Concerning the quenchings by the organic buffers, it is the first time, to our knowledge, that such effects have been pointed out with the Ru(II) complexes. Since these quenchings occur in a pH domain where quenching by protonation is absent, they are attributed to formation of hydrogen bonds between the acid and the free nitrogen atoms of the excited complex. The associated mechanism is rather complicated because ion pairs participate in the quenching process; therefore, the reaction scheme that we propose is certainly oversimplified. Under the conditions of linear SV plots, the quenching rate constant by the organic acid increases with the basicity of the excited complex and the acidity of the buffer but never reaches the diffusion limit. This quenching seems characteristic of carboxylic acid since a phosphoric acid–phosphate buffer, alcohols, and sugars such as glucose have no effect on the luminescence lifetimes of the TAP complexes.

Acknowledgment. We are grateful to A. Masschelein and Professor E. Vander Donckt for their helpful discussions about this work. L.J. thanks the IRSIA (Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture) for a fellowship.

Registry No. $[Ru^{2+}(TAP)_3](PF_6^-)_2$, 88181-61-7; $[Ru^{2+}(bpy)(TAP)_2](PF_6^-)_2$, 117183-29-6; $[Ru^{2+}(bpy)_2(TAP)](PF_6^-)_2$, 117183-31-0; $[Ru^{2+}(TAP)_3]H^+$, 117183-32-1; $[Ru^{2+}(bpy)(TAP)_2]H^+$, 117183-33-2; $[Ru^{2+}(bpy)_2(TAP)_2]H^+$, 117183-34-3; $[Ru^{2+}(bpy)_2(TAP)_2](H^+)_2$, 117183-35-4; oleum, 8014-95-7; SO_3, 7446-11-9; sodium acetate, 127-09-3; sodium citrate, 68-04-2; citric acid, 77-92-9; sodium hydroxide, 1310-73-2; sulfuric acid, 7664-93-9; acetic acid, 64-19-7; ammonium persulfate, 7727-54-0; tartaric acid, 87-69-4; sodium tartrate, 868-18-8.

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Characterization of Copper–Nickel and Silver–Nickel Bovine Superoxide Dismutase by ^1H NMR Spectroscopy

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Received April 7, 1988

Nickel(II) substitution for Zn^{2+} in bovine Cu,Zn-superoxide dismutase has provided information concerning the configurational and spectroscopic properties of Ni^{2+} in the zinc site and also of Cu^{2+} in the copper site (Ming, L.-J.; Valentine, J. S. *J. Am. Chem. Soc.* **1987**, *109*, 4426–4428). The "effective" electronic relaxation time of Cu^{2+} in this derivative is greatly decreased from 2×10^{-9} s in native protein to about 3×10^{-12} s by the magnetic coupling between the paramagnetic Ni^{2+} and Cu^{2+} ions. Consequently, an isotropically shifted ^1H NMR spectrum of this species has been obtained, consisting of resonances from amino acid residues bound to both metal ions. On the basis of azide titration and proton relaxation time measurements on this species, a full assignment of the isotropically shifted signals is presented. The smaller nuclear relaxation rates of the histidyl protons and the smaller differences in the relaxation rates between ortho-like and meta-like protons of the coordinated histidine residues in the copper site in Cu_2Ni_2SOD as compared to those in Cu_2Co_2SOD result from different correlation times and different contributions of relaxation mechanisms in these two derivatives. A reasonable physical picture of proton relaxation in this magnetically coupled system is proposed, and a theoretical fitting is reported. A comparison of the spectra and relaxation rates of this derivative and of M_2Ni_2SOD ($M = Ag^+, Cu^+$) has provided further information on the bridging ligand and on the relaxation mechanisms.

The high-spin Ni^{2+} ion has two unpaired electrons with a spin-triplet ground state. There are low-lying spin-triplet excited states in its four- or five-coordinated complexes, which allow the unpaired electrons to relax rapidly. However, the energy level of the first excited spin triplet is high above that of the ground state in six-coordinated complexes, where the electronic relaxation mechanism is less efficient.[1] As a consequence, the ^1H NMR spectra of nickel complexes with coordination number <6 usually show well-resolved isotropically shifted NMR signals of reasonable narrow line width.[1]

Bovine copper–zinc superoxide dismutase (Cu_2Zn_2SOD)[2] is a dimeric metalloenzyme with two equivalent subunits, each of which binds a Cu^{2+} and a Zn^{2+} ion at a distance of 6.3 Å with the imidazolate ring of the histidine-61 residue serving as a bridging ligand.[3] A scheme of the metal binding site of this protein from X-ray crystallography studies is shown in Figure 1. The native metal ions have been replaced by various other metal ions in different studies.[4] Most recently, Ni^{2+} has been substituted for Zn^{2+} at the zinc site, forming two new metal-substituted derivatives, Cu_2Ni_2SOD and Ag_2Ni_2SOD, and the former was found to have 26–45% of the SOD activity relative to the native enzyme.[5] It was also shown that both derivatives had relatively sharp isotropically shifted ^1H NMR signals. Moreover, the signals of the protons in the copper binding site can be detected and distinguished. We report here a full characterization of these derivatives by means of ^1H NMR spectroscopy and relaxation.

Experimental Section

Bovine Cu_2Zn_2SOD was purchased from DDI Pharmaceuticals, Inc. (Mountain View, CA), as lyophilized powder and was used without further purification. All other chemicals used are commercially available. The two Ni^{2+}-substituted derivatives, Cu_2Ni_2SOD and Ag_2Ni_2SOD, were prepared in 50 mM phosphate buffer at pH 6.5 as previously reported.[5] All the samples for NMR study were concentrated by ultrafiltration using a Centricon microconcentrator with a molecular mass cutoff of 10 000 Da (Amicon Corp., Danvers, MA).

