indicate that a histidine detaches from coordination to a paramagnetic center; since the anions bind at the copper site as shown by charge-transfer spectra (see later), it is reasonable that anions displace a histidine coordinated to copper(II).

There are other sizable changes in the spectra which indicate a rearrangement of other histidine ligands upon anion binding, but we feel that the shift variations of signals K and L are quite meaningful. They move from 34.5 and 28.4 to 17 and 14 ppm downfield in the N_3^- adduct, respectively. They substantially reach the diamagnetic region, thus indicating that a histidine almost does not feel the paramagnetic center anymore when the anions are coordinated. This is an important feature of the anion binding which we had already proposed.[7]

The changes in shifts of the ^{1}H signals as a function of anion concentration are shown in Figure 4. The affinity constants estimated from such plots are consistent with the more accurate values obtained from spectral titration (see later). Although NCS^- binds with a smaller constant, the pattern of the isotropic shifts is analogous to the pattern of N_3^- and NCO^- adducts.

pH Dependence of the ^{1}H NMR Spectra of Cu_2Co_2SOD. The spectra of Figure 2 are pH-dependent in the sense that the three signals assigned to NH protons of histidines which are capable to be deuterated upon dissolving the compound in D_2O disappear with increasing pH; they decrease in intensity without broadening until they eventually disappear. This happens between pH 7.5 and 9.3 with a pK_a of about 8.2. Such behavior, which was observed also for liver alcohol dehydrogenase,[24] can be due either to a proton dissociation or to an increase of the proton-exchange rate at high pH. Since it is not reasonable that all the coordinated histidines give rise to proton dissociation in this pH range, the present data indicate that the proton-exchange rate with solvent increases with increasing pH. Since the diamagnetic protons are saturated, the NH signals may decrease in intensity through

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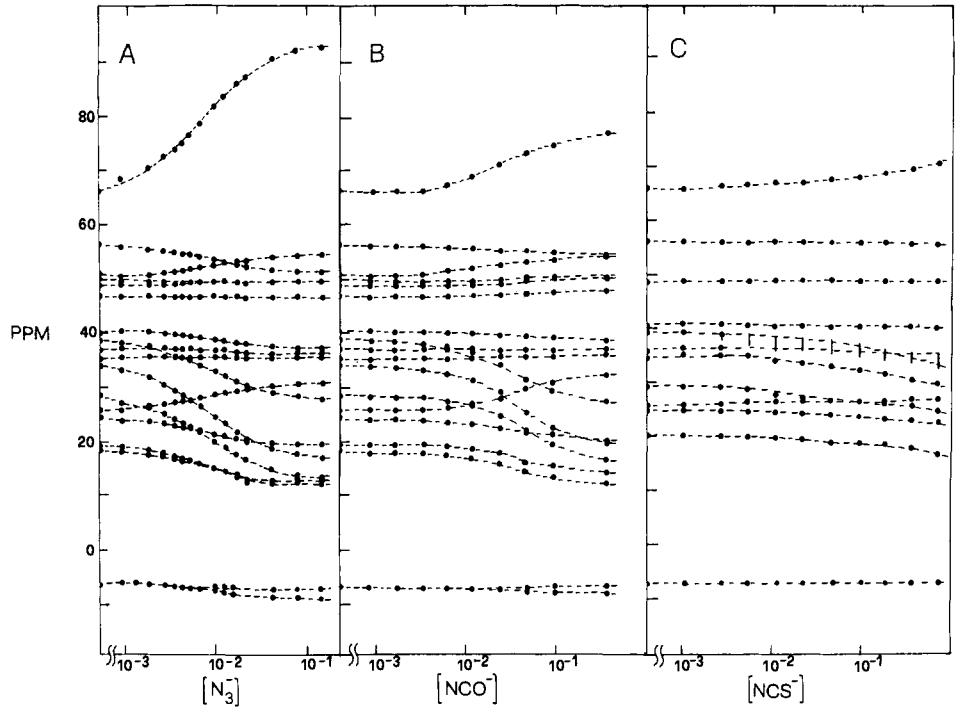

Figure 4. Chemical shift dependence of ^1H NMR signals of Cu_2Co_2SOD in 10 mM acetate buffer at pH 5.5 on N_3^- (A), NCO^- (B), and NCS^- (C) concentrations. The titrations with N_3^- and NCO^- have been performed at 300 MHz; that with NCS^- has been performed at 60 MHz. In the latter case, only the resolved signals have been followed.

