(C) Salt compounds of ArF^+: The bond energy of ArF^+ in its $^1\Sigma^+$ ground state has recently been predicted by us as 49 ± 3 kcal/mol.[8] It was estimated that this should be sufficient to form stable salts such as $ArFAuF_6$ or $ArFSbF_6$.[8] This might be achieved by electric oxidation of F_2 in the presence of AuF_5 and Ar.

(D) NgBeO compounds, which have been predicted[2,3] to be stable for Ng = He, Ne, and Ar, might be detected by pyrolysis of polymeric BeO and trapping the monomeric BeO in fluid Ng. Another approach might be laser desorption of monomeric BeO from polymeric BeO in a Ng atmosphere.[54]

(E) Perutz and Turner[53] have shown that spectroscopic interactions of Ar with metal pentacarbonyls at 20 K can yield a substantial frequency shift which can only be explained by assuming that Ar occupies the sixth ligand position at $Cr(CO)_5$, $Mo(CO)_5$, and $W(CO)_5$. They conclude that "these stereospecific interactions are tantamount to the formation of a chemical bond".[53] While these observations have been made by accident, a systematic search for better acceptor species could result in even more stable transition metal-argon complexes.

Our suggestions for appropriate experiments are based on traditional laboratory techniques, but other ways will certainly be found by the inventive chemist. The chemistry of the light noble gases is a new field which is ideally suited for a combined theoretical/experimental approach.

Acknowledgment. Stimulating discussions with Prof. Christian K. Jørgensen, Dr. Jürgen Gauss, and Prof. Joel F. Liebmann are gratefully acknowledged. Part of this research has been supported by a grant of computer time from the San Diego Supercomputer Center. G.F. and D.C. thank the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for financial support. Calculations in Götegorg have been carried out using the CRAY XMP 48 of the NSC, Linköping, Sweden. W.K. thanks the IBM Düsseldorf computing center for providing computer time.

Supplementary Material Available: Table of calculated harmonic vibrational frequencies for **1-20** (2 pages). Ordering information is given on any current masthead page.

(53) Perutz, R. N.; Turner, J. J. *J. Am. Chem. Soc.* **1975**, *97*, 4791.
(54) This has been suggested by M. Devries.

NMR Studies of Cobalt(II)-Substituted Derivatives of Bovine Copper-Zinc Superoxide Dismutase. Effects of pH, Phosphate, and Metal Migration

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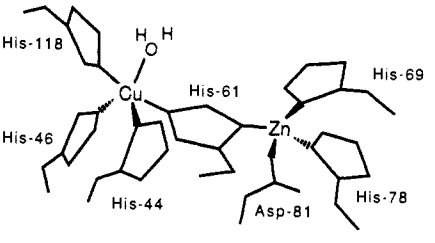
Abstract: The effects of changing pH and phosphate concentration on a number of Co^{2+}-substituted derivatives of bovine copper-zinc superoxide dismutase (Cu_2Zn_2SOD) have been studied by means of electronic spectroscopy, isotropically shifted ^1H NMR spectroscopy, and NMR relaxation. For the derivative E_2Co_2SOD, a pH-dependent Co^{2+} migration from the zinc site to the empty copper site was observed, forming subunits containing Co^{2+} in both metal binding sites. The presence of phosphate was observed to faciliate the cobalt migration process, presumably due to the fact that the anion causes an enhancement of Co^{2+} binding to the copper site. Some related studies of pH-dependent effects in the presence and absence of phosphate were carried out on Co_2Co_2SOD and Co_2Zn_2SOD, derivatives with Co^{2+} in the copper site, and on $Cu^I_2Co_2SOD$ and $Ag^I_2Co_2SOD$, derivatives with Co^{2+} in the zinc site. Phosphate had earlier been reported to have a strong influence on the geometry of Co^{2+} in the copper site under neutral pH conditions. We found, however, that this effect was not present under highly alkaline conditions where no influence of phosphate was observed. We also found that pH was an important factor in determining whether or not the imidazolate bridge was present between the two metal ions in each subunit in the derivatives with Co^{2+} bound in the copper site. Under high pH conditions, the bridging imidazolate was present in the derivative $Ag^I_2Co_2SOD$, but not in the derivative $Cu^I_2Co_2SOD$ (an analogue of reduced native SOD), which retained its metal binding configuration, at least in the zinc site, over a wide range of pH.

Bovine copper-zinc superoxide dismutase (Cu_2Zn_2SOD)[1] is a dimeric metalloenzyme containing a Cu^{2+} ion and a Zn^{2+} ion in each of its identical subunits.[2] The two metal ions are bound in close proximity, 6.3 Å apart, and are bridged by the imidazolate ring of histidine-61.[2] The copper binding site is known to be the active site for the disproportionation of superoxide anion to oxygen and hydrogen peroxide ($2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$). A variety of metal ions, including Co^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ag^+, and Hg^{2+}, have been shown to substitute for the native metal ions, Cu^{2+} and Zn^{2+}, in the enzyme, but only derivatives with Cu^{2+} in the copper site have substantial SOD activity.[2] Recently, some Ni^{2+}-substituted derivatives have also been prepared and char-



(1) Abbreviations: SOD, superoxide dismutase; $M_2M'_2SOD$, M- and M'-substituted SOD with M in the copper site and M' in the zinc site (M and M' are divalent metal ions unless otherwise noted), and an E represents an empty site; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; DEFT, driven equilibrium Fourier transform; FID, free induction decay; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; TMS, trimethylsilane.
(2) For a general reference, see: Valentine, J. S.; Pantoliano, M. W. In *Copper Proteins*; Spiro, T. G., Ed.; Wiley: New York, 1981; Vol. 3, Chapter 8.

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acterized and have been found to have anion binding properties and structural configurations similar to those of the native enzyme.[3] The studies of these metal-substituted SOD's have provided much information about the nature of the metal-binding region of the protein.

pH-dependent binding of a metal ion to a ligand results from competition between the metal ion and a proton at the metal binding site. As a consequence, a larger apparent affinity constant for metal ion binding to a protein can be obtained at higher pH.[4] The binding of different metal ions to SOD has been reported to be strongly pH dependent in certain cases,[5] e.g. the occurrence of a Cu^{2+} migration from the copper site to the empty zinc site in Cu_2E_2SOD at pH >8[6] and the release of metal ions from the zinc site in $Cu_2M'_2SOD$ ($M' = Cu^{2+}$, Zn^{2+}, Co^{2+}) at pH 4.[7] The deprotonation of N_ϵ2-H of His-61 residue at high pH to form an imidazolate-bridged biscopper pair was considered to be a significant factor in inducing the Cu^{2+} migration.[6]

The binding of different metal ions to metal-depleted derivatives of SOD is also influenced by the presence of certain anions, such as CN^- and phosphate.[8-12] Phosphate is unique in its influence on the properties of native SOD[9,10] and Co^{2+}-substituted SOD's[11,12] relative to other anions. In the case of native SOD, phosphate binds near to the copper site but not directly to the metal ion and does not cause changes in the visible or EPR spectra due to Cu^{2+}.[10] By contrast, the presence of phosphate in solution has a pronounced effect on the binding of Co^{2+} to the protein in certain cases and also on spectroscopic properties of some Co^{2+}-substituted derivatives in which Co^{2+} is bound at the copper site of the protein.[11,12] These effects have been attributed to direct binding of phosphate to Co^{2+} in the copper site.[11,12]

The zinc binding sites of metalloproteins are in large part spectroscopically "silent" owing to the d^{10} electronic configuration of Zn^{2+}. However, Co^{2+} substitution for Zn^{2+} has provided a particularly useful method of probing the zinc binding site by electronic and magnetic resonance spectroscopies.[13,14] Some of the effects of changing pH on spectroscopic properties of Co^{2+}-substituted SOD's have been noted previously.[15] We report here our more extensive observations of the effect of changing pH on a number of Co^{2+}-substituted SOD's in the presence and absence of phosphate using electronic and NMR spectroscopies and NMR relaxation. The results provide more information concerning the nature of Co^{2+} and phosphate binding to SOD and the effect of changing pH. In addition, we report one observation