The isotropically shifted ^1H NMR spectra were obtained on an IBM AF200 spectrometer at 200 MHz, a Bruker CXP300 at 300 MHz, and a Bruker CXP90 at 90 MHz. The modified DEFT multipulse sequence was used to suppress water and diamagnetic protein signals.[6] Typical spectra consisted of about 10 000 scans with 8K data points. Chemical shifts were measured from the water signal, which was assumed to be 4.8

[†] University of California, Los Angeles.
[‡] University of Florence.

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(2) Abbreviations: SOD, superoxide dismutase; $M_2M'_2SOD$, M- and M'-substituted SOD with M in the copper site and M' in the zinc site (the metal ions in the above derivatives have oxidation states of 2+ unless otherwise specified); NMR, nuclear magnetic resonance; FID, free induction decay; NOE, nuclear Overhauser effect; DEFT, driven equilibrium Fourier transform.
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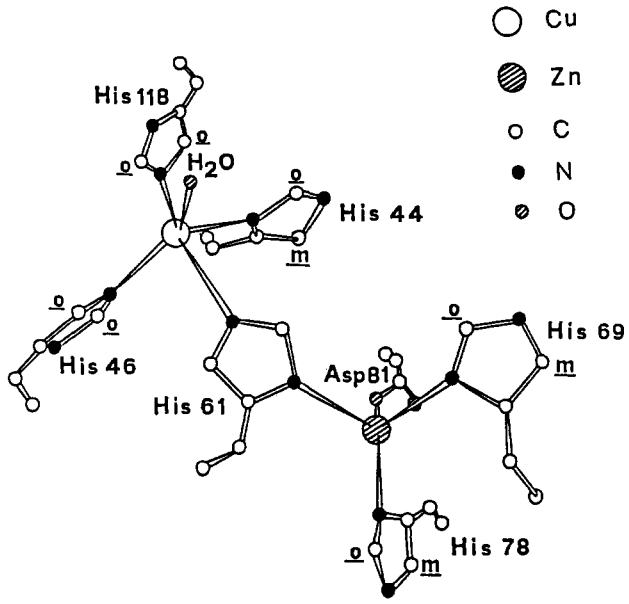

Figure 1. Active-site structure of bovine Cu_2Zn_2SOD.

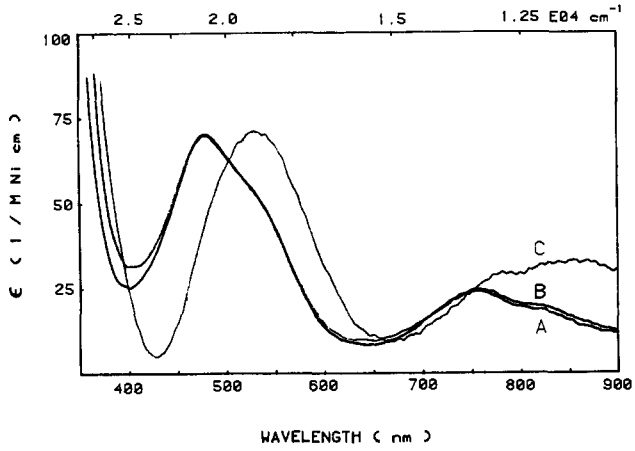

Figure 2. Electronic spectra of $Ag^I_2Ni_2SOD$ (A) and $Cu^I_2Ni_2SOD$ (B) and the difference spectrum of Cu_2Ni_2SOD minus Cu_2Zn_2SOD (C).

ppm downfield from TMS. Selective excitation ^1H NMR spectra of the derivative in H_2O were recorded on the IBM AF200 spectrometer at 200 MHz using the 1-3̄-3-1̄ hard-pulse sequence.[7] The maximum excitation was centered around the signals of interest, while the null was set on H_2O. A 20-Hz line broadening was introduced by exponential multiplication of FID's to improve the signal-to-noise ratio. Proton spin–lattice relaxation times were measured by the modified DEFT pulse sequence by varying the delay time between subsequent pulses,[6] and a nonlinear least-squares fitting of the signal intensity as a function of the delay time was performed to obtain the values.

Electronic spectra were obtained at ambient temperatures on a Beckman UV5270 spectrometer referenced against deionized water. The pH values of protein and buffer solutions were measured on a Corning Model 12 pH meter equipped with a combined microelectrode (Wilmad Glass Co., Inc., NJ).

Results and Discussion

Electronic Spectra of Nickel-SOD Derivatives. The electronic spectra of $Ag^I_2Ni_2SOD$ and $Cu^I_2Ni_2SOD$ are virtually identical as shown in Figure 2. Two absorption bands occur at 13 200 and 21 000 cm^{-1} with molar absorptivities of 25 and 70 $(M\ cm)^{-1}$ per subunit, respectively, with shoulders at 18 500 and 12 300 cm^{-1}. The absorptions at 18 500 and 21 000 cm^{-1} are in the region of typical ^3F-^3P transitions, and those in the near-IR region are consistent with the highest ^3F-^3F transitions of four-coordinated high-spin Ni^{2+} complexes.[8] However, both transitions are at the high-energy margin for a four-coordinate complex with three aromatic nitrogen donor ligands such as the imidazole side chain of histidine. The higher energy transitions and low molar absorptivity of these derivatives may result from Asp-81 acting as a bidentate ligand, giving rise to a pseudo-five-coordinate chromophore. The bidentate binding of a carboxylate amino acid residue to a metal ion has been observed for carboxypeptidase A and its Co^{2+}- and Ni^{2+}-substituted derivatives by X-ray crystallography.[9]

