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NMR Study of Cobalt(II)-Substituted Yeast and Human Copper-Zinc Superoxide Dismutase

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A recent study of yeast Cu,Zn superoxide dismutase (Cu_2Zn_2YSOD) came to the surprising conclusion that the two subunits of YSOD, unlike those of bovine Cu,Zn-SOD, were inequivalent. This conclusion was based on studies by Dunbar et al. of the addition of cobaltous ion to apo-YSOD using optical absorption spectroscopy. We report here the results of a new study of cobaltous-substituted derivatives of Cu_2Zn_2YSOD and of human Cu_2Zn_2SOD using isotropically shifted ^1H NMR spectroscopy and ^{31}P relaxation. Results similar to those obtained previously in studies of bovine Cu,Zn-SOD were obtained; i.e., apo-YSOD binds 2 equiv of Co^{2+} in acetate buffer at pH 5.5 and 4 equiv in phosphate (or HEPES) at pH 7.0. We find that the incorporation of 2 equiv of Cu^{2+} into E_2Co_2YSOD has a strong effect on both the electronic absorption and NMR spectra due to Co^{2+} bound at the zinc-binding site. This result differs from that reported by Dunbar et al., but it is consistent with the results obtained with Cu,Zn-SOD's from other species. It is our conclusion that yeast Cu_2Zn_2SOD is similar to bovine and human Cu_2Zn_2SOD in that it is comprised of equivalent metal-binding sites in each subunit.

Copper-zinc superoxide dismutase (Cu,Zn-SOD)[1] is a metalloenzyme found in almost all eukaryotic cells and in some bacteria that catalyzes the disproportionation of superoxide to give dioxygen and hydrogen peroxide ($2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$). Most studies of this enzyme, including an X-ray crystal structure,[2] have been carried out on the bovine enzyme, which has been found to consist of two equivalent subunits, each of which contains a Cu^{2+} and a Zn^{2+} ion bound in close proximity, bridged by the imidazolate ring of a histidyl residue.

It is clear from the amino acid sequences of Cu,Zn-SOD's from other species that the protein has been highly conserved throughout evolution.[3] In particular, the amino acid sequence of human Cu,Zn-SOD shows 82% homology[4] and that of yeast 55%[5] to the sequence of the bovine enzyme.[6] In addition, the physical and catalytic properties of Cu,Zn-SOD's from other eukaryotic sources appear to be quite similar and their spectroscopic properties, and those of metal-substituted derivatives, indicate that the metal-binding regions are highly conserved.[7] For these reasons, it has generally been assumed that most, if not all, of the Cu,Zn-SOD's from various sources are very similar to the bovine protein and, therefore, that they have similar three-dimensional structures and contain two equivalent subunits. However, a series of papers has appeared since 1980 in which it has been proposed that yeast Cu,Zn-SOD is comprised of two *inequivalent* subunits.[8-11] The principal pieces of evidence presented to support this conclusion were based on a study of reactions of yeast apo-Cu,Zn-SOD with Co^{2+} followed by optical absorption spectroscopy.[11] These authors concluded that yeast apo-Cu,Zn-SOD binds only 1 equiv of Co^{2+} in acetate and 2 equiv of Co^{2+} in phosphate per protein dimer. They concluded from these results that the metal-binding regions of the two subunits of each protein dimer were inequivalent.

Isotropically shifted ^1H NMR studies of Co^{2+}-substituted proteins have been quite useful in elucidating the nature of the metal-binding sites.[12,13] Moreover, it has recently been shown that substitution of Co^{2+} for Zn^{2+} in native bovine Cu,Zn-SOD gives a derivative, Cu_2Co_2SOD, that has a ^1H NMR spectrum consisting of isotropically shifted signals from amino acid residues bound to both Cu^{2+} and Co^{2+} ions.[14] An isotropically shifted proton NMR spectrum of a paramagnetic species is highly dependent upon the distance between a resonating nucleus and metal ion, the geometry of the metal-binding site, and the electronic structure of the metal ion;[13,15] therefore, it provides an elegant method to investigate a minor perturbation at the metal-binding region of a metalloprotein and also to compare the metal-binding regions of a metalloprotein from different species. We have reinvestigated the Co^{2+}-substituted derivatives of yeast Cu,Zn-SOD using NMR spectroscopy and relaxation studies. The results obtained by these methods are similar to those obtained with the bovine[14,16,17] and human proteins.[18] We conclude from these results that the two subunits in each of these Cu,Zn-SOD's are equivalent. In addition, we have obtained evidence from NMR relaxation studies that the metal ion in the native copper site, but not that in the native zinc site, has an open coordination position for binding of anions.

Experimental Procedures

Yeast Cu,Zn-SOD was purchased from Pharmacia, Copenhagen, Denmark. Human Cu,Zn-SOD, made from the human SOD gene expressed in yeast, was a gift from Dr. R. A. Hallewell (Chiron Corp.). All other chemicals are commercially available. The preparation of apoproteins followed that of bovine Cu,Zn-SOD,[19] and the concentration was determined by Lowry assay.[20] The metal content of apoproteins prepared as above was less than 5% of that of the native proteins, measured

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(1) Abbreviations: Cu,Zn-SOD, copper-zinc superoxide dismutase; NMR, nuclear magnetic resonance; YSOD, yeast SOD; $M_2M'_2$SOD, M- and M'-substituted SOD with M in the copper site and M' in the zinc site (E in the above derivatives represents an empty site); DEFT, driven equilibrium Fourier transform; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N'*-ethanesulfonic acid; T_1, spin-lattice relaxation time; EXAFS, extended X-ray absorption fine structure.