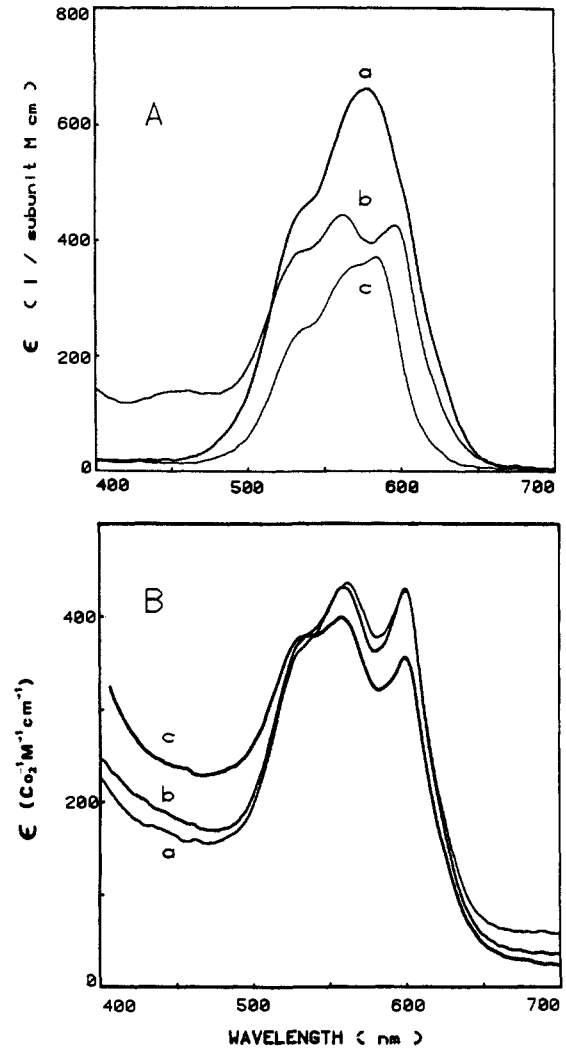


Figure 1. (A) Electronic spectra of (a) Co_2Co_2SOD in 50 mM phosphate at pH 6.5, (b) Co_2Co_2SOD in 50 mM acetate pH 7.5, and (c) E_2Co_2SOD in 50 mM phosphate at pH 6.5. (B) Electronic spectra of (a) Co_2Co_2-SOD in 50 mM phosphate at pH 10.4, (b) Co_2Co_2SOD in 50 mM acetate at pH 10.1, and (c) E_2Co_2SOD in 50 mM phosphate at pH 10.4. The absorption of the spectra in (B) was normalized according to the Co^{2+} concentration in each derivative. All of the spectra were taken at room temperature referenced against deionized water.

of a reversible pH-dependent migration of Co^{2+} to the empty copper site in E_2Co_2SOD which reaches completion at pH 10 and is facilitated by the presence of phosphate.

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. All the Co^{2+}-substituted derivatives of Cu_2Zn_2SOD were prepared as reported with minor modification.[2] Co_2Co_2SOD and Co_2Zn_2SOD in acetate buffer solution were prepared either by directly infusing Co^{2+} into E_2Co_2SOD and E_2Zn_2SOD, respectively, at pH 7.5[11,12] or by ultrafiltration of solutions of Co_2Co_2SOD and Co_2Zn_2SOD prepared in phosphate buffer with several changes of acetate solution at pH 7.5. The latter procedure is preferred over the former, which requires several days.

The isotropically shifted ^1H NMR spectra were obtained on Bruker WP200 and IBM AF200 spectrometers at 200 MHz and a Bruker AM500 spectrometer at 500 MHz using the modified DEFT multipulse sequence[16] to suppress water and diamagnetic protein signals. Typical spectra obtained in D_2O consisted of about 2000 to 8000 scans while those in H_2O consisted of about 10000 to 20000 scans with 8 K or 16 K data points and spectral width sufficiently wide to cover all the isotropically shifted signals. Chemical shifts were measured from water signal which was assumed to be 4.8 ppm downfield from TMS. A 10-30

(3) (a) Ming, L.-J.; Valentine, J. S. *J. Am. Chem. Soc.* **1987**, *109*, 4426-4428. (b) Ming, L.-J.; Valentine, J. S. *Recl.: J. R. Neth. Chem. Soc.* **1987**, *106*, 362 (Abstract for 3ICBIC). (c) Ming, L.-J.; Valentine, J. S. *J. Am. Chem. Soc.*, in press (see also: P17-14, XIII International Conference on Magnetic Resonance in Biological Systems, Madison, Wisconsin, August 14-19, 1988).

(4) Breslow, E. In *Inorganic Biochemistry*; Eichhorn, G. L., Ed.; Elsevier: New York, 1973; Vol. 1, Chapter 7.

(5) See ref 2, pp 327-333.

(6) Valentine, J. S.; Pantoliano, M. W.; McDonnell, P. J.; Burger, A. R.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4245-4249.

(7) Pantoliano, M. W.; McDonnell, P. J.; Valentine, J. S. *J. Am. Chem. Soc.* **1979**, *101*, 6454-6456.

(8) Beem, K. M.; Richardson, D. C.; Rajagopalan, K. V. *Biochemistry* **1977**, *16*, 1930-1936.

(9) (a) Rotilio, G.; Morpurgo, L.; Giovagnoli, C.; Calabrese, L.; Mondovi, B. *Biochemistry* **1972**, *11*, 2187-2192. (b) Rigo, A.; Viglino, P.; Rotilio, G.; Tomat, R. *FEBS Lett.* **1975**, *50*, 86-88. (c) Strothkamp, K. G.; Lippard, S. J. *Biochemistry* **1981**, *20*, 7488-7493.

(10) (a) Mota de Freitas, D.; Valentine, J. S. *Biochemistry* **1984**, *23*, 2079-2082. (b) Mota de Freitas, D.; Luchinat, C.; Banci, L.; Bertini, I.; Valentine, J. S. *Inorg. Chem.* **1987**, *26*, 2788-2791.

(11) Banci, L.; Bertini, I.; Luchinat, C.; Monnanni, R.; Scozzafava, A. *Inorg. Chem.* **1987**, *26*, 153-156.

(12) (a) Calabrese, L.; Cocco, D.; Desideri, A. *FEBS Lett.* **1979**, *106*, 142-144. (b) Banci, L.; Bertini, I.; Luchinat, C.; Monnanni, R.; Scozzafava, A. *Gazz. Chim. Ital.* **1986**, *116*, 51-54. (c) Ming, L.-J.; Banci, L.; Luchinat, C.; Bertini, I.; Valentine, J. S. *Inorg. Chem.* **1988**, *27*, 2328-2334.

(13) Bertini, I.; Luchinat, C. *Adv. Inorg. Biochem.* **1984**, *6*, 71-111.

(14) Bertini, I.; Luchinat, C. *NMR of Paramagnetic Molecules in Biological Systems*; Benjamin/Cummings: Menlo Park, CA, 1986.

(15) (a) Calabrese, L.; Cocco, D.; Morpurgo, L.; Mondovi, B.; Rotilio, G. *Eur. J. Biochem.* **1976**, *64*, 465-470. (b) Desideri, A.; Cocco, D.; Calabrese, L.; Rotilio, G. *Biochim. Biophys. Acta* **1984**, *785*, 111-117.

(16) Hochmann, J.; Kellerhals, H. *J. Magn. Reson.* **1980**, *38*, 23-29.

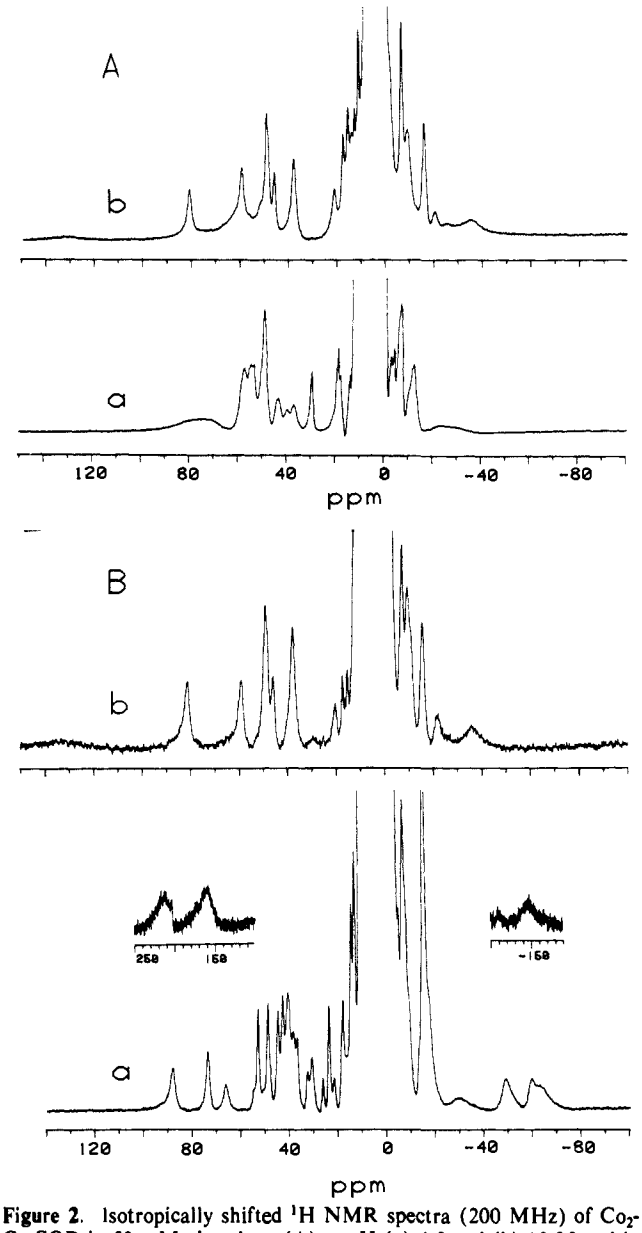


Figure 2. Isotropically shifted ^1H NMR spectra (200 MHz) of Co_2-Co_2SOD in 50 mM phosphate (A) at pH (a) 6.5 and (b) 10.85 and in 50 mM acetate (B) at pH (a) 7.2 and (b) 10.9 at ambient temperature (~23 °C) in H_2O.