Table II. Spectral Data and Affinity Constants for Cu_2Zn_2SOD and Cu_2Co_2SOD

	absorption maxima ($cm^{-1} \times 10^{-3}$)				
	electronic spectra		CD spectra		
derivative					K,[a] M^{-1}
Cu_2Zn_2SOD	29.4	14.7	29.4	24.0	
$+NCS^-$	29.0	14.7		23.2	4.3 ± 0.3
$+NCO^-$	32.2	15.1		24.8	51 ± 1
$+N_3^-$	27.0	15.1	28.2	24.7	138 ± 4
Cu_2Co_2SOD[b]	29.4	14.7	29.4	24.0	
$+NCS^-$[b]	28.6	14.7		23.2	7 ± 1
$+NCO^-$[b]	31.2	14.9		24.8	51 ± 2
$+N_3^-$[b]	27.0	14.9	28.2	24.5	339 ± 10

[a] Affinity constants measured at pH 5.6. The values for the N_3^-–Cu_2Zn_2SOD system at pH 7.8 and 8.9 are 190 and 160 M^{-1}, respectively. [b] The absorptions of the cobalt(II) ion are not reported.

saturation transfer.[33] At pH >10, also the other two NH signals start decreasing in intensity.

At pH >9, the isotropic shifts become pH-dependent. The patterns are similar to those observed upon addition of increasing amounts of anions; in particular, signal L shows a marked decrease in the isotropic shift, whereas signal A increases in shift. Apparently OH^- behaves like the other anions although the titration cannot be followed in full. Above pH 11, the intensity of the signals decreases, indicating that the sample undergoes denaturation.

Electronic Spectra of Cu_2Co_2SOD with Anions. Comparison with the Native Enzyme. The electronic absorption spectra of both the native and the Cu_2Co_2SOD derivatives change upon anion binding.[3,7,34] In each derivative, an absorption is present in the region 25–33 × 10^3 cm^{-1} (see Figure 5), which is probably due to a ligand-to-metal charge transfer. The position and intensity of this band depend on the presence and nature of the anion (Table II). The d–d region is less sensitive to anion binding. The native enzyme shows a very broad band with a maximum at 14.7 × 10^3 cm^{-1} which in the azide and cyanate adducts undergoes small hypsochromic shifts. Also the molar absorption is slightly sensitive to the different derivatives. The electronic absorption spectrum of Cu_2Co_2SOD shows in the visible region the d–d transitions of the pseudotetrahedral cobalt(II) ion superimposed to the previously mentioned copper absorption (see Figure 6). In the anion adducts, the maximum of the copper absorption band shows small hypsochromic shifts again in the case of N_3^- and NCO^- derivatives, whereas the energy of the cobalt(II) transitions remains essentially unchanged. However, the molar absorption coefficients of the cobalt transitions vary appreciably in the different derivatives.

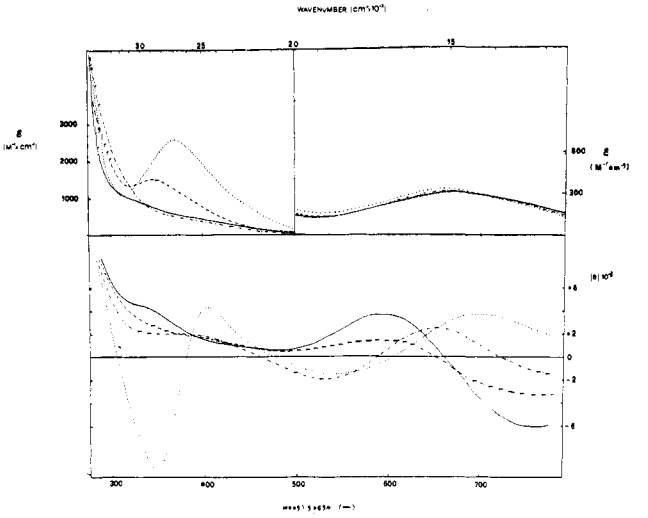

Figure 5. Room-temperature electronic absorption (top) and CD (bottom) spectra of Cu_2Zn_2SOD in 10 mM acetate buffer at pH 5.5 (—) and of its derivatives with N_3^- (···), NCO^- (-·-), and NCS^- (---).