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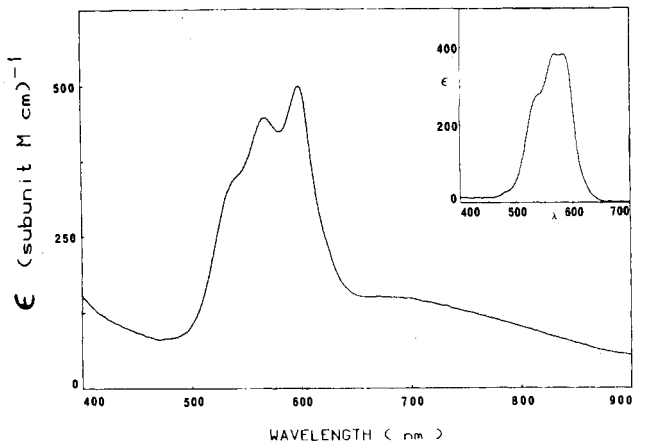

Figure 1. Electronic spectrum of yeast Cu_2Co_2SOD in 10 mM acetate at pH 5.5 at room temperature referenced against deionized water. The inset is yeast E_2Co_2SOD under the same conditions.

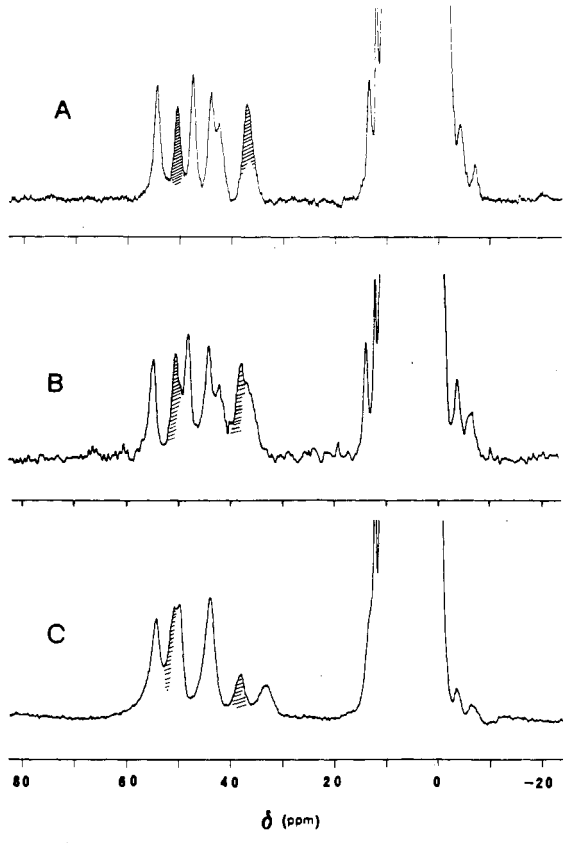

Figure 2. ^1H NMR spectra (200 MHz, ambient temperature) of bovine (A), human (B), and yeast (C) E_2Co_2SOD in 10 mM acetate at pH 5.5. The shaded signals are solvent-exchangeable.

by atomic absorption spectroscopy on a Varian SpectAA 30 spectrometer. Varying amounts of Co^{2+} from a standard 1000 ppm $CoCl_2$ solution (Ricca Chemical Co., Arlington, Texas) were infused into apo-SOD's to prepare Co^{2+}-substituted derivatives with different metal ion contents for different studies. The Cu_2Co_2SOD's were prepared by directly infusing 2 equiv of Co^{2+} into apoproteins in 10 mM acetate buffer at pH 5.5 and incubating overnight at 4 °C, followed by addition of 2 equiv of Cu^{2+}. The reconstitution of human Cu_2Co_2SOD under these conditions is a very slow process, which takes days to reach completion. All the derivatives were concentrated by ultrafiltration prior to NMR experiments using a Centricon microconcentrator with a molecular weight cutoff of 10 000 D (Amicon Corp., Danvers, MA). The electronic spectra were taken on a Beckman UV5270 spectrometer at room temperature.

The proton NMR spectra were recorded on Bruker WP200, IBM AF200, and Bruker CXP300 spectrometers using the modified DEFT pulse sequence[21] in order to suppress H_2O (HDO) and bulk protein

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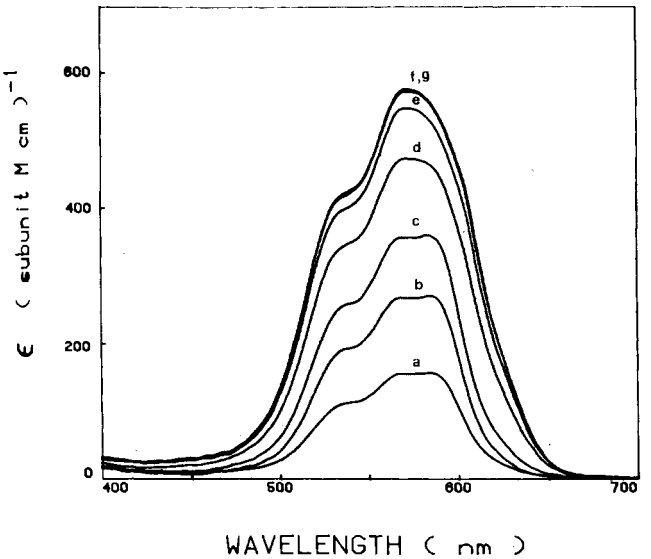

Figure 3. Electronic spectra of yeast apo-SOD in 20 mM phosphate buffer at pH 7.0 immediately after infusing, from a to g, 0.68, 1.2, 1.8, 2.7, 3.6, 4.2, and 5.0 equiv of Co^{2+} into the solution. The ^{31}P relaxivity of a, b and d, e after a few days' incubation at 4 °C are reported in Table I.

signals. Typical spectra consisted of 5000–20 000 scans with 8K or 16K data points over a sufficiently wide bandwidth to cover all the signals, which varied from 40 000 to 100 000 Hz. Chemical shifts were measured from the H_2O (HDO) signal, assumed to be at 4.8 ppm from TMS. A 20-Hz additional line broadening was introduced to all the spectra by exponential multiplication of the FID's to improve the signal-to-noise ratio. Proton spin–lattice relaxation time at 300 MHz was determined by the modified DEFT sequence by varying the delay time between subsequent pulses, and the values were determined by a nonlinear least-squares fitting of the intensity as a function of the delay time.[21] Phosphorus spin–lattice relaxation times were determined at 202.5 MHz on a Bruker AM500 spectrometer at 298 K using the inversion-recovery pulse sequence.