Hz additional line broadening was introduced to all the spectra by exponential multiplication of the FID's to improve the signal-to-noise ratio.

The ^{31}P nuclear spin-lattice relaxation times (T_1) of phosphate in different solutions of Co^{2+}-substituted SOD were determined on a Bruker AM500 spectrometer at 25 °C operated at 202.5 MHz by the inversion-recovery pulse sequence. The measurements of the T_1 values of phosphate in E_2Co_2SOD solution at different pH's were carried out in a short period of time in order to avoid a pH-dependent redistribution of Co^{2+} in the two metal binding sites (see Results section).

Electronic spectra were obtained at room temperature on a Beckman UV 5270 spectrometer referenced against deionized water. The pH (or pH*, uncalibrated pH reading in D_2O) of protein solutions was adjusted with dilute HCl (DCl) or NaOH (NaOD) in appropriate buffer solution and was measured on a Corning Model 12 pH meter with a combined microelectrode (Wilmad Glass Company, Inc., NJ).

Results

Co_2Co_2SOD. The electronic and the isotropically shifted ^1H NMR spectra of Co_2Co_2SOD in 50 mM phosphate and 50 mM acetate solutions under neutral pH conditions[11,12] were found to be very different from each other as shown in Figures 1A and 2. The electronic spectrum of this same derivative in 50 mM acetate solution was not significantly changed when the pH was raised to 10.1. However, dramatic changes in the electronic spectrum

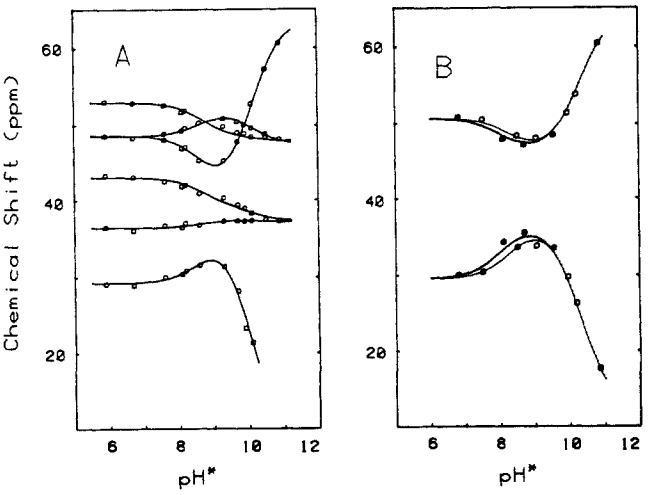


Figure 3. Plot of the chemical shifts (at 200 MHz) versus pH* and the simultaneous numerical fitting of each signal to eqs 1-4 of (A) Co_2-Co_2SOD and (B) Co_2Zn_2SOD in 50 mM phosphate at 25 °C. The dotted lines and solid circles in (B) were in a 25 mM phosphate solution.

of this derivative in 50 mM phosphate solution were observed when the pH was raised to pH 10.4, and the resulting spectrum was virtually identical with that observed in acetate (Figure 1B).

Similar effects were seen in the isotropically shifted ^1H NMR spectra of Co_2Co_2SOD, i.e., the spectra at high pH were very similar in both phosphate and acetate solutions while they were markedly different at neutral pH (Figure 2). Specifically, at pH 7.2, the derivative in acetate had a well-shaped and well-separated ^1H NMR spectrum consisting of more than 20 signals spread over a range of about 400 ppm. When the pH was raised to 10.9, only seven signals were observed in the downfield region greater than 30 ppm in which the signals at 80.7 and 46.1 ppm disappeared in deuterium oxide and therefore can be assigned to the solvent-exchangeable imidazole N-H protons of coordinated histidines. The low intensity of these signals in H_2O is due to saturation of the solvent by the modified DEFT pulse followed by saturation transfer from the solvent to the signals.

The isotropically shifted ^1H NMR spectrum of Co_2Co_2SOD in phosphate solution is simpler than that observed in acetate solution (Figure 2). Phosphate binding to Co^{2+} in the copper site has been reported to change the geometry of the Co^{2+} binding site from five-coordinate to distorted tetrahedral while at the same time the imidazolate bridge between the two Co^{2+} ions is broken.[11] This phenomenon is presumably the cause of the dramatic effect of phosphate on the spectra of solutions of Co_2Co_2SOD near neutral pH. However, the differential influence of phosphate relative to acetate vanishes completely at pH >10 as indicated by the observation of essentially the same isotropically shifted ^1H NMR spectra of this derivative in the presence or absence of phosphate (Figure 2). The changes of the spectra of this derivative with pH in both solutions were observed to be completely reversible.

A plot of the chemical shifts of the isotropically shifted signals of Co_2Co_2SOD in 50 mM phosphate solution against pH* is shown in Figure 3A. (Similar behavior was not observed in acetate buffer.) A minor perturbation of the signals was observed at pH* <9 and a significant influence was shown on some of the signals at pH* >9, especially the signals at 49 and 29 ppm. We tentatively assign these signals to protons on ligands of Co^{2+} in the copper site since Co^{2+} in this site is known to have an open coordination position for anion binding and would be expected to be perturbed more by changes in the medium (such as the addition of anions) as compared to the Co^{2+} in the zinc site which is inaccessible to solvent. A conformational change of the Co^{2+} in the copper site caused by increasing pH as observed by electronic spectroscopy (vide supra) could also explain the shifts of the proton signals with pH in the copper site. The pH-titration profiles of the chemical shifts of the isotropically shifted signals can be fitted by using the following equations (eqs 1-4) along with the three

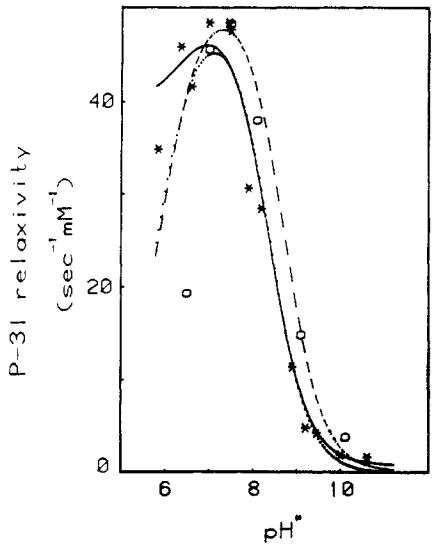


Figure 4. ^{31}P relaxivities of 50 mM phosphate (202.5 MHz) in the presence of Co_2Co_2SOD (*) and Co_2Zn_2SOD (O) at different pH*'s. The solid line is the numerical fitting of ^{31}P relaxivities in Co_2Co_2SOD solution to eqs 1 and 2 with affinity constants of 50 and 0.3 M^{-1} respectively for $H_2PO_4^-$ binding to the species A and for HPO_4^{2-} binding to the species C in eq 2. The dotted line (for Co_2Co_2SOD) and the dashed line (for Co_2Zn_2SOD) are the fittings obtained by assuming that the HPO_4^{2-} binding to the species A in eq 2 is the only phosphate interaction for the Co^{2+}-substituted derivatives.

ionization constants of phosphate assuming that the dibasic phosphate is the major species binding to the protein and that phosphate binding to the deprotonated forms (C and D in eqs 2 and 3) of the protein is small. (K is the apparent affinity constant

$$\underset{A}{\text{CoCoSOD}} + \text{Pi} \overset{K}{\rightleftharpoons} \underset{B}{\text{CoCoSOD-Pi}} \quad (1)$$

$$\underset{A}{\text{CoCoSOD}} \overset{K_{a1}}{\rightleftharpoons} \underset{C}{\text{CoCoSOD}}(-H^+)^- + H^+ \quad (2)$$

$$\underset{C}{\text{CoCoSOD}}(-H^+)^- \overset{K_{a2}}{\rightleftharpoons} \underset{D}{\text{CoCoSOD}}(-2H^+)^{2-} + H^+ \quad (3)$$

$$CS = \frac{CS_B[B] + CS_C[C] + CS_D[D]}{[SOD]_0} \quad (4)$$

for phosphate (Pi) binding to CoCoSOD, the subunit of Co_2Co_2SOD, CS_B, CS_C, and CS_D are the chemical shifts of the species B, C, and D, respectively, and $[SOD]_0$ is the total concentration of the derivative.) Two pK_a's (uncalibrated pH reading in D_2O) of Co_2Co_2SOD of 7.5 and 10.1 are obtained by a simultaneous fitting of the chemical shifts of all the signals with respect to pH*.