The CD spectroscopy is by far more sensitive for monitoring anion binding both in the native and in the cobalt-substituted SOD. From the analysis of the CD spectra (Figures 5 and 6), it appears that a transition assigned to a histidine–metal charge transfer[28] at 29 × 10^3 cm^{-1} decreases in ellipticity until it becomes strongly negative in the N_3^- derivative. Furthermore, the shape of the CD spectra in the near-IR region changes dramatically, showing that

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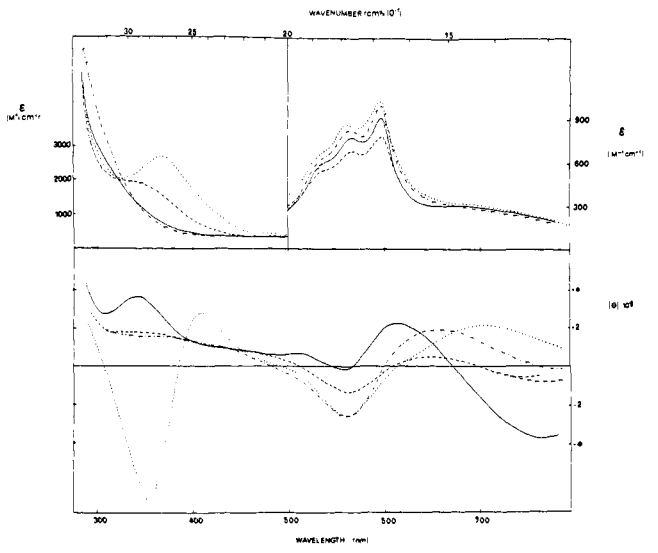

Figure 6. Room-temperature electronic absorption (top) and CD (bottom) spectra of Cu_2Co_2SOD in 10 mM acetate buffer at pH 5.5 (—) and of its derivatives with N_3^- (···), NCO^- (-·-), and NCS^- (---).

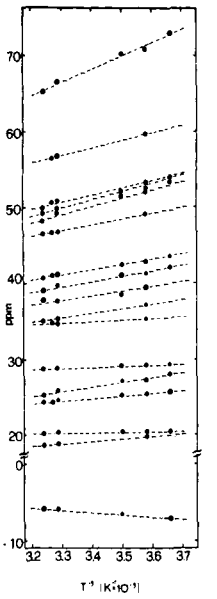

Figure 7. Chemical shift vs. $1/T$ for proton signals of Cu_2Co_2SOD at 300 MHz.

under the envelope of the absorption at 14.7×10^3 cm^{-1} in the absorption spetrum, there are several transitions. This peculiarity has been used for determining the values of the anion affinity constants (Table II). It appears that the three anions have somewhat larger affinities for the Cu_2Co_2SOD than for the native enzyme. The affinity varies in the order N_3^-, NCO^-, NCS^-. The last anion was found to interact with the paramagnetic center of the native enzyme with an affinity constant of 40 M^{-1} through ^{13}C NMR spectroscopy;[7] such a value has been reconfirmed during the present research by measuring the ^{13}C line-width enhancement with respect to reduced SOD. The CD spectra give a quite lower value, i.e., 4 M^{-1} which becomes 7 for the Cu_2Co_2SOD. The latter value is the one consistent with the histidine detachment monitored through ^1H NMR spectroscopy.

Tentative Assignment of the ^1H NMR Spectra of Cu_2Co_2SOD. A tentative assignment of the signals can be proposed on the basis of T_1 and T_2 of the signals and on chemical grounds. Proton T_1^{-1} have been shown to have a predominant contribution due to the dipolar coupling with unpaired electrons;[22-24,35-37] the extent of such coupling depends on r^{-6} (where r is the metal–proton distance). T_2^{-1} depends on dipolar coupling but also on a contact contribution[38] and is magnetic-field-dependent (Curie relaxation);[39,40] furthermore, when T_2^{-1} is measured through the line width, also chemical exchange can affect the line width. We now assume that the protons of ligands coordinated to copper(II) predominantly feel this ion; the same holds for ligands coordinated to cobalt(II). In the case of bridging histidine protons, the effects of both ions are additive. T_1^{-1} depends on r^{-6} and on the number of transitions between levels separated by an energy $\hbar\omega < \hbar\tau_c^{-1}$. Such a number is not easy to predict in the coupled system, and, therefore, the ratio between T_1^{-1} values of protons feeling only cobalt(II) and those of protons feeling only copper(II) at the *same* distance is not known. However, we expect that protons feeling only copper(II) be less relaxed than the others in the coupled system because of the smaller $S(S+1)$ value of the isolated ion.