Results

Cobalt-Substituted Yeast SOD's. The apo-YSOD prepared as described in the Experimental Procedures had essentially the same $E^{1\%}$ value at 279 nm of 1.4 as reported by Dunbar et al.[11] Addition of 2 equiv of Co^{2+} to apo-YSOD in 10 mM acetate buffer at pH 5.5 gives a solution with the characteristic spectrum of E_2Co_2SOD (see insert, Figure 1), i.e. an absorptivity of 380 M^{-1} cm^{-1} per subunit at 580 nm, which is very close to that of bovine E_2Co_2SOD, 370 ± 30 M^{-1} cm^{-1} per subunit at 583 nm.[22] The proton NMR spectrum of this derivative is also very similar to those of the bovine[14] and human proteins as shown in Figure 2. All the correlated resonances among the derivatives have a standard deviation of chemical shift of <1 ppm (out of a spectral width of about 70 ppm),[23a] except the one closest to the diamagnetic region (2.3 ppm). Neither the electronic nor the NMR spectra

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(23) (a) A ±2 ppm change of an isotropically shifted signal at 50 ppm could be caused by a 4% change of spin density in a proton orbital if the contact shift mechanism were the only factor dominating the isotropic shifts, a situation that usually applies to complexes having an A ground state.[15] However, sizable dipolar shifting may be observed in tetrahedral high-spin Co^{2+} complexes with a $^4A_2$ ground state because of spin–orbital coupling of the ground-state level with the low-lying excited level $^4T_2$.[23c] Thus, the change of dipolar shifts with geometry factors[13,15] $\langle(3\cos^2\theta - 1)/r^3\rangle$ and $\langle\sin^2\theta\cos 2\Omega/r^3\rangle$ may make a significant contribution to the difference in the isotropic shifts of the three derivatives. (b) The nuclear relaxation rate of protons in the coordination sphere of a paramagnetic metal ion, T_M^{-1}, is proportional to r^{-6}, where r is the distance between the resonating proton and the metal ion. An increase of the relaxation rate from 1/5.3 ms^{-1} to 1/3.6 ms^{-1} (signal K) may be accounted for by a 6.6% decrease in the distance r (e.g. from 5.0 to 4.7 Å). (c) La Mar, G. N.; Horrocks, W. D., Jr.; Allen, L. C. *J. Chem. Phys.* **1964**, *41*, 2126–2134. La Mar, G. N. *J. Chem. Phys.* **1964**, *41*, 2992–2998. La Mar, G. N. *J. Chem. Phys.* **1965**, *43*, 235–239.

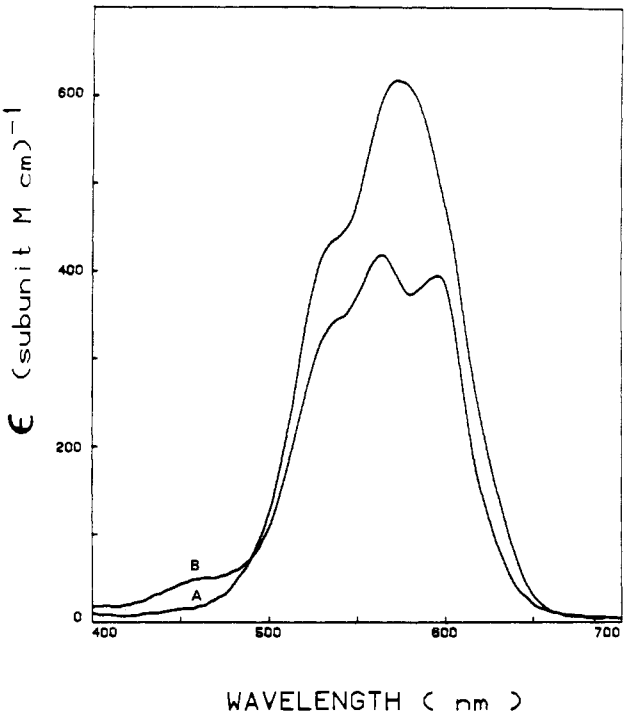

Figure 4. Electronic spectra of yeast Co_2Co_2SOD in 20 mM phosphate at pH 7.0 (A) and in 20 mM HEPES at pH 7.0 (B) after infusion of 4 equiv of Co^{2+} into apo-SOD and incubation in a cold room for several days.

Table I. ^{31}P Relaxivity (202.5 MHz, 298 K) of Yeast apo-Cu,Zn-SOD in 20 mM Phosphate Buffer, pH 7.0, with Various Amounts of Co^{2+}

amt of Co(II), equiv	[apo], mM	T_1, s	$1/T_{1p}$,[a] s^{-1}	$(1/T_{1p})/[Co_2Co_2SOD]$,[b] $s^{-1}\ M^{-1}$
0.68	0.432	5.271	0.0101	34.4
1.2	0.426	5.133	0.0152	29.7
2.7	0.410	0.082	12.02	8.35×10^4
3.6	0.401	0.058	17.06	5.32×10^4

[a] Paramagnetic contribution to the relaxation rate defined as $1/T_{1p} = 1/T_1 - (1/T_1)_0$, where $(1/T_1)_0$ (=0.1796 s^{-1}) is the diamagnetic contribution to the relaxation rate obtained by using a 0.38 mM yeast E_2Zn_2SOD solution under the same conditions. [b] ^{31}P relaxivity of the derivatives with Co^{2+}:apo < 2 is simply calculated as $(1/T_{1p})/[Co(II)]$ for comparison.

of yeast E_2Co_2SOD were found to be affected by the presence of phosphate. The electronic spectrum of Cu_2Co_2YSOD (see Figure 1) was also observed to be very similar to that of the same derivative of the bovine protein[22] and (vide infra) that of human Cu_2Co_2SOD, which had a molar absorptivity of 500 M^{-1} cm^{-1} per subunit at 600 nm after overnight incubation (which was 18% more than that of the sample immediately after addition of the metal ions, i.e. 425 M^{-1} cm^{-1} per subunit).