The ^{31}P relaxivities (i.e. the normalized ^{31}P relaxation rates) of phosphate solution in the presence of Co_2Co_2SOD at various pH*'s were obtained as $T_{1p}^{-1}/[Co_2Co_2SOD]$ (Table I and Figure 4), where T_{1p}^{-1} is the paramagnetic enhancement of the ^{31}P relaxation rate of bulk phosphate in the presence of the paramagnetic metalloprotein, Co_2Co_2SOD, and is defined in eq 5, where $(T_1^{-1})_{obs}$

$$T_{1p}^{-1} = (T_1^{-1})_{obs} - (T_1^{-1})_0 \quad (5)$$

and $(T_1^{-1})_0$ are the measured ^{31}P relaxation rates of phosphate in the presence of Co_2Co_2SOD and E_2Co_2SOD, respectively. E_2Co_2SOD is used as the reference to obtain $(T_1^{-1})_0$ in order to correct for small enhancement of the ^{31}P relaxation rate by the paramagnetic Co^{2+} in the zinc site of Co_2Co_2SOD. (The outer-sphere interaction between phosphate and Co^{2+} buried in the solvent-inaccessible zinc site of E_2Co_2SOD was observed to result in only a minor enhancement of the ^{31}P relaxation rate as shown in Table I.) The ^{31}P relaxivities of 47.8 and 1.62 (s mM)$^{-1}$ were obtained at pH* 7.5 and pH* 10.6, respectively, using values of 0.252 and 0.134 s^{-1} as the diamagnetic contributions to the relaxation rate (Table I).

Table I. Phosphate ^{31}P (202.5 MHz) Nuclear Magnetic Resonance Relaxation in the Presence of Co^{2+}-Substituted Derivatives of SOD at 25 °C and Different pH*s

derivative	pH*	[protein][a], mM	$1/T_1$, s^{-1}	$(1/T_{1p})/[protein]$,[b] s^{-1} mM^{-1}
E_2Zn_2SOD	5.9	0.489	0.251	
	6.9		0.239	
	8.1		0.168	
	9.0		0.142	
	10.2	0.480	0.076	
	7.4[c]	0.38	0.18	
E_2Co_2SOD	6.35	0.300	0.245	
	7.5		0.252	
	9.0		0.146	
	10.55	0.270	0.134	
Co_2Zn_2SOD	7.5	0.589	28.6	48.2
	8.1	0.556	21.3	38.0
	9.1		8.37	14.8
	10.1		2.18	3.78
	6.5	0.434	8.62	19.3
	7.0		20.0	45.5
	7.4[c]	0.37	15.6	41.7
Co_2Co_2SOD	6.6	0.370	15.6	41.6
	7.0	0.358	17.0	48.4
	7.45	0.345	17.0	48.4
	8.2	0.336	9.80	28.4
	8.9	0.333	3.92	11.3
	9.45	0.331	1.55	4.24
	10.0	0.312	0.714	1.86
	10.6	0.260	0.554	1.62
	9.2	0.236	1.27	4.78
	7.9	0.312	9.80	30.6
	7.5		15.2	47.8
	6.35	0.302	14.1	45.8
	5.85		10.8	34.8

[a] Concentration of Co^{2+} per dimer in the copper site for Co_2Zn_2SOD and Co_2Co_2SOD. [b] Values for the two paramagnetic derivatives with Co^{2+} in the copper site are obtained according to eq 5 by using the values of $(1/T_1)_0$ of their diamagnetic analogues under the closest pH conditions. [c] Hirose, J.; Hayakawa, C.; Noji, M.; Kidani, Y. *Inorg. Chim. Acta* **1985**, *107*, L7–L10.

The general trend of the ^{31}P relaxivities of phosphate at different pH*'s in the presence of Co_2Co_2SOD can be fitted by using eqs 1 and 2 assuming that the dibasic phosphate is the major species interacting with the protein in eq 1 (Figure 4). The success of using eqs 1 and 2 in fitting the relaxation data indicates that phosphate binding to the protein decreases when the protein is deprotonated (i.e., B $\rightleftharpoons$ C + H^+ + Pi with an equilibrium constant of K_{a1}/K). By using a phosphate molar relaxivity of 48.4 × 10^3 (s M)$^{-1}$ (Table I) and the affinity constant[11] of 300 M^{-1} at pH 7.5, a group in the vicinity of the active site with a pK_a of 7.0 is found to be important in influencing the phosphate–protein interaction and thereby affecting the ^{31}P relaxation rates of phosphate. The affinity constants of monobasic phosphate binding to (A) and dibasic phosphate binding to (C) in eqs 1 and 2 were found to be negligibly small compared to dibasic phosphate binding to (A) (Figure 4), indicating that the assumption made above for the fitting of the chemical shifts versus pH* was appropriate.

The Solomon–Bloembergen equation[17] can be applied to estimate the distance between phosphate and Co^{2+} in the copper site when T_{1p}^{-1} of phosphate and the affinity constant for its binding to the protein are obtained. Briefly, the equation can be written as in eq 6 assuming a dominant dipolar relaxation, where

$$T_{1M}^{-1} = (T_{1p}f)^{-1} = Cr^{-6}f(\tau_c,\omega) \quad (6)$$

T_{1M}^{-1} is the inner-sphere relaxation rate of the phosphate, f is the molar fraction of bound phosphate which can be obtained from the affinity constant, C is a group of physical constants, r is the distance between the resonating nucleus and the paramagnetic metal ion, and $f(\tau_c,\omega)$ is the correlation function. Assuming the smallest value for the electronic relaxation rate of the high-spin

(17) (a) Solomon, I. *Phys. Rev.* **1955**, *99*, 559–564. (b) Bloembergen, N. *J. Chem. Phys.* **1957**, *27*, 572–573.

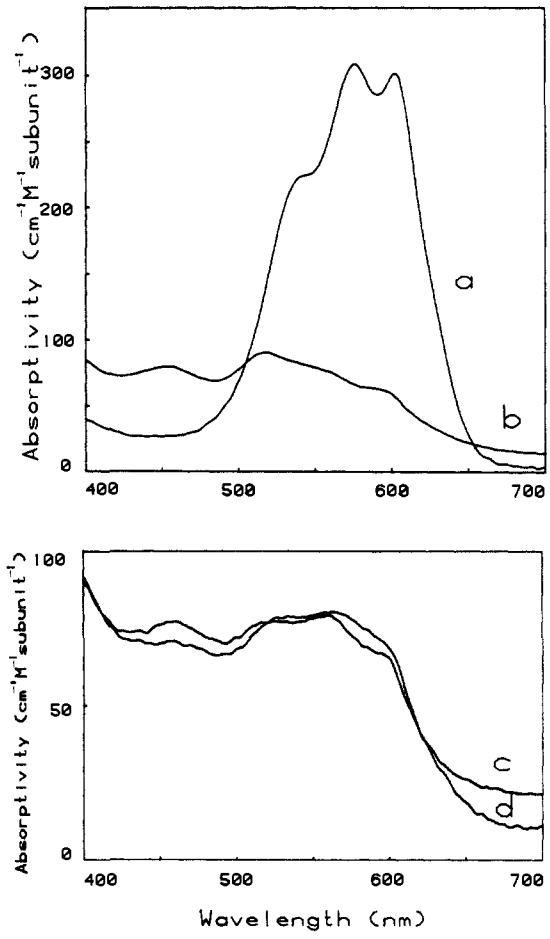


Figure 5. Electronic spectra of Co_2Zn_2SOD in 50 mM phosphate solution at pH 7.5 (a) and pH 10.4 (d) and in 50 mM HEPES solution at pH 7.0 (b) and pH 10.3 (c). The spectra were obtained at room temperature referenced against deionized water.