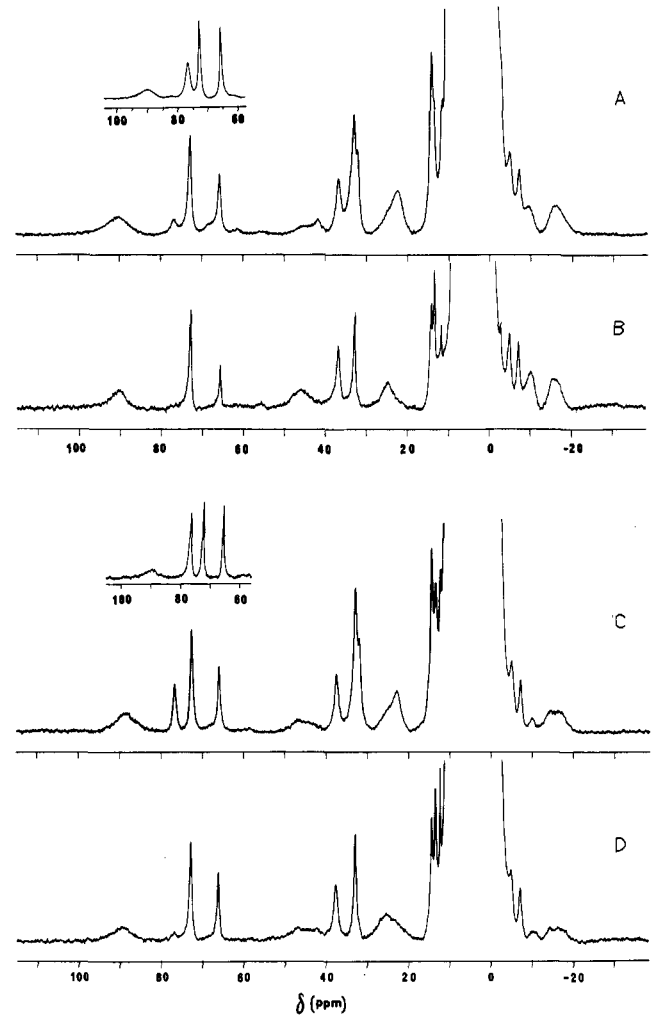

Figure 3. ^1H NMR spectra (200 MHz) at ambient temperatures (~23 °C) of Ag_2Ni_2SOD in H_2O (A) and in D_2O (B) and of $Cu^I_2Ni_2SOD$ in H_2O (C) and in D_2O (D). The insets are the spectra in H_2O taken by using a selective excitation hard-pulse sequence under the same conditions.

Electronic difference spectra have been used as a criterion to determine the binding status of His-61 in a number of Co^{2+}-substituted derivatives of Cu_2Zn_2SOD.[10] The formation of the bridging His-61 in Co^{2+} derivatives changes the spectral shape of the Co^{2+} chromophore in the zinc site as reported for Cu_2Co_2SOD and its reduced form.[10] An electronic difference spectrum of Cu_2Ni_2SOD and Cu_2Zn_2SOD, both of which contain His-61 as a bridging ligand,[3,5] shows an absorption band at 18 800 cm^{-1} (~70 $M^{-1}\ cm^{-1}$ per subunit) (Figure 2C). This absorption band is presumed to be due to Ni^{2+} in the zinc site when the imidazolate ring of His-61 bridges between Cu^{2+} and Ni^{2+}. The lack of agreement between this spectrum and those of $M^I_2Ni_2SOD$ ($M^I = Ag^+, Cu^+$) indicates that the coordination structure of Ni^{2+} in Cu_2Ni_2SOD changes when Cu^{2+} is reduced or replaced by Ag^+.

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We attribute this difference to the absence of a bridging imidazolate in the derivatives $M^I_2Ni_2SOD$ ($M^I = Ag^+, Cu^+$).

^{1}H NMR Spectra of $Ag^I_2Ni_2SOD$ and $Cu^I_2Ni_2SOD$. The 200-MHz ^{1}H NMR spectra of these derivatives show a number of isotropically shifted signals in both the upfield and the downfield regions and are very similar to each other, indicating a very similar metal binding environment in both derivatives (Figure 3). Since both Ag^+ and Cu^+ ions are diamagnetic (d^{10} electronic configuration), we conclude that the isotropically shifted ^{1}H NMR signals originate from the protons of the amino acid residues coordinated to the high-spin Ni^{2+} in the zinc site.

The N–H protons of coordinated histidine residues in a metalloprotein can be easily deuteriated by using deuterium oxide as the solvent and consequently are not detected in ^{1}H NMR spectra. This process has been used to determine the number of coordinated histidines in a few Co^{2+}-substituted metalloproteins by Bertini and co-workers.[1a] When the spectra of these two derivatives are recorded in D_2O, two signals at 32 and 21.7 ppm in $Ag^I_2Ni_2SOD$, as well as the two at 32.1 and 23 ppm in $Cu^I_2Ni_2SOD$, disappear and are tentatively assigned to the N–H protons of two histidines coordinated to Ni^{2+} in the zinc site.

A signal of low intensity at 77.5 ppm ($Ag^I_2Ni_2SOD$) or at 76.5 ppm ($Cu^I_2Ni_2SOD$) also disappears in D_2O and can be assigned to the third N–H proton of the coordinated histidines in the Ni^{2+} binding site. The low intensity of the signal is due to saturation of the solvent by the modified DEFT pulse sequence followed by saturation transfer from the solvent to the signal.[11] The intensity of this signal in $Ag^I_2Ni_2SOD$ is significantly lower than that in $Cu^I_2Ni_2SOD$ under the same experimental conditions, implying different environments for this N–H proton in these two derivatives presumably as a consequence of the nature of the monovalent metal ion, Cu^+ or Ag^+, in the copper site. The lower intensity of this N–H proton in the $Ag^I_2Ni_2SOD$ derivative reflects its faster exchange with the saturated solvent. A reasonable candidate for this proton would be the N_ϵ–H proton of His-61, which is coordinated to Ni^{2+} in the zinc site through the N_δ1 nitrogen, causing the N_ϵ–H proton to be pointed toward the monovalent metal in the copper site. Unlike hydrogen bonding of the N–H protons to other electrostatically negative moieties in a protein or a (poly)nucleotide molecule, which decreases the solvent-exchanging lability of the N–H protons,[11] an electrostatic repulsive interaction between N–H protons and a positive metal ion may increase the lability of the N–H protons. With different metal ions in the copper site, electrostatic interaction between the N_ϵ–H proton and the metal ion may occur to different degrees, which may result in differences in the solvent-exchange rate and, therefore, in the intensity of the signal.