In 20 mM phosphate buffer at pH 7.0, apo-YSOD was observed to bind up to a total of 4 equiv of Co^{2+} (Figures 3 and 4), giving an electronic spectrum that is very similar, both in its shape and in its absorptivity of 610 M^{-1} cm^{-1} per subunit to that of bovine protein at similar conditions.[24] It is clearly shown in Figure 3 that the spectra with Co:apoprotein < 2 resemble the spectrum reported for "Co_1SOD" and those with Co:apoprotein > 2 resemble the spectrum reported for "Co_2SOD" by Dunbar et al.[11b] The paramagnetic contribution to the relaxation rate of ^{31}P in phosphate buffer, after the addition of various amounts of Co^{2+} to apo-YSOD, is shown in Table I. The paramagnetic contribution is comparatively small when Co:apoprotein < 2. There is a dramatic enhancement when the ratio is greater than 2. An

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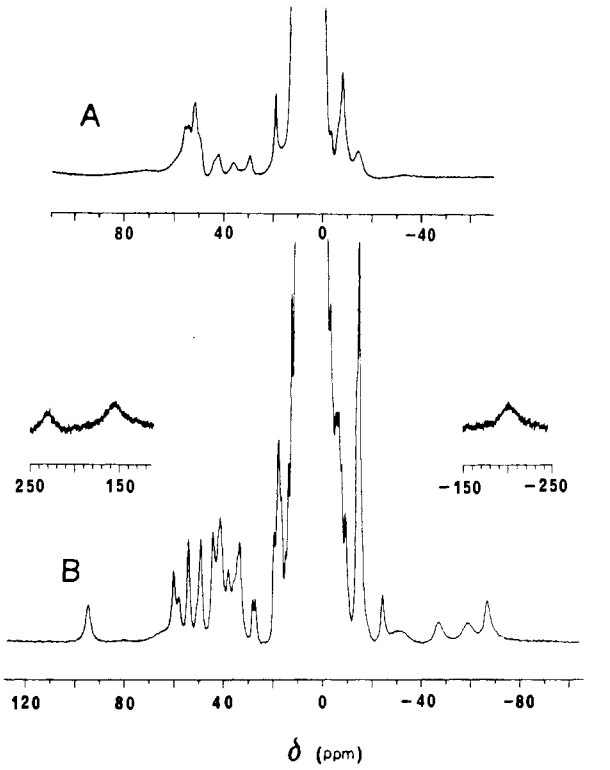

Figure 5. ^1H spectra (200 MHz, ambient temperature) of yeast Co_2Co_2SOD in 20 mM phosphate at pH 7.0 (A) and in 20 mM HEPES at pH 7.0 (B) after infusion of 4 equiv of Co^{2+} into apo-SOD and incubation in a cold room for several days.

incorporation of 4 equiv of Co^{2+} to apo-YSOD in 20 mM HEPES buffer at pH 7.0 yields a derivative, in a very slow process, that has an electronic spectrum similar to that of bovine protein[16] with absorption at 598 and 565 nm (418 M^{-1} cm^{-1} per subunit) and shoulders at 535 and 460 nm (Figure 4). This spectrum, again, is similar to that reported for "Co_2SOD" under the same conditions by Dunbar et al.[11b]

Proton NMR spectra of the four-cobalt derivatives of YSOD in the presence and absence of phosphate are shown in Figure 5. The derivative in 50 mM phosphate buffer has a spectrum with less than 10 well-separated signals between 60 and −20 ppm. This is the typical region for isotropically shifted proton signals in tetrahedral-like cobalt(II) coordination.[12,13] In the absence of phosphate, this derivative has a spectrum rich in signals that are spread over a range of 500 ppm. Some of the signals disappear when the derivative is in D_2O and can be assigned to N–H protons of the coordinated ligands or of other groups near the metal ion. In comparison to the spectrum of bovine Co_2Co_2SOD,[16] the solvent-exchangeable signals at 94.5, 59.8, and 57.7 ppm can be assigned to the coordinated histidines in the copper site; the signals at 40.9 and 34.1 ppm can be assigned to the coordinated histidines in the zinc site. The other solvent-exchangeable signals at 26.8, 17.2, and −25.1 ppm are considered to be peptide N–H protons in the proximity of Co^{2+} ion in the five-coordinated copper site on the basis that five-coordinate high-spin Co^{2+} complexes give larger dipolar shifts than comparable four-coordinate complexes.[13,15] This mechanism also explains the shifts at 232, 158, and −198 ppm. It should be noted that addition of phosphate to the solution used for Figure 5B converts that spectrum to that of Figure 5A and that removal of phosphate from the solution used for Figure 5A by ultrafiltration converts that spectrum to that of Figure 5B. Similar observations have previously been reported for bovine Co_2Co_2SOD.[17]

^1H NMR Study of Cu,Co Derivatives. The electronic spectra of all the Cu_2Co_2SOD's are essentially the same independent upon the sources of SOD[22] (Figures 1 and 6). The spectra of these derivatives show a tetrahedral environment of Co^{2+} ions and also the presence of a bridging histidine.[17]