Signals K and B are the sharpest with the longest T_1, and they are exchangeable NH. The T_1 values are long, consistent with their large distance from the metal. They are largely affected by anion binding and, therefore, are assigned to histidines coordinated to copper(II). It is possible that the next sharpest NH, signal C, for which T_1 could not be estimated, belongs to the third histidine coordinated to copper(II).

The only meta CH proton, i.e., the m-H of histidine 44, is expeced to have long T_1 and to be relatively sharp, i.e., with a line width comparable to those of signals B, K, and C. Signal L with $T_1 = 4.2$ ms and half-height line width 190 s^{-1} is a good candidate for this proton. The next T_1 values are those of signals D and E (3.1 ms). They are assigned to m-H of histidines coordinated to cobalt(II). The NH signals F and J which are assigned to histidines coordinated to cobalt(II) have comparable line widths. The ratio between T_1 values of protons feeling the two metal ions is close to unity, although the T_1 values of protons feeling copper(II) are smaller. All other signals with T_1 varying from 1.1 to 2.8 ms are ortho CH protons.

With this assignment, histidine 44 could be monitored by signal L which is the only meta CH among the histidines coordinated to copper(II). Since this belongs to a set of three signals (L, K, and O) which upon anion binding move to the diamagnetic positions, and signal K is assigned to an NH and signal O to an ortho CH proton, we can propose that (i) the three signals belong to His 44 and (ii) His 44 is the one detached upon anion binding.

We can tentatively further proceed with the assignment among the ortho CH signals; those with the shortest T_1 (signals A, I, P, and J′) are assigned to three CH's ortho to cobalt(II) and one CH of the bridging histidinate ortho to copper(II) and meta to cobalt(II), the other signals (M, N, and G) to protons feeling the copper(II) ion.

It is interesting to note that all the signals assigned to protons feeling copper(II) experience sizable shift upon addition of anions. They are signals B, C, K, L, H, G, M, N, and O. Among the fast relaxing signals (A, I, P, and J′), there are two which experience shift variation upon anion binding, i.e., A and P. They are assigned as protons of the bridging histidine. The signals I and J′ (ortho CH) and D, E, F, and J (meta CH and NH), belonging to histidines coordinated to cobalt(II), are essentially insensitive to anion binding. This is consistent with chemical sense, since anions bind copper(II) and cause a change in the ESR and electronic spectra of the metal ion.

The line widths are also consistent with the present assignment: m-H and NH protons of histidines coordinated to copper(II) have line widths ranging from 105 to 240 Hz; m-H and NH of histidines coordinated to cobalt(II) have line widths ranging from 295 to 380 Hz. o-H's feeling copper(II) have line widths in the range 210–280 Hz; o-H's feeling cobalt(II) have line widths of 530 Hz. And finally the two histidinate protons have line widths of 430 and 480 Hz.

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Table III. Magnetic Susceptibility Data[a] and Calculated μ_{eff} Values[b] for Cu_2Zn_2SOD, E_2Co_2SOD, and Cu_2Co_2SOD

sample	T, K	χ_M[a]	μ_{eff}[b]
Cu_2Zn_2SOD	280	18.5 (±1.5)	1.81
	306	15.9 (±1.2)	1.80
E_2Co_2SOD	280	106 (±3)	4.34
	306	98 (±3)	4.38
Cu_2Co_2SOD	280	124 (±5)	
	306	114 (±4)	

[a] From the Evans method.[31] χ_M values are in $m^3\ mol^{-1} \times 10^9$ and inclusive of diamagnetic correction. [b] In μ_B units. $\mu_B = 9.724 \times 10^{-24}$ J T^{-1}.

The shifts of protons of histidines bound only to cobalt(II) are in the same range as those observed in E_2Co_2SOD. This is expected if the electronic levels arising from magnetic coupling are separated by an energy smaller than kT. Indeed, magnetic susceptibility measurements between 0 and 38 °C on Cu_2Co_2SOD provided values which are, within the experimental uncertainty, equal to the sum of the magnetic susceptibilities of E_2Co_2SOD and native SOD (see Table III). These data, which are consistent with measurements previously made between 1.4 and 200 K,[41,42] indicate that the high temperature limit is a pertinent description of the Cu_2Co_2SOD.