Co^{2+} in the copper site, i.e., 10^{11} s^{-1} [13] an upper limit of the metal–nucleus distance can be estimated by eq 6. A ^{31}P–Co distance of 2.49 Å is obtained by using eq 6, which is obviously shorter than a possible Co–O–P bond distance (>3 Å). The shorter distance obtained by the Solomon equation may be explained by the existence of covalency,[18] i.e. an extensive spin delocalization of the unpaired electrons from Co^{2+} to the orbitals of oxygen and phosphorus. This result also implies direct phosphate binding to Co^{2+} in the copper site. A similar observation was also reported for the derivative Co_2Zn_2SOD in which a phosphate directly binding to the Co^{2+} in the copper site was observed and showed a shorter Co–P distance by the relaxation data.[11]

Co_2Zn_2SOD. The electronic and isotropically shifted ^1H NMR spectra of Co_2Zn_2SOD in 50 mM phosphate solution are significantly different from those in 50 mM acetate (or HEPES) solution at neutral pH[11,12] as shown in Figures 5 and 6. Under highly alkaline conditions, the absorption bands of the electronic spectrum of this derivative in acetate solution became less resolved without significant changes in the intensity while dramatic changes, in both the intensity and the shape, of the spectrum were observed in phosphate solution (Figure 5). The changes in the electronic spectrum of this derivative in phosphate were attributed to deprotonation of an amino acid residue with a pK_a of about 8.2 near the metal binding site as estimated from the changes of the electronic absorptivity with pH.[15] However, such an estimation should be treated with care in a system with complex equilibria as in the Co_2Co_2SOD system indicated above, in which a direct

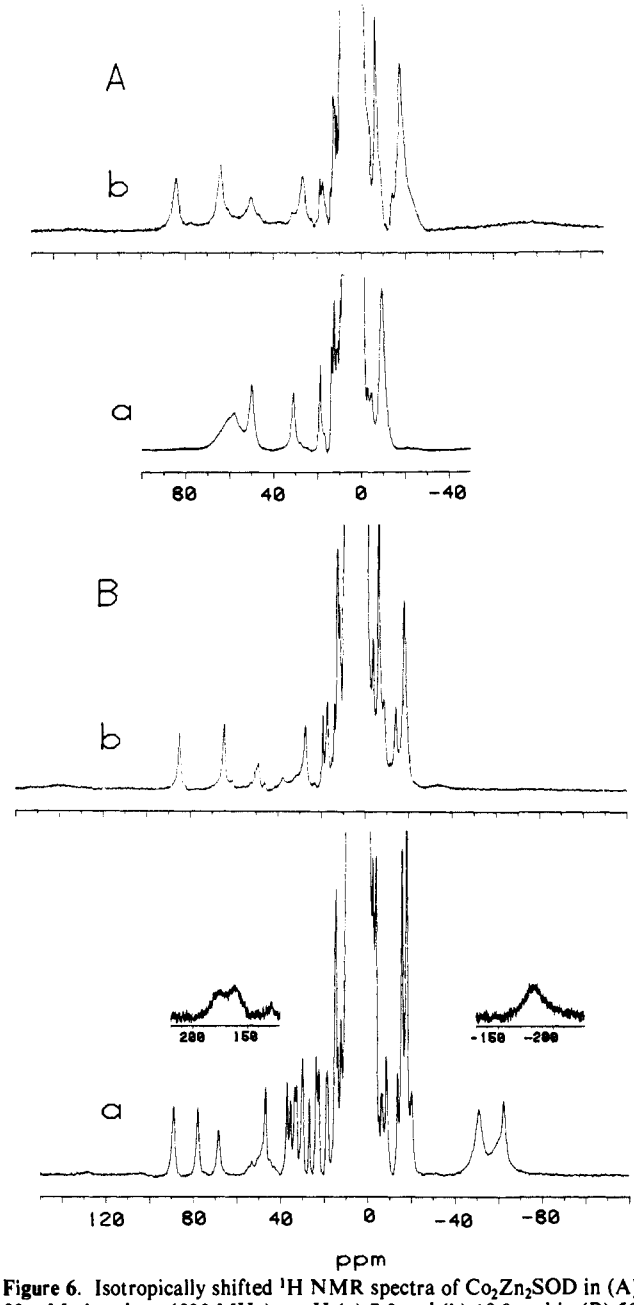


Figure 6. Isotropically shifted ^1H NMR spectra of Co_2Zn_2SOD in (A) 50 mM phosphate (500 MHz) at pH (a) 7.5 and (b) 10.8 and in (B) 50 mM acetate (200 MHz) at pH (a) 7.2 and (b) 11.0, at ambient temperature (~23 °C) in H_2O.

estimation would give a pK_a value close to 8.5 rather than the values (7.5 and 10.1) obtained by the numerical fittings (cf. Figures 3 and 4).

The pH-dependent changes in the NMR spectra of this derivative are similar to those of Co_2Co_2SOD, but the analysis is simpler because only the protons on the histidines directly coordinated to Co^{2+} in the copper site are isotropically shifted. Similar pH-dependent changes of the isotropically shifted signals in Co_2Zn_2SOD (Figure 3B) and of the two signals at 49 and 29 ppm in Co_2Co_2SOD (Figure 3A) suggest that similar geometric changes with pH occur in these two derivatives.

The pH–titration profiles of the chemical shifts of the isotropically shifted signals of Co_2Zn_2SOD can also be fitted by using eqs 1–4. These equations indicate a significant dependence of the chemical shifts on the concentration of phosphate with respect to the change of pH as shown in Figure 3B where two different phosphate concentrations, 25 and 50 mM, were chosen. Two pK_a's (uncalibrated pH reading in D_2O) of 7.2 and 10.3 are obtained by a simultaneous fitting of the chemical shifts of the signals versus pH* at two different phosphate concentrations, which are close

(18) Navon, G.; Valensin, G. In *Metal Ions in Biological Systems*; Sigel, H., Ed.; Marcel Dekker: New York, 1987, Vol. 21, Chapter 1 and references therein.

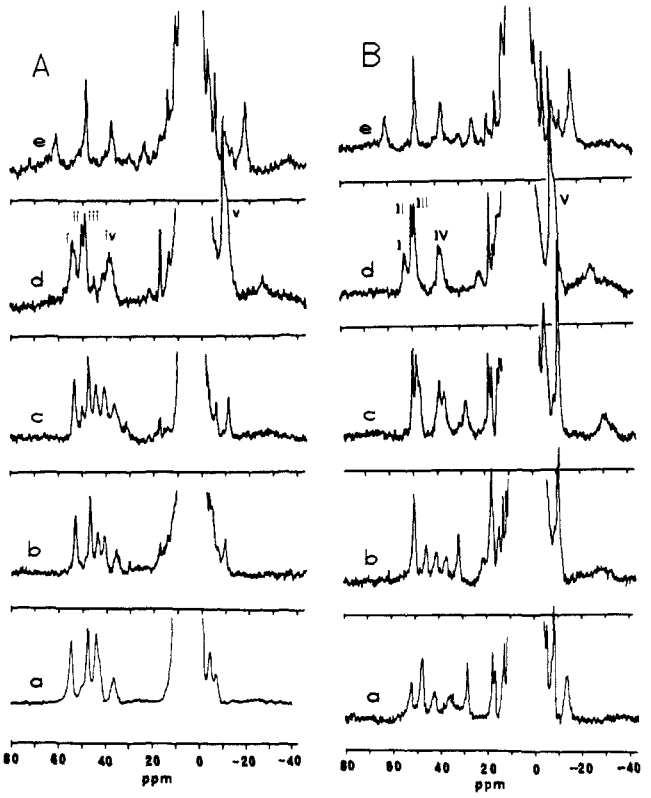


Figure 7. Isotropically shifted ^1H NMR spectra (200 MHz, 50 mM phosphate and 25 °C) of E_2Co_2SOD (A) at pH* (a) 6.5, (b) 8.4, (c) 9.5, (d) 10.1, and (e) 10.9 and of Co_2Co_2SOD (B) at pH* (a) 6.7, (b) 8.6, (c) 9.7, (d) 10.1, and (e) 10.9. The signals at 30 and −9 ppm in E_2Co_2SOD at pH* 8.4 (A.b) correspond to signals occurring at the same positions in Co_2Co_2SOD at pH* 8.6 (B.b).

to those obtained in Co_2Co_2SOD (vide ante).

The ^{31}P relaxivities of phosphate were obtained at different pH's in the presence of Co_2Zn_2SOD with E_2Zn_2SOD as the diamagnetic reference. Similar results as those of Co_2Co_2SOD were obtained (Table I and Figure 4) showing a significant decrease of the ^{31}P relaxivity under alkaline conditions with a maximum at about pH* 7.2. A fitting of the relaxivity with pH* using eqs 1 and 2 with a pK_a of 7.4 is also shown in Figure 4.