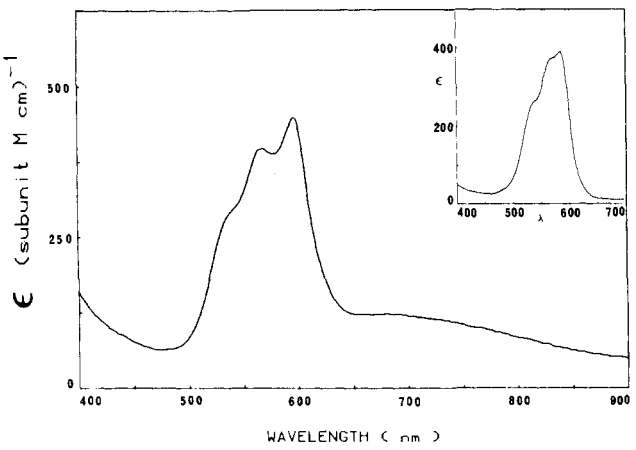

Figure 6. Electronic spectrum of human Cu_2Co_2SOD in 10 mM acetate at pH 5.5 at room temperature referenced against deionized water. The inset is human E_2Co_2SOD under the same conditions.

Table II. NMR Parameters of the ^1H Signals of Bovine, Human, and Yeast Cu_2Co_2SOD at 300 MHz and 303 K

		shift[a] (T_1)[b]		
signal	proton	bovine	human	yeast
A	His-61 Hδ2	66.2 (1.1)	66.0 (1.0)	66.3 (1.3)
B	His-118 Hδ1	56.5 (4.1)	56.4 (6.2)	57.6 (5.5)
C	His-46 Hδ1	50.3 (*c*)	~50	46.9 (2.5)
D	His-78 Hδ2	49.4 (3.1)	49.4 (2.7)	49.2 (3.0)
E	His-69 Hδ2	48.8 (3.1)	48.8 (*c*)	49.2
F	His-78 Hε2	46.7 (*c*)	46.2 (1.9)	46.9 (2.5)
G	His-46 Hε1	40.6 (2.8)	40.9 (3.0)	41.5 (2.0)
H	His-118 Hε1	39.0 (1.7)	38.5 (1.4)	~41 (2.0)
I	His-78 Hε1	37.4 (1.4)	36.3 (*c*)	37.5 (1.2)
J′	His-69 Hε1	35.6 (1.6)	~36 (*c*)	~33 (*c*)
J	His-69 Hε2	35.4 (*c*)	35.6 (*c*)	~35 (*c*)
K	His-44 Hε2	34.5 (4.5)	34.6 (3.6)	34.5 (5.3)
L	His-44 Hδ2	28.4 (4.2)	28.3 (3.6)	28.1 (3.9)
M	His-46 Hδ2	25.3 (2.5)	25.5 (2.4)	24.2 (2.5)
N	His-118 Hδ2	24.1 (2.5)	23.6 (2.3)	25.4 (2.4)
O	His-44 Hε1	19.6 (2.4)	19.6 (2.0)	19.8 (2.5)
P	His-44 Hβ1	18.7 (1.2)	18.3 (1.5)	17.7 (<1)
Q, R		−6.2 (2.2)	−5.6 (2.4)	−6.1 (2.9)

[a] In ppm from TMS. [b] In ms. [c] Not measurable because the signal is within a complex envelope.

The ^1H NMR spectrum of human Cu_2Co_2SOD in 10 mM acetate at pH 5.5 is quite similar to that of the bovine protein with respect to both chemical shift and T_1 values (Figure 7 and Table II). The ^1H NMR spectrum of Cu_2Co_2YSOD under the same conditions looks slightly different from those of the other two proteins. Specifically, the pairs of resonances D and E, C and F, G and H, and J and K all overlap in the YSOD spectrum. However, the chemical shifts and T_1 values[23b] of the signals of Cu_2Co_2YSOD are still close to those of the correlated signals of bovine and human SOD's (Table II). All the correlated signals of the three derivatives have standard deviations of the chemical shift of <1 ppm, except those at 50.3, 39.0, and 35.6 ppm (of the bovine derivative), which have standard deviations of <2 ppm (out of a spectral width of about 80 ppm).[23a]

Both human and yeast Cu_2Co_2SOD's bind azide (N_3^-), which exchanges rapidly with free azide in solution on the NMR time scale. The shift of each signal with respect to different amounts of azide can be followed and is reported in Figure 8. A fitting of the data to the simple equilibrium

$$SOD + N_3^- \overset{K}{\rightleftharpoons} SOD\text{–}N_3^- \qquad (1)$$

gives $K = 151 \pm 5$, 154 ± 8, and 521 ± 31 M^{-1} for bovine, human, and yeast proteins, respectively. This fitting assumes the equilibrium occurs rapidly as compared to the NMR time scale. The presence of azide causes signals A, K, and L of human protein to split (Figure 9); however, this phenomenon is less significant

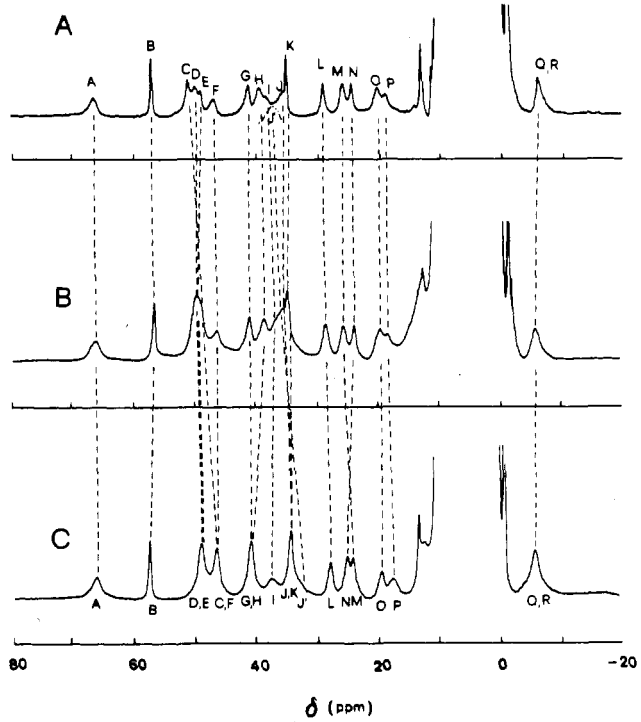

Figure 7. ^1H NMR spectra (300 MHz, 303 K) of bovine (A), human (B), and yeast (C) Cu_2Co_2SOD in 10 mM acetate at pH 5.5. Signals, B, C, F, J, and K are solvent-exchangeable. The correlation of individual signals of human and yeast derivatives with those of bovine protein is shown by dotted lines.