The temperature dependence of the isotropic shifts has also been measured between 0 and 38 °C (Figure 7). The slope for the various signals is different, indicating a complex temperature dependence of the shifts. The narrow temperature range, however, prevented us from obtaining more detailed information. It may be noted that signals L, K, and O are almost temperature independent.

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Conclusions

Substitution of zinc(II) with cobalt(II) in bovine erythrocyte SOD provides a derivative which allows isotropically shifted proton signals to be detected. From the ^1H NMR spectra, it can be guessed that the correlation time τ_c experienced by protons relaxed by the copper electron is about the same experienced by protons relaxed by the cobalt electrons, since comparable line widths are observed for protons interacting with either metal ion. In macromolecules, the correlation time is likely to be determined by the electronic relaxation time. Therefore, the electronic relaxation times of the two ions are about the same, although they are $\sim 10^{-9}$ s for isolated copper(II) and $\sim 10^{-11}$ s for isolated cobalt(II) ions. The comparison between the ^1H NMR spectra of E_2Co_2SOD and Cu_2Co_2SOD indicates that the overall electronic relaxation time of the latter system is of the same order of magnitude of the former system, i.e., of the order of 10^{-11} s. This is expected to occur when the magnetic-exchange coupling constant J between the two ions is larger than $\hbar$ multiplied by the higher electronic relaxation rate. This sets $J > 0.5$ cm^{-1}. On the other hand, the magnetic susceptibility measurements give $J < kT$.

Observation of the ^1H NMR signals provides a tool for monitoring the interaction of the enzyme with inhibitors. Anions like N_3^-, NCO^-, and NCS^- interact with copper(II), causing removal of a histidine from the coordination sphere of the metal. This is presumably what happens also with the native enzyme since all the spectroscopic (optical absorption and CD) data underline the essentially identical behavior of the two derivatives with anions. A tentative assignment of the NMR signals seems to indicate His 44 as the one which is removed upon anion binding; anions would therefore push away such histidine, providing a more planar CuN_3-anion moiety.

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Registry No. SOD, 9054-89-1; N_3^-, 14343-69-2; NCO^-, 661-20-1; NCS^-, 302-04-5; L-histidine, 71-00-1.

Exploratory Study of the Intermolecular Reactivity of Excited Diphenylmethyl Radicals[1]

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Abstract: A series of intermolecular reactions of excited diphenylmethyl radicals has been examined with laser flash photolysis techniques. The radicals, $Ph_2\dot{C}H$, were generated by 308-nm photodecomposition of 1,1,3,3-tetraphenylacetone or diphenyldiazomethane. In the latter system the radicals result from reaction of diphenylcarbene with hydrogen-donor solvents. The radicals were then excited with the 337-nm pulses from a nitrogen laser, leading to the formation of the readily detectable excited state of the radical, $Ph_2\dot{C}H^*$ (λ_{max} 355 nm). The excited radical is an excellent electron donor, reacting with methyl viologen with $k_q^* = 1.3 \times 10^{10}$ M^{-1} s^{-1} in wet acetonitrile and leading to the formation of methyl viologen radical cation. $Ph_2\dot{C}H^*$ is readily quenched by amines, but no evidence for full electron transfer could be obtained, e.g., for triethylamine $k_q^* = 5.2 \times 10^8$ M^{-1} s^{-1} in cyclohexane. The excited radical is 14 times more reactive toward oxygen than its ground state, but the process seems to involve a different mechanism, probably leading to singlet oxygen generation. Halogenated substrates react readily with $Ph_2\dot{C}H^*$; for example, for CCl_4 $k_q^* = 1.6 \times 10^8$ M^{-1} s^{-1} and leads to Ph_2CHCl in a process that is presumed to involve charge transfer. Excited diphenylmethyl is not a good hydrogen abstractor in spite of the fact that its hydrogen abstraction reactions would be more exothermic than those of phenyl radicals.

The photophysics and photochemistry of organic reaction intermediates is a subject of current interest. Experiments of this type at ambient temperatures usually require two pulsed sources: one is used for "synthesis" (a laser or radiolytic pulse), while the second source (usually a laser) is used to excite the transient at a wavelength where all other reagents are transparent. Accurate and reliable timing sequences are critical, since the delay between pulses must be adjusted as a function of the intermediate's lifetime and its rate of formation. Recent studies by Meisel et al.,[4,5]

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