When a selective excitation 1-$\bar{3}$-3-$\bar{1}$ hard pulse[7] is applied, signals of very similar integrated area can be detected at 90, 77.5, 72.7, and 65.5 ppm in $Ag^I_2Ni_2SOD$, as well as at 88.9, 76.5, 72.7, and 66 ppm in $Cu^I_2Ni_2SOD$ (insets in Figure 3), indicating that each of the signals can be assigned to each individual proton on the coordinated ligand. The signals at 77.5 ppm (of $Ag^I_2Ni_2SOD$) and at 76.5 ppm (of $Cu^I_2Ni_2SOD$), with much lower intensities by the modified DEFT pulse, are clearly detected and can be unambiguously assigned to an N–H proton by this method. This signal is almost completely saturated at lower magnetic field (90 MHz) due to the longer T_1 values experienced by the protons at low magnetic field (vide infra), which result in their being saturated more effectively.

The existence of three solvent-exchangeable signals in these two derivatives indicates that three histidines are bound to Ni^{2+} in the zinc site. This result therefore implies that His-61 is not deprotonated and that it does not form an imidazolate bridge between Ni^{2+} in the zinc site and the monovalent metal in the copper site. We conclude that the derivatives $M^I_2Ni_2SOD$ ($M^I = Cu^+, Ag^+$) have the bridging ligand detached from the copper site and protonated. Similar conclusions have been drawn for the analogous Co^{2+}-substituted derivatives $Cu^I_2Co_2SOD$[12] and $Ag^I_2Co_2SOD$[13] under similar conditions.

There are four sharp signals at 72.7, 65.5, 36.7, and 32.7 ppm in $Ag^I_2Ni_2SOD$ (72.7, 66, 37.5, and 32.7 ppm in $Cu^I_2Ni_2SOD$) with 300-MHz T_1 values between 6 to 18 ms (Table I). Three of them are assigned to the meta-like protons of the coordinated histidines, which have the metal–proton distances to exhibit the T_1 values in that range by dipolar relaxation. The half-height line widths of these signals, $\Delta\nu_{1/2}$, in the two derivatives are very similar (about 150–200 Hz at 200 MHz), while those of the solvent-exchangeable signals, also meta-like protons, at 77.5 ppm in Ag_2Ni_2SOD and at 76.5 ppm in $Cu^I_2Ni_2SOD$ are quite different (430 and 210 Hz, respectively, by the selective excitation pulse). This observation implies that the larger $\Delta\nu_{1/2}$ of this N–H signal in $Ag^I_2Ni_2SOD$ is due to the fast exchange of this proton with solvent and is consistent with the conclusion proposed above, based on the different intensities of this signal when the solvent signal was saturated.

The fourth sharp signal is difficult to assign at this stage. Tentatively, it might originate from one of the β-CH_2 protons of the Asp-81 ligand. From the analysis of the Cu_2Ni_2SOD data (see later) the choice is restricted to either the signal at 65.5 ppm (66 ppm in $Ag^I_2Ni_2SOD$) or the signal at 72.7 ppm. If this is the case, such a large downfield shift for a carboxylate β-CH_2 proton deserves comment. Indeed, a number of carboxylate complexes have been studied by NMR spectroscopy and were reported to have different chemical shifts resulting from different spin-delocalization mechanisms, which may be modulated by the coordination of the carboxylate (i.e. monodentate vs bidentate).[1,14,15] In the case of monodentate carboxylate complexes, both the spin delocalization through the σ bond and the spin polarization may in principle contribute to the isotropic shifts. The spin-delocalization mechanism, which is often dominant, would give moderate downfield isotropic shifts. In the case of bidentate carboxylate complexes, however, the contribution of a third mechanism, the spin delocalization through π orbitals, was found to produce remarkable downfield shifts on the α-CH_2 protons of the coordinated carboxylate (or the methyl of acetate) of some iron complexes.[15] The mechanism of spin delocalization through π orbitals would favor a bidentate character for the Asp-81 ligand. However, the other geminal β-CH_2 proton of the Asp-81 residue is not apparent in the isotropically shifted NMR spectrum. It should also experience a downfield shift, although possibly a smaller one if the C–H is nearly coplanar with the COO^- plane. Preliminary NOE experiments show that irradiation of the signal at 65.5 ppm gives no appreciable nuclear Overhauser effect (NOE) on any signal closer to the diamagnetic region, while irradiation of the one at 72.2 ppm gives a strong negative NOE on a signal at 11.7 ppm downfield. These results would favor the assignment of the signals at 72.7 and 11.7 ppm as β-CH_2 protons of a bidentate Asp-81. The bidentate nature of Asp-81 proposed here on the basis of NMR spectroscopy is consistent with a similar conclusion based on the results of electronic spectroscopy (see above).