Table III. Chemical Shifts[a] and T_1 Values[b] of Cu_2Co_2SOD in the Presence of Saturating Amounts of N_3^- at 300 MHz and 303 K

	shift[c] (T_1)[d]		
signal	bovine	human	yeast
A	95.1 (<1)	94.6 (<1)	98.6 (<1)
B	51.2 (5.8)	51.3 (7.1)	50.5 (5.4)
C	55.0 (1.4)	54.9 (2.8)	51.3 (3.4)
D	49.7 (1.9)	49.9 (3.1)	49.7 (3.8)
E	50.1 (1.9)	50.1 (3.1)	48.9 (3.4)
F	46.9 (1.8)	46.9 (3.1)	46.5 (3.2)
G	37.3 (2.9)	37.8 (3.4)	37.2 (3.6)
H	27.3 (2.3)	27.1 (2.4)	27.1 (2.1)
I	36.3 (-)		35.2 (1.8)
J′	35.7	35.8	33.7
J			33.7
K	15.9 (19.1)	16.3, 16.0[e] (18, 20)	13.6 (19.2)
L	12.5 (10.9)	12.8, 12.6[e] (10)	10.5 (7.3)
M	31.4 (2.3)	31.9 (1.8)	30.1 (2.3)
N	18.9 (2.9)	18.9 (3.3)	18.7 (3.5)
O	10.5 (2.8)	9.9 (2.6)	10.6 (7.3)
P	11.6 (8.7)	9.7	10.5
Dia′	13.1	13.1	
Dia′		12.4	
Q	−9.2 (-)	−6.9 (2.4)	−8.1 (-)
R	−7.4 (-)	−8.3 (-)	−10.0 (-)

[a] Obtained from the best fit analysis of the data in Figure 8. [b] Measured on the spectra reported in Figure 10. [c] In ppm from TMS. [d] In ms. [e] The splitting of this signal is discussed in the text (see also Figure 9).

at high azide concentrations. The relative intensity of the split signals is not consistent from various samples. Even an azide titration performed on the same protein sample after a period of time, after the azide was dialyzed from the protein, had a different relative intensity of the split signals. When this process was performed on a derivative prepared from the SOD obtained directly from human erythrocytes, no splitting of those signals was observed. With a saturating amount of azide, the NMR spectra of all the derivatives were quite similar, especially the human and the bovine Cu_2Co_2SOD's as shown in Figure 10 and Table III. Some of the signals of YSOD move into a more crowded region

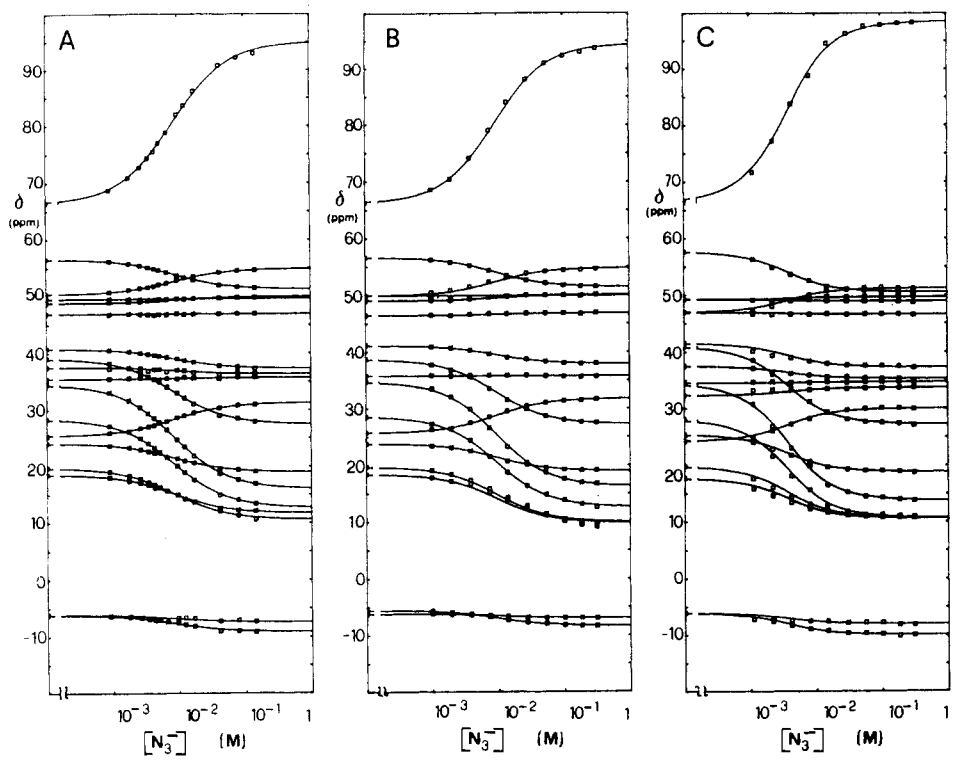

Figure 8. Azide titration of bovine (A), human (B), and yeast (C) Cu_2Co_2SOD under the same conditions as in Figure 7. The curves are the best fit of the data to eq 1 in text.