E_2Co_2SOD. The electronic and isotropically shifted ^1H NMR spectra of this derivative in phosphate solution at pH 6.5 (Figures 1A and 7A) are identical with those in acetate solution at pH 5.5.[11,12] All the NMR spectra were taken in D_2O solution for simplification. The NMR spectra of Co_2Co_2SOD in D_2O at different pH's are also reported for comparison (Figure 7B).

At pH 10.4, the electronic spectrum of E_2Co_2SOD has absorption bands at 598 and 557 nm with a shoulder at 530 nm (Figure 1B). Both the shape and the absorptivity of the spectrum at pH 10.4 are dramatically different from those at pH 6.5, indicating a change of the geometric configuration of the metal binding site. However, the spectrum is very similar to that of Co_2Co_2SOD under the same experimental conditions. (The relatively high absorption of this derivative at high pH observed in the near-UV region may result from coagulation of the protein which scatters light more at shorter wavelength.)

The changes in the NMR spectrum of E_2Co_2SOD with pH were significant at pH* >9.5 (Figure 7A) and became similar to that of Co_2Co_2SOD within several hours at pH* >10. Thereafter, identical changes in the spectra with pH were observed for both derivatives, which reached an equilibrium state immediately after the pH was raised (Figure 7): signals I and i shifted further downfield; signals II and III as well as ii and iii collapsed to form one peak; signals IV and iv were little affected by pH; and signals V and v splitted and shifted to different positions. When the pH of E_2Co_2SOD in 50 mM acetate solution was raised to pH* 10.4, there was no significant change in its NMR spectrum in 2 days (spectrum not shown). The spectrum was similar to that observed in phosphate solution at pH* 8.4 (Figure 7A) except that the small signals at 30 and −9 ppm were not observed.

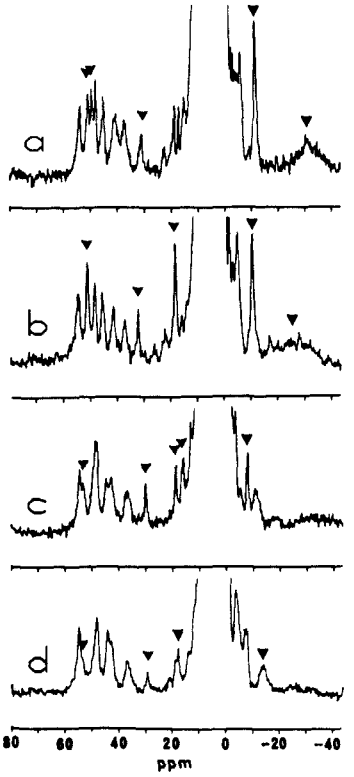


Figure 8. Isotropically shifted ^1H NMR spectra (200 MHz) of E_2Co_2SOD in 50 mM phosphate at 25 °C under different pH* conditions by adjusting pH* from 10.9 to (a) 9.6, (b) 8.7, (c) 7.5, and (d) 5.6. The "extra signals" mentioned in the text in each spectrum are marked.

The changes of this derivative with pH were reversible; however, the rate of the reverse reaction (i.e., from high pH to low pH condition) was much slower than that of Co_2Co_2SOD (vide ante). Some "extra signals", in addition to the signals of E_2Co_2SOD, were detected during the recovery process (Figure 8). A decrease of the relative intensity of the "extra signals" with lowering pH implies that an "extra species" is disappearing. By comparing the "extra signals" to the isotropically shifted ^1H NMR signals of Co_2Co_2SOD in phosphate (Figure 7B), the "extra signals" are clearly shown to be the same as the signals of Co_2Co_2SOD under similar conditions.

$Ag^I_2Co_2SOD$ and $Cu^I_2Co_2SOD$. The electronic spectra of $Ag^I_2Co_2SOD$ and $Cu^I_2Co_2SOD$ in acetate solution at pH 5.5 are almost identical with each other[8] and both spectra are not influenced by the presence of phosphate (Figure 9). The isotropically shifted ^1H NMR spectrum of $Cu^I_2Co_2SOD$ at 200 MHz, pH 5.5 (Figure 10A), is the same as reported at 300 MHz[19] and is very similar to that of $Ag^I_2Co_2SOD$ reported here under similar conditions (Figure 10B). Since both Ag^+ and Cu^+ have d^{10} electronic configuration and are diamagnetic, the isotropically shifted ^1H NMR signals of these two derivatives are expected to be due solely to the protons in the distorted tetrahedral Co^{2+} binding site. A broad signal at 129 ppm is detected in Ag_2Co_2SOD and is tentatively assigned to an ortho-like proton of a ligated histidine, which may be too broad to be detected in $Cu^I_2Co_2SOD$. A downfield-shifted signal at about 130 ppm was also observed in Co^{2+}-substituted carbonic anhydrase with a distorted tetrahedral geometry at pH 5.9 by Bertini et al. and was proposed to be due to an ortho-like proton of a coordinated histidine.[20]

When the ^1H NMR spectrum of Ag_2Co_2SOD was obtained in a D_2O solution, the signals a, c, and g disappear and can be assigned to the three imidazole N-H protons of the coordinated

(19) Bertini, I.; Luchinat, C.; Monnanni, R. *J. Am. Chem. Soc.* **1985**, *107*, 2178–2179.

(20) Bertini, I.; Canti, G.; Luchinat, C.; Mani, F. *J. Am. Chem. Soc.* **1981**, *103*, 7784–7788.

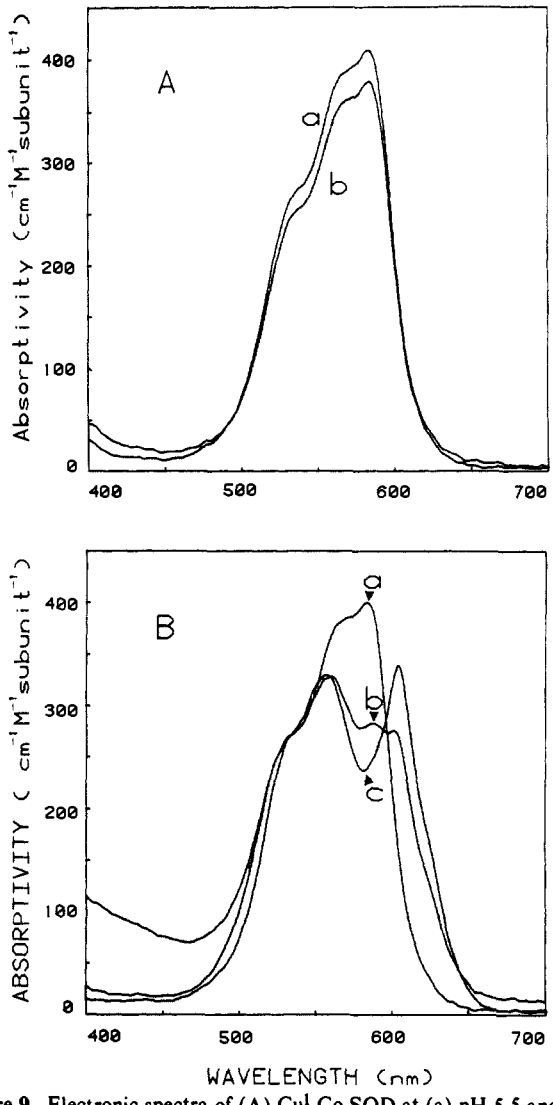


Figure 9. Electronic spectra of (A) $Cu^{I}_2Co_2SOD$ at (a) pH 5.5 and (b) 10.5 and of (B) $Ag^{I}_2Co_2SOD$ at (a) pH 5.5, (b) 9.9 (adjusted from pH 10.6), and (c) 10.6 in 50 mM phosphate solutions referenced against deionized water at room temperature.