The three broad signals at 90, 45, and 24.2 ppm in $Ag^I_2Ni_2SOD$ (88.9, 44.8, and 25.4 ppm in $Cu^I_2Ni_2SOD$), having T_1 values of 1–2 ms at 300 MHz (Table I), can be assigned to ortho-like protons of the coordinated histidines due to their proximity to Ni^{2+} in the zinc site. The observation of signals from three ortho- and meta-like protons and three N–H protons indicates that the histidine ligation in the Ni^{2+} binding site of these two derivatives is the same as that of the native zinc site, i.e., that all three histidines are coordinated to the metal ion through the N_δ1 nitrogen. The signals in the upfield region may be due to the histidine β-CH_2 protons if spin density is in the p_π orbital of the imidazole ring.[1]

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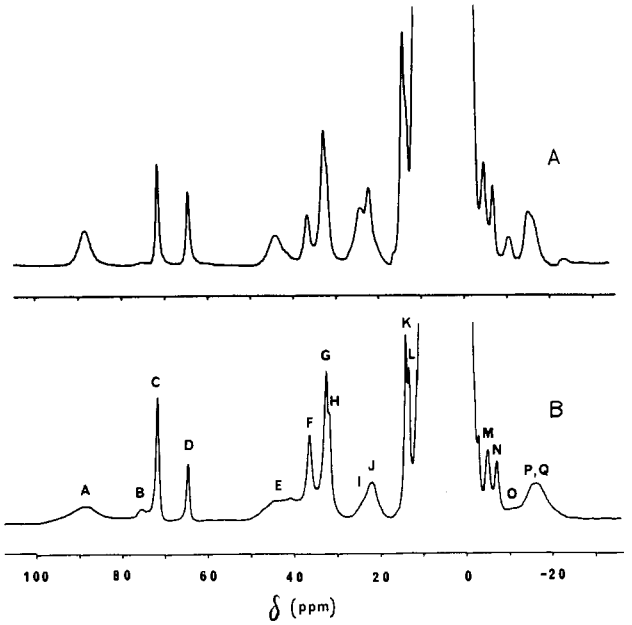

Figure 4. ^1H NMR spectra of Ag_2Ni_2SOD at (A) 90 and (B) 300 MHz and 30 °C. T_1 values of the signals at the two fields are shown in Table I.

Table I. Chemical Shifts and T_1 Values for $Cu^I_2Ni_2SOD$ (A) and $Ag^I_2Ni_2SOD$ (B) at 90 and 300 MHz[a]

shift, ppm		T_1, ms (90 MHz)		T_1, ms (300 MHz)
A	B	A	B	B
88.9	90	9.9	5.9	1.2
76.5	77.5	*b*	*b*	1.1[c]
72.7	72.7	40.8	31.3	6.5
66	65.5	72.9	73.0	18.1
44.8	45	9.7	5.0	2.0
37.5	36.7	41.9	33.3	12.3
32.7	32.7	52.7		9.8
32.1	32			11.7
25.4	24.2	14.8	8.9	
23	21.7		12.7	4.8
14.2				20
13.3				18
-5.5	-5			11.3
-7.2	-7.1	54.4		23.8
-10.4	-11		11.4	<2
-14.3	-15.5		~10.9	~4.6
-17.6	-16.5			

[a] In 50 mM phosphate buffer at pH 6.5, 30 °C. [b] Not observed due to fast exchange with solvent; see text. [c] The small relaxation time of the signal is due to fast solvent exchange.[11]

The spectra of $Ag^I_2Ni_2SOD$ in H_2O at 90 and 300 MHz are reported in Figure 4. An increase in the line width of various signals with magnetic field may arise from Curie relaxation, which is more effective at higher magnetic field.[1] As a consequence, the protons closest to the paramagnetic center (i.e. the ortho-like protons) would display a larger increase of the line width with the field. The assignment proposed above that the broad signals at 90, 45, and 24.2 ppm (of Ag_2Ni_2SOD) are ortho-like protons is consistent with this theoretical prediction.

The T_1 values of $Ag^I_2Ni_2SOD$ at 90 and 300 MHz, along with those of $Cu^I_2Ni_2SOD$ at 90 MHz, are reported in Table I. It shows that the T_1 values are much longer at low field and decrease significantly at 300 MHz. This observation is unprecedented in previous NMR studies on paramagnetic metalloproteins with either mono- or dimetallic centers. It has been shown that water proton relaxation rates in some paramagnetic Ni^{2+} complexes are low and substantially field-independent up to about 10 MHz and then increase dramatically with magnetic field.[16-19] The small

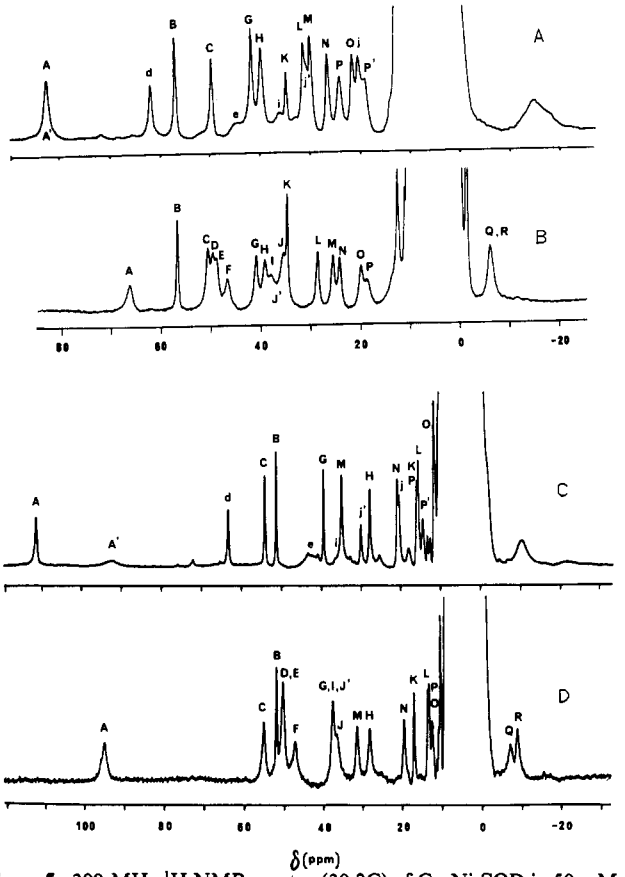

Figure 5. 300-MHz ^1H NMR spectra (30 °C) of Cu_2Ni_2SOD in 50 mM phosphate buffer at pH 6.5 (A) and Cu_2Co_2SOD in 10 mM acetate buffer at pH 5.5 (B) and those of $Cu_2Ni_2SOD + N_3^-$ (C) and $Cu_2Co_2SOD + N_3^-$ (D) under the same conditions as the above derivatives.