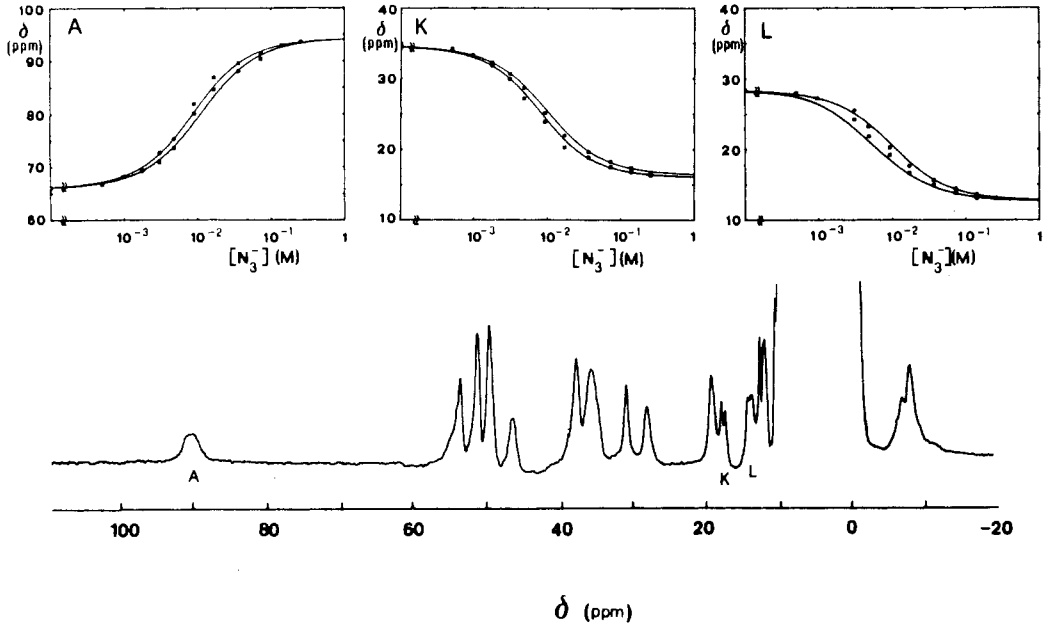

Figure 9. ^1H NMR spectrum of a sample of human Cu_2Co_2SOD in the presence of 7.2×10^{-2} M azide (about 90% saturation) showing splitting of signals A, K, and L. Experimental conditions are the same as in Figure 7. The insets show the chemicals shifts versus various amounts of azide of the three splitting pairs.

at the edge of the bulk protein signals under these conditions, and therefore, their T_1 values are difficult to obtain with high accuracy.

Discussion

Cobalt-Substituted Yeast SOD's. The similarity of the electronic spectra of the yeast Co_2Co_2SOD prepared by us and the yeast "Co_2SOD" described by Dunbar et al.[11b] in phosphate or HEPES buffer to those of bovine Co_2Co_2SOD under the same conditions leads us to the conclusion that the sample studied by Dunbar contained 4 rather than 2 equiv of cobalt per protein dimer. We conclude further, therefore, that the metal-binding regions of the yeast, human, and bovine proteins are very similar and that the subunits are equivalent. From the study of bovine Cu,Zn-SOD by Banci et al.,[16,17] it is believed that the two cobalt ions in each subunit of the four-cobalt derivative are bridged by the imidazolate ring of histidine-61, giving five-coordinate Co^{2+} in the copper site and four-coordinate Co^{2+} in the zinc site. In the presence of phosphate, it is believed that the bridge is broken and both Co^{2+} ions have tetrahedral geometry.

The phosphorus relaxivity (i.e. normalized paramagnetically enhanced relaxation rate) shown in Table I provides further information on Co^{2+}-substituted YSOD in phosphate buffer. The ^{31}P relaxation rate is dramatically enhanced by high-spin Co^{2+} in the copper site while it is almost identical with that of its diamagnetic analogues when Co^{2+} resides in the zinc site. A comparably large ^{31}P relaxivity was also reported for bovine Co_2Zn_2SOD, with Co^{2+} in the copper site, in 50 mM phosphate buffer at pH 7.4 by Banci et al.[17] and Hirose et al.[25] The small