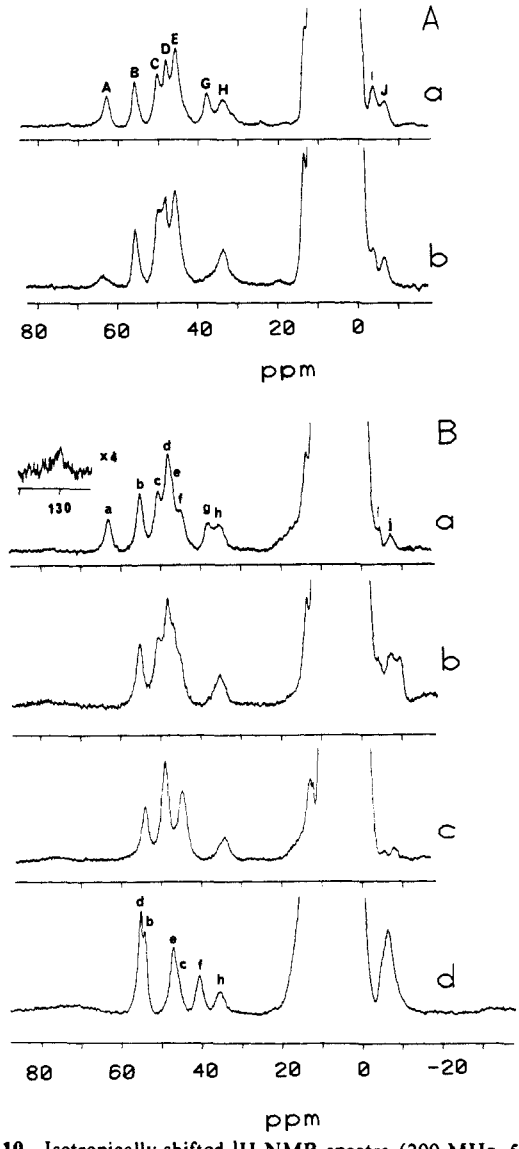


Figure 10. Isotropically shifted ^1H NMR spectra (200 MHz, 50 mM phosphate in H_2O) of $Cu^{I}_2Co_2SOD$ (A) at (a) pH 5.5 and (b) 10.0 and of $Ag^{I}_2Co_2SOD$ (B) at (a) pH 6.1, (b) 8.9 (c), 10.4, and (d) 11.2 at ambient temperature (~23 °C).

histidines in the zinc site. Of these, signal c is slowly solvent-exchangeable, reflecting its lesser solvent accessibility or its stabilization relative to other imidazole N-H protons by ligation to the metal ion.[21] The presence of three solvent-exchangeable signals in Ag_2Co_2SOD under neutral conditions is strong evidence that histidine-61 is not a bridging ligand in this derivative. Otherwise, only two isotropically shifted solvent-exchangeable signals would be detected in the Co^{2+} binding site due to the deprotonation of the N_ϵ2-H proton of hisitidine-61 upon formation of the imidazolate bridge. A similar NMR experiment was performed on $Cu^{I}_2Co_2SOD$ to obtain direct evidence for the breakage of the bridging His-61 in the reduced form of SOD.[19] The similarity of the spectra between Ag^+ and Cu^+ derivatives was also observed for $Ag^{I}_2Ni_2SOD$ and $Cu^{I}_2Ni_2SOD$, where the detachment of the bridging His-61 was also shown by three solvent-exchangeable ^1H NMR signals.[3a,22] The similarity between the Ag^+ and the Cu^+ derivatives was also suggested by the similar ^1H NMR N-H signals of the histidyl imidazoles of the derivative $Ag^{I}_2Zn_2SOD$[23] and the reduced native protein $Cu^{I}_2Zn_2SOD$[24] and by the observation of similar NOE results on the N-H protons.[23,24a] All of the above results strongly suggest the use of $Ag^{I}_2M'_2SOD$ ($M' = Co^{2+}$, Ni^{2+}, Zn^{2+}) as good spectroscopic models for the reduced SOD.

The electronic spectrum of $Cu^{I}_2Co_2SOD$ at pH 10.5 is virtually identical with that at pH 5.5 (Figure 9A). However, a significant change of the spectrum of Ag_2Co_2SOD with pH was observed and was more pronounced at pH >9, where the absorption band at 580 nm decreased and a new band at 605 nm appeared (Figure 9B).

The isotropically shifted N-H signals A, C, and G of $Cu^{I}_2Co_2SOD$ are strongly affected by increasing pH while other signals are virtually unchanged (Figure 8A). The decrease of the intensity of these N-H signals under alkaline conditions is due to the faster exchange rate of these signals with water protons at higher pH followed by a saturation transfer from the saturated water magnetization (caused by the modified DEFT multipulse sequence) to the signals.[25]

Most of the isotropically shifted signals of Ag_2Co_2SOD at pH 9 were the same as those at pH 6 except that the N-H signals a and g were wiped out by their more rapid exchange with the

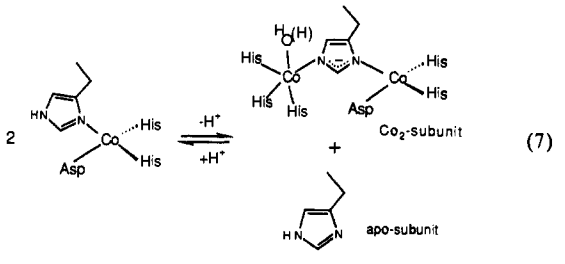
(21) Bertini, I.; Lanini, G.; Luchinat, C.; Messori, L.; Monnanni, R.; Scozzafava, A. *J. Am. Chem. Soc.* **1985**, *107*, 4391–4396.
(22) Ming, L.-J.; Banci, L.; Luchinat, C.; Bertini, I.; Valentine, J. S. *Inorg. Chem.* **1988**, *27*, 4458–4463.
(23) Ming, L.-J. Ph.D. Dissertation, University of California, Los Angeles, 1988.
(24) (a) Stoesz, J. D.; Malinowski, D. P.; Redfield, A. G. *Biochemistry* **1979**, *18*, 4669–4675. (b) Cass, A. E. G.; Hill, H. A. O.; Smith, B. E.; Bannister, J. V.; Bannister, W. H. *Biochemistry* **1977**, *16*, 3061–3066.
(25) Jardetzky, O.; Roberts, G. C. K. *NMR in Molecular Biology*; Academic: New York, 1981.

solvent at higher pH (Figure 8B).[25] Larger changes in the signals were observed at pH >9 where signals b, c, and f had upfield shift while signal d moved to the further downfield region. The pH-dependent changes of the electronic and the NMR spectra of both derivatives were completely reversible.

Discussion

The changes in the spectra of the Co^{2+}-substituted proteins and the NMR relaxation rate of phosphate in the protein solutions can be concluded to be due to changes in the Co^{2+}-binding environment[13,14] and in the phosphate binding affinity to the proteins. We propose here some explanations for the pH-dependent changes of these Co^{2+} derivatives, including a pH-dependent redistribution of Co^{2+} in the two metal binding sites and the important role of the bridging His-61, and will discuss the significance and some implications of the study as follows.

Cobalt(II) Migration. The similarity of the electronic and NMR spectra of E_2Co_2SOD to those of Co_2Co_2SOD at high pH's in phosphate solution suggests that the geometric configurations of the metal binding sites of these two cobalt derivatives are the same under those conditions. We conclude here that 1 equiv of Co^{2+} in E_2Co_2SOD has migrated from the zinc site to the empty copper site to form 1 equiv of two-cobalt (Co_2) subunit with a five-coordinate cobalt (see reaction 8 in next section) in the copper site under high pH conditions as shown in reaction 7.



(7)

This conclusion also implies that 1 equiv of apoprotein subunits is produced as well. The similarity of the Co_2-subunit derivative to the directly prepared Co_2Co_2SOD was shown by the resemblance of the isotropically shifted ^1H NMR spectra of both species at pH >10 (Figure 7). The recovery of E_2Co_2SOD from the Co_2 subunit upon lowering the pH is quite slow and is accompanied by the gradual disappearance of the Co_2 subunit (i.e. the marked signals in Figure 8). This result further confirms that the Co_2 subunits are formed at high pH. However, whether the species formed is a one-to-one mixture of Co_2Co_2SOD and *apo*-SOD, an *apo*-(Co_1Co_1)SOD (i.e., a dimer containing a Co_2 subunit associated with an apo subunit), or a mixture of all three possible derivatives cannot be distinguished by the evidence available.

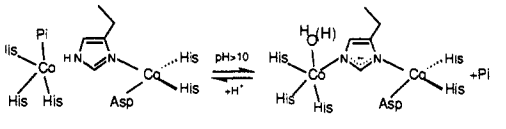
The possibility that Co^{2+} is lost from Co_2Co_2SOD at high pH can be simply ruled out by comparing the absorptivity of their electronic absorptions at different pH's (Figure 1), in which it is clearly shown that the absorptivity of Co_2Co_2SOD is double that of E_2Co_2SOD. In addition, the absorptivity of Co_2Co_2SOD in acetate is virtually unchanged at high pH, which indicates that Co^{2+} is still bound to the protein (Figure 1B).

The signals at 30 and −9 ppm of E_2Co_2SOD in phosphate observed at pH* 8.4 (Figure 7A) with lower intensity were consistent with two signals of Co_2Co_2SOD under the same conditions (Figure 7B). However, these signals were not observed either at lower pH in phosphate solution or at pH* 10.4 in acetate solution. The intensity of these signals increased with pH, suggesting that the migration of Co^{2+} has occurred at pH* 8.4.