and almost constant relaxation rate at low magnetic field may be due to the presence of a sizable static zero-field splitting,[18,19] and the increase in the relaxation rate at higher field is due to the decrease in the electronic relaxation rates that results from the less efficient modulation of the zero-field splitting.[20] Therefore, the dependence of the nuclear relaxation rate on field in $Ag^I_2Ni_2SOD$ is likely due to an increase in the electronic relaxation time of Ni^{2+} in the zinc site, and the increase in the line width with field mentioned above would obviously be accounted for by the contribution of the dipolar coupling to T_2.

^1H NMR Spectrum of $Cu^{II}_2Ni^{II}_2SOD$. The proton NMR spectrum of Cu_2Ni_2SOD shows many well-resolved signals at 300 MHz, as shown in Figure 5. It is known that the protons of a ligand coordinated to Cu^{2+} would be excessively broad to be detected in most cases because of the relatively slow electronic relaxation rates.[1] However, the protons of histidines coordinated to both Cu^{2+} and Ni^{2+} ions are detected in this derivative because of the presence of magnetic coupling between these two metal ions, which provides an efficient relaxation mechanism for the copper(II) unpaired electron. According to the Solomon–Bloembergen equation, the line widths of the signals are proportional to the $S(S + 1)$ value of metal ions and can be used as a criterion for assigning the resonances.[1,21] However, it is difficult to differentiate between the ^1H NMR signals of the histidines coor-

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Table II. NMR Parameters for Bovine Cu_2Ni_2SOD and Cu_2Co_2SOD at 300 MHz and 30 °C

		shift $(T_1)^b$		
			Cu_2Ni_2SOD	
signal	proton[a]	Cu_2Co_2SOD	Cu^{2+} site	Zn^{2+} site[c]
A	His-61 $H_\delta 2$	66.2 (1.1)	82.4 (3.9)	
A′	His-61 $H_\epsilon 1$	*d*	83	
B	His-118 $H_\delta 1$	56.5 (4.1)	56.5 (11.5)	
C	His-46 $H_\delta 1$	50.3 (*e*)	49.1 (14.5)	
D	His-78 $H_\delta 2$	49.4 (3.1)		61.5 (14.8)
E	His-69 $H_\delta 2$	48.8 (3.1)		44.7 (3.1)
F	His-78 $H_\epsilon 2$	46.7 (*e*)		
G	His-46 $H_\epsilon 1$	40.6 (2.8)	41.1 (14.9)	
H	His-118 $H_\epsilon 1$	39.0 (1.7)	39.2 (6.9)	
I	His-78 $H_\epsilon 1$	37.4 (1.4)		35.8 (3.0)
J′	His-69 $H_\epsilon 1$	35.6 (1.6)		30.2 (*e*)
J	His-69 $H_\epsilon 2$	35.4 (*e*)		19.8 (14.2)
K	His-44 $H_\epsilon 2$	34.5 (4.5)	34.4 (28.7)	
L	His-44 $H_\delta 2$	28.4 (4.2)	30.8 (15.3)	
M	His-46 $H_\delta 2$	25.3 (2.5)	29.3 (8.8)	
N	His-118 $H_\delta 2$	24.1 (2.5)	26.0 (9.6)	
O	His-44 $H_\epsilon 1$	19.6 (2.4)	21.1 (12.2)	
P	His-44 $H_\beta 1$	18.7 (1.2)	23.7 (3.8)	
P′	His-44 $H_\beta 2$	*d*	18.6 (10.9)	

[a] These assignments are tentative and are based on the relative T_1 values measured for Cu_2Co_2SOD. The assignment of each group of protons to a single histidine is based on their behavior during anion titrations. [b] Chemical shift in ppm from TMS; T_1 in ms. [c] Not assigned; correlated to the protons in the Co^{2+} binding site of Cu_2Co_2SOD (see text). [d] Not observed. [e] Not measurable because of signal overlap.

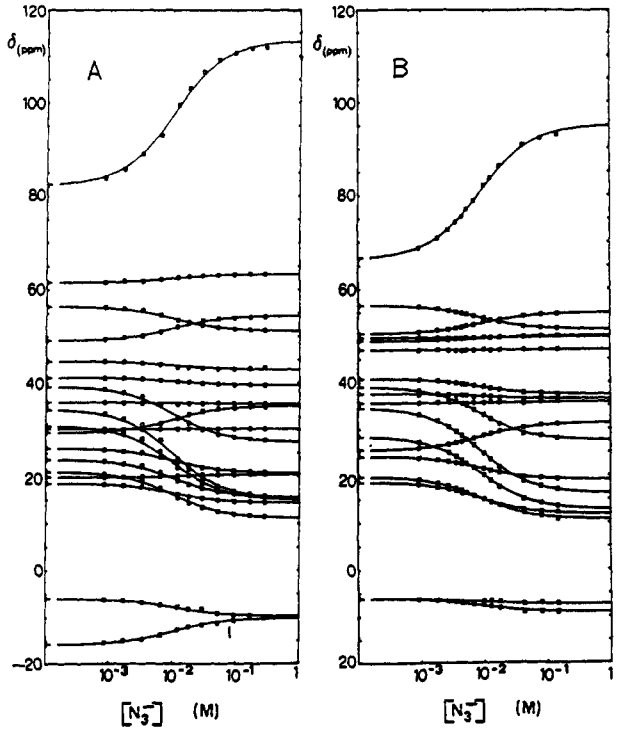

Figure 6. Plots of ^1H NMR chemical shift (300 MHz, 30 °C) against $[N_3^-]$ for Cu_2Ni_2SOD (A) in 50 mM phosphate buffer, pH 6.5, and for Cu_2Co_2SOD (B) in 10 mM acetate buffer, pH 5.5. Signal A′ is not shown in (A).