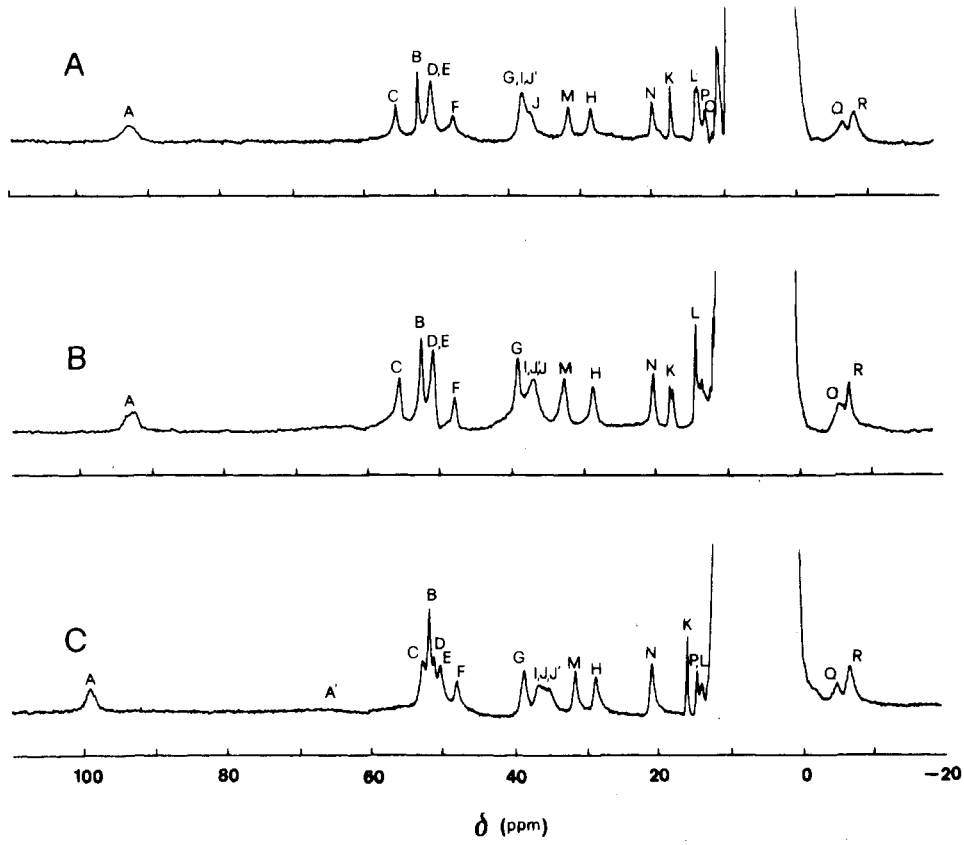

Figure 10. ^{1}H NMR spectra (300 MHz, 303 K) of bovine (A), human (B), and yeast (C) Cu_2Co_2SOD in 10 mM acetate at pH 5.5 in the presence of 1.4×10^{-1} M (A), 3.1×10^{-1} M (B), and 3.0×10^{-1} M (C) azide. These concentrations correspond to 95% (A), 98% (B), and 99% (C) saturation.

^{31}P relaxivities of apo-YSOD reconstituted with 0.68 and 1.2 equiv of Co^{2+} and the greatly increased values with 2.7 and 3.6 equiv of Co^{2+} confirms that the "Co_1SOD" derivative described by Dunbar et al.[11b] is actually E_2Co_2YSOD with Co^{2+} in the zinc site and that the "Co_2SOD" derivative is actually Co_2Co_2YSOD with Co^{2+} in both the copper and the zinc sites.

The proton NMR spectra of E_2Co_2YSOD and Co_2Co_2YSOD (Figures 2C and 5) show a great similarity to those of bovine derivatives.[17] The effects of phosphate on the spectra of Co_2-Co_2SOD, along with the ^{31}P relaxivity study, provide evidence that anions can interact with Co^{2+} in the copper site but not with Co^{2+} in the zinc site, suggesting that Co^{2+} in that latter site is not accessible to solvent. These results suggest that the model for metal coordination in yeast SOD with asymmetric subunits and an open coordination position on the metal ion in the zinc site, which was proposed on the basis of EXAFS data fitting,[10] should be reexamined. The conclusion that yeast Cu,Zn–SOD is comprised of two asymmetric subunits[11] is not consistent with the results presented here.

^{1}H NMR Study of Cu_2Co_2SOD's. The strong similarity of the spectra of all of the Cu_2Co_2SOD derivatives indicates that all of their metal-binding regions are very similar, and we find, furthermore, that the two subunits of Cu,Zn-YSOD are spectroscopically identical. In the presence of azide as well, the two subunits have the same characteristics without any detectable difference induced by azide binding (Figures 8 and 10). However, azide ions cause a splitting of three signals of human SOD (Figure 9), which we explain by the equilibrium

$$[SOD]_2 \overset{+N_3^-\,(K_1)}{\rightleftharpoons} SOD'\ SOD(N_3^-) \overset{+N_3^-\,(K_2)}{\rightleftharpoons} [SOD(N_3^-)]_2$$

A single signal would be observed under conditions of fast exchange unless one of the subunits, once it is bound with azide, induces a change on the other subunit that is irreversible with respect to the NMR time scale. As shown in Figure 9, only the signals experiencing large shifts upon azide binding undergo detectable splitting. The difference between the two affinity constants (130 and 180 M^{-1}) is relatively small, indicating a very small induced modification of the metal-binding sites by azide. The absence of this phenomenon in bovine and yeast Cu,Co-SOD's may be due to the fact that the interconversion between the unaltered and the modified forms is fast on the NMR time scale, or that the induced modification is very small.

In the presence of a saturating amount of azide in the protein solutions, signals K, L, O, and P of all the proteins move into the diamagnetic region, indicating that a coordinated histidine in the copper site has been displaced. This histidine is believed to be His-44 in bovine SOD.[26,27] There are two signals at 12.5 and 13.0 ppm, which can be detected for all the proteins (Figure 7). The one at 12.5 ppm is a solvent-exchangeable proton. It cannot be assigned to a histidine residue because that yeast SOD has no other histidine outside the metal-binding sites.[5] Its T_1 value (30 ms) indicates that it is in close proximity to the metal center, possibly Co^{2+}. A candidate could be a peptide N–H proton, such as that of the Asp-83 residue.

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Registry No. SOD, 9054-89-1; Cu, 7440-50-8.

(25) Hirose, J.; Hayakawa, C.; Noji, M.; Kidani, Y. *Inorg. Chim. Acta* **1985**, *107*, L7–L10.

(26) Banci, L.; Bertini, I.; Luchinat, C.; Scozzafava, A. *J. Am. Chem. Soc.* **1987**, *109*, 2328–2334.

(27) (a) Recently, Blackburn et al.[27b] have used a multiple-scattering approach to X-ray absorption spectroscopy to study anion binding to SOD. They concluded that the bonding of an azide molecule to the bound copper causes displacement of an imidazole from a Cu–N bond length of 2.00 Å to 2.27 Å. Our results can be reconciled with theirs only if we assume that the histidine that has been displaced is no longer bonded to copper. (b) Blackburn, N. J.; Strange, R. W.; McFadden, L. M.; Hasnain, S. S. *J. Am. Chem. Soc.* **1987**, *109*, 7162–7170.