dinated to either metal ion by this method because the different electronic relaxation times of the two metal ions in the magnetically coupled system result in different values for the correlation function, $f(\tau_c)$, of the two metal ions. This difficulty has been resolved in the study of Cu_2Co_2SOD by Bertini and co-workers by modifying the Solomon–Bloembergen equation to account for the different electronic states in the magnetically coupled species with total spin S'. In the modified equation, reduction coefficients were applied to the $S(S+1)$ values of each metal ion (i.e. $^3/_8$ for Cu^{2+} and $^7/_8$ for Co^{2+}) and the electronic relaxation times of the $S'=2$ and the $S'=1$ multiplets for this magnetically coupled system were assumed to be the same.[22]

Assuming that the dipolar contribution to the isotropic shifts of the protons in the copper site caused by Ni^{2+} in the zinc site is relatively minor,[23] the signals of the coordinated histidines in the copper site of this derivative should fall in the same spectral region as those of the Cu_2Co_2SOD derivative as shown in Table II. The assignment of the signals to the histidines in the copper site is also supported by the shifting of the signals upon titration with azide, N_3^-, which is known to bind copper and cause the detachment of a coordinated histidine.[21,22] The exchange rate between free and bound N_3^- is fast on the NMR time scale; therefore, the signals related to the copper site shift in a typical sigmoidal manner during the titration (Figure 6). Fitting of the data to a simple equilibrium containing two species gives an azide binding constant of 111 ± 5 M^{-1} at 30 °C. The value is very close to that of Cu_2Co_2SOD (150 ± 5 M^{-1}) in 10 mM acetate at pH 5.5 and 30 °C, indicating similar mechanisms of azide interacting with both derivatives.

The signals in Figure 5 are labeled in such a way that each label indicates the correlated signals in both Cu_2Ni_2SOD and Cu_2Co_2SOD. Signals B, C, and K are assigned to N–H protons of the three histidines bound to copper, signals G, H, M, N, and O are assigned to the ortho-like histidine protons, and signal L is assigned to a meta-like histidine proton. This assignment is tentative and is based on the relative T_1 values measured for the Cu_2Co_2SOD derivative.[22] Signal L of the latter compound, having the longest T_1 value among the protons of the copper domain, was assigned to the unique meta-like histidine ring proton belonging to His-44.[22] Signals L, O, P, and K are considered to be from the same histidine because of similar characteristics upon azide titration, moving into the diamagnetic region at the edge of the protein backbone resonances. Signal P is assigned to one of the β-CH_2 protons of His-44. Most of the signals have chemical shifts close to, within ±1 ppm out of a total spectral width of about 100 ppm, those of the correlated signals in Cu_2Co_2SOD (Table II). This observation indicates that the isotropic shifts of the coordinated histidines in the copper site have obtained little dipolar contribution from Ni^{2+} in the zinc site, which is consistent with the assumption made above.

If the T_1 values are taken into account, signal G would be assigned to a proton of the coordinated histidines in the nickel binding site owing to its relatively long T_1 value compared to that of other ortho-like protons in the copper site. However, the coincidence of its chemical shift and its properties upon azide titration with those of signal G in Cu_2Co_2SOD suggests that this signal is preferentially assigned to a proton in the copper site. Also at variance with the results for Cu_2Co_2SOD, signal L for Cu_2Ni_2SOD has a T_1 value similar to those of the other protons of the copper domain. The signal labeled P′, which has no correlated pair in the Cu_2Co_2SOD derivative, is significantly influenced upon N_3^- titration and moves into the diamagnetic region, which strongly suggests that the signal can be assigned to a proton in the copper site, possibly to the second β-CH_2 proton of the His-44 residue.

The isotropically shifted signals associated with the protons in the nickel binding site should not be greatly influenced by the presence of anions. Therefore, the signals at 19.8 and 30.2 ppm are assigned to N–H protons of the histidines coordinated to Ni^{2+}; however, the assignment of the latter one is obscured owing to its smaller reduction in intensity in D_2O. The observation of two N–H signals at 23 and 32.1 ppm in the $M^1_2Ni_2SOD$ derivatives also suggests that N–H protons of the histidines coordinated to Ni^{2+} in the zinc site may be found in this region. Three broad and ill-shaped signals are observed at 44.7, 35.8, and 32.6 ppm in this derivative, which could be assigned to the ortho-like protons of coordinated histidines in the nickel binding site. The azide titration reveals that there is a broad signal under signal H, which

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Table III. Experimental and Calculated[a] T_1 Values for the Copper-Domain Protons in Cu_2Ni_2SOD

	T_1, ms			T_1, ms	
	expt	calc		expt	calc
His-44 $H_\epsilon 1$	12.2	11.0	His-46 $H_\delta 2$	8.8	11.6
His-44 $H_\epsilon 2$	28.7	16.7	His-61 $H_\delta 2$	3.9	4.0
His-44 $H_\delta 2$	15.3	16.4	His-61 $H_\epsilon 1$		3.1
His-44 $H_\delta 1$	3.8	3.8	His-118 $H_\delta 1$	11.5	19.1
His-46 $H_\delta 1$	14.5	19.5	His-118 $H_\epsilon 1$	6.9	8.6
His-46 $H_\epsilon 1$	14.9	12.1	His-118 $H_\delta 2$	9.6	11.6

[a] Calculated by using the equation for magnetically coupled systems (see ref 27); $\tau_c(Cu) = 3 \times 10^{-12}$ s; $\tau_c(Ni) = 1 \times 10^{-12}$ s; $LC(Cu) = 4.5 \times 10^{44}$ cm^{-6}. The correspondence is based on the assignment of Cu_2Co_2SOD.

may also be due to one of the ortho-like protons in the nickel binding site.

There is only one signal, signal d at 61.5 ppm, originating from the nickel site in this derivative above 60 ppm downfield, while $M^I_2Ni_2SOD$'s have three, one of which is solvent exchangeable. Therefore, it could correspond to the signal at either 72.7 or 65.5 (66) ppm in $M^I_2Ni_2SOD$. It may be concluded that the solvent-exchangeable signal at 76.5 (77.5) ppm and one of the two sharp signals above 60 ppm in $M^I_2Ni_2SOD$ are from the protons of His-61 that shift to different resonating positions upon the formation of the bridge in $Cu^{II}_2Ni_2SOD$. Signal A, after the assignment of all other signals in the copper binding site, can be assigned to the bridge His-61. The difference of the isotropic shifts of signal A between Cu_2Ni_2SOD and Cu_2Co_2SOD derivatives results from the fact that isotropic shifts are not determined by the copper ion alone, but by the metal ion in the zinc site as well. There is a broad signal, A', underneath signal A, which can be easily observed in the presence of azide (Figure 5C). This signal can be assigned to the $H_\epsilon 1$ proton of the bridge His-61, which is at the ortho-like position with respect to both metal ions. It was not detected in Cu_2Co_2SOD presumably due to its broadness in this derivative (Figure 5).[21]

When the spectrum of Cu_2Ni_2SOD was taken at various pH's, a shifting of the signals was observed at pH > 10, especially the signals assigned to His-44. This result indicates that the hydroxide anion may have the same characteristics as other anions upon binding to SOD, i.e. replacing a basal histidine (not the bridging His-61[21,24]) without displacing the axial bound water.[25]

The proton-relaxation rates of the histidines coordinated to copper in Cu_2Ni_2SOD are much smaller than those in Cu_2Co_2SOD (Table II). This is due to a shorter correlation time of the proton–unpaired electron coupling in the Cu_2Ni_2SOD derivative. Assuming that the coupling of the unpaired electron with the ortho-like protons of the coordinated histidines is dipolar in origin,[26] an electronic relaxation time, τ_s, for copper of 3×10^{-12} s and that for nickel of 1×10^{-12} s can be estimated by using appropriate coefficients for the Solomon equation.[22,27] The meta-like protons are proposed to be relaxed by both metal-centered and ligand-centered mechanisms because the ratios of the relaxation rates of CH_{ortho}/CH_{meta} protons are smaller than those expected for a purely metal-centered relaxation mechanism.

The experimental and calculated T_1 values for the protons in the copper domain are reported in Table III, the Cu–proton distances being determined from the X-ray structure of the native protein.[3] The fitting of the nuclear relaxation times is quite satisfactory, which suggests that the theoretical model proposed is substantially correct. Larger discrepancies are observed for signals B, C, and K, which are assigned to the N–H protons of His-118, His-46, and His-44. These differences may be explained by the changes in H-bonding distances with other residues or with the protein backbone, by changes in ligand-centered effects on different nuclei, or by different solvent-exchange rates.[11] Theoretical fitting of T_1 was not performed for the protons in the nickel binding site because the two resolved ortho-like signals had similar T_1 values (3.0 vs 3.1 ms) and the only resolved meta-like signal at 19.8 ppm was an N–H proton that would not give a satisfactory fitting to the experimental data, as was the case also for the N–H protons in the copper site indicated above.

Concluding Remarks

The four- or five-coordinate nickel(II) ion is known to have very short τ_s values due to an almost orbitally degenerate ground state. It is estimated to be 1×10^{-12} s for Cu_2Ni_2SOD in this study at 300 MHz. It is also found that the electronic correlation time in these species is magnetic field dependent. The magnetic coupling between nickel and copper changes the "effective" electronic relaxation time of Cu^{2+}, which is 2×10^{-9} s in native enzyme, to about 3×10^{-12} s. This result indicates that the lower limit of the magnetic coupling constant in this derivative is ~ 2 cm^{-1}. Since the isotropic shifts experienced by the protons of histidines in the copper domain are the same or slightly larger in Cu_2Ni_2SOD compared with those in Cu_2Co_2SOD, we may conclude that the magnetic coupling constant of the former derivative is much smaller than kT. We note here that the separation between the $S' = 1$ ground state and the $S' = 2$ excited state is 33 cm^{-1} in Cu_2Co_2SOD.[28] The isotropically shifted NMR signals are sharper in Cu_2Ni_2SOD than in Cu_2Co_2SOD because the τ_s of copper is shortened more in the former derivative by the magnetic coupling. The neglect of the zero-field splitting of Ni^{2+}, as well as of the magnetic coupling, does not permit a more accurate estimation of the above values.

Native metal ion(s) in most metalloproteins can be replaced by other foreign metal ions, including Ni^{2+}. Study of Ni^{2+}-substituted derivatives of Cu,Zn-SOD shows that the metal binding environment is not significantly changed as a consequence of Ni^{2+} substitution and that the mode of histidine binding to the metal ion, i.e. $N_\delta 1$ or $N_\epsilon 2$ coordination, can be unambiguously assigned by signal widths and relaxation time measurements. Therefore, this study provides a model for investigation of the nature of the metal binding site(s) of other metalloproteins by use of Ni^{2+} as a paramagnetic probe.

Acknowledgment. Financial support from the USPHS (GM 28222) is gratefully acknowledged. This work has also been performed with a contribution from the Progetto Strategico Biotecnologie del CNR and a travel grant from NATO.

Registry No. SOD, 9054-89-1; Ni, 7440-02-0; Ag, 7440-22-4.

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