The migration of Co^{2+} may be promoted by two different factors, i.e., an increase in the affinity constant for Co^{2+} binding to the copper site when phosphate is present as well as the effect of high pH. The significance of phosphate and pH on the Co^{2+} binding to SOD has been demonstrated by two observations:[12] first, when *apo*-SOD was dialyzed against Co^{2+} in 50 mM acetate buffer at pH 5.5, only the derivative E_2Co_2SOD was obtained; second, Co^{2+} was observed to bind to the copper site of E_2Co_2SOD and E_2Zn_2SOD very slowly in acetate solution only at pH >7; however, the rate was greatly accelerated by phosphate. The Co^{2+}-migration phenomenon was not observed in the absence of phosphate in 2 days by NMR spectroscopy, indicating that the migration either does not occur or is extremely slow under highly alkaline conditions without phosphate. E_2Co_2SOD is not stable at pH >10 for more than 2 days, which prevented us from investigating the possibility of a very slow Co^{2+}-migration phenomenon in acetate solution.

The Co^{2+} migration we observe is similar to that reported previously for Cu_2E_2SOD where Cu^{2+} moves from the copper site to the empty zinc site at high pH.[6] A change of the metal binding affinity of either or both metal binding sites of the protein with pH[5] may result in the redistribution of the metal ions from one metal binding site to the other. However, whether or not the Zn^{2+} in E_2Zn_2SOD has similar pH-dependent redistribution (from the zinc site to the copper site) is not easily resolved owing to the absence of suitable spectroscopic properties for Zn^{2+}. The use of Co^{2+} substitution in the study of zinc proteins is usually quite successful since both native enzymatic activity and structure appear to be preserved.[13,14] The observation of the pH-dependent redistribution of Co^{2+} ion from a single metal binding site to both sites in the derivative E_2Co_2SOD therefore implies that a similar situation may occur in the Zn^{2+} derivative as well.

Co_2Co_2SOD and Co_2Zn_2SOD. The line shapes of the electronic spectra of Co^{2+} in the zinc site have been analyzed on a number of Co^{2+}-substituted SOD's in the presence and absence of phosphate by Banci et al.,[11] and the results were interpreted as indicating whether or not His-61 was bridging between the metal ions in the copper and zinc sites. The appearance of a distinct band at about 600 nm was correlated to the existence of an imidazolate bridge, such as in the derivatives Cu_2Co_2SOD and Co_2Co_2SOD in acetate solutions.[11] Using this criterion, Banci et al. observed that in the presence of phosphate in $Co_2M'_2SOD$ (M' = Co^{2+}, Zn^{2+}), the imidazolate bridge was broken and both metal sites became 4-coordinate.[11] In this paper, we show that the influence of phosphate on the electronic and NMR spectra of $Co_2M'_2SOD$ vanishes at pH >10 and the spectra resemble those taken in the absence of phosphate under the same conditions as shown in Figures 1 and 5. We conclude here, based on the electronic spectroscopic studies, that the imidazolate bridge is present in the derivatives $Co_2M'_2SOD$ at high pH and that the Co^{2+} in the copper site becomes 5-coordinate and is not affected by the presence of phosphate. Whether the fifth ligand of the Co^{2+} in the copper site of $Co_2M'_2SOD$ is a water or a hydroxide is not directly evident based on our observations, although the second pK_a* of about 10 in these derivatives might be due to the deprotonation of the coordinated water. Our conclusions also indicate that the imidazolate bridge can successfully compete with phosphate for binding to Co^{2+} in the copper site in the derivatives $Co_2M'_2SOD$ at high pH but fails to do so at neutral pH as shown in reaction 8.



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If a simple acid–base equilibrium were the only perturbation on these derivatives, sigmoidal curves would be shown on the chemical shifts versus pH plot as reported for reduced native SOD[24] and several other protein systems.[25] The "doubly sigmoidal" curves observed in this study imply the existance of several different pH-dependent perturbations at the metal binding site, including the interaction of phosphate with the protein and the deprotonation of certain groups, such as the water molecule and some amino acid residues in the proximity of the active site. This assumption was confirmed by numerical fittings using three equilibria of the proteins in addition to the equilibria of phosphate (eqs 1–4 and Figure 3).

A change in the charge of the proteins due to deprotonation of specific amino acid residues in the active site channel may alter the apparent affinity constant for phosphate binding to the active site of the proteins. The interaction of phosphate with the Co^{2+} derivatives can be monitored by means of nuclear relaxation as

shown in eqs 5 and 6, in which a higher relaxation rate obviously results from a larger molar fraction of bound phosphate. As shown in Figure 4, the phosphate relaxation rate, which reflects the apparent phosphate binding affinity to the proteins, decreases dramatically at pH* >7.5 and is accompanied with the deprotonation of an amino acid residue(s) with pK_a's of 7.0–7.5 (uncalibrated pH readings in D_2O). The significant influence of the deprotonation of the protein in phosphate binding to Co^{2+} in the copper site also suggests that the deprotonation may occur near the metal binding site in the active site channel. A similar conclusion was also proposed for the studies of phosphate binding to native SOD at different pH's by ^{31}P nuclear relaxation.[10b] Although the phosphate binding situation for native SOD (bound to Arg-141)[10] is different from that for Co^{2+} derivatives (bound to Co^{2+}),[11,12] the similar conclusion can be used for both situations, i.e., that the larger affinity constant for phosphate binding to native SOD and the Co^{2+}-substituted derivatives at pH 7 relative to that at pH 6.3 is due to a higher affinity of HPO_4^{2-} binding to the proteins, and a smaller affinity constant at pH 8 (or higher pH in this study) is due to the deprotonation of a group(s) in the proximity of the active site.

$Ag^I_2Co_2SOD$ and $Cu^I_2Co_2SOD$. The influence of pH on the Co^{2+} binding site of $Cu^I_2Co_2SOD$ is not significant. Co^{2+} in the zinc site retains its tetrahedral geometry under highly alkaline conditions as shown by the virtually unchanged electronic spectra indicating the resistance of this derivative to high pH. The results also indicate that His-61 residue remains as a monodentate ligand, i.e. non-bridging, over a wide range of pH. The Co^{2+} migration which occurred for E_2Co_2SOD is clearly blocked by the presence of either Cu^+ or Ag^+ ion in the copper site.

Because the zinc site has a closed coordination sphere, any perturbation at this site caused by changing the conditions of the medium is relatively minor compared to that at the copper site as reported on Cu_2Co_2SOD[21] and Cu_2Ni_2SOD[3,22] in the presence of different anions. However, deprotonation of the imidazole N_ϵ2-H of His-61 forming a bridge between the Co^{2+} in the zinc site and the metal ion in the copper site may have a significant influence on the inner sphere of Co^{2+} in the zinc site which results in changing of its electronic spectra as mentioned above for $Co_2M'_2SOD$ (M' = Co^{2+}, Zn^{2+}).[11,12] The line shape of the electronic spectrum (with a distinct band at 605 nm) of $Ag^I_2Co_2SOD$ at pH >10 suggests the possibility that His-61 residue may be a bridging ligand between Co^{2+} in the zinc site and Ag^+ in the copper site under highly alkaline conditions.

While hydrogen bonding can decrease the lability of N-H protons of a histidine residue,[25] an electrostatic interaction between the N_ϵ2-H proton of His-61 (which points toward the copper site) and the metal ion in the copper site of $M^I_2Co_2SOD$ may dramatically increase the lability of this proton. This electrostatic interaction may result in the deprotonation of the N_ϵ2-H proton of His-61 forming a more stable imidazolate-bridged configuration under highly alkaline conditions. An early study of Co^{2+}–histidine complexes at different pH's showed that the N_ϵ2-H proton of the imidazole ring was completely deprotonated at about pH 12 (while the pK_a of the N_ϵ2-H proton of free histidine is about 14) to form a more favorable tetrahedral configuration.[26] This result implies that deprotonation of N_ϵ2-H of His-61 residue (a ligand of Co^{2+} in the zinc site) in Ag_2Co_2SOD may also occur at about pH 12 and result in formation of the His-61 bridge. This phenomenon might also occur in $Cu^I_2Co_2SOD$ at higher pH's; however, denaturation of this reduced protein at pH 12 prevented us from observing any further spectral changes under those conditions.

The spectroscopic similarity between Cu^+ and Ag^+ derivatives under neutral conditions has allowed us to use the Ag^+ derivatives as models for the metal binding structure of reduced SOD.[3a,8,23] However, both the electronic and the isotropically shifted ^1H NMR spectra of the derivative Ag_2Co_2SOD are dramatically different from those of the reduced Cu_2Co_2SOD under high pH conditions, indicating the structure of the metal binding site between these two derivatives is significantly different from each other under those conditions. It is noted here that caution must be considered when using silver derivatives as models to study the reduced form of SOD's under different pH conditions.

Summary

The significant influences of pH on the Co^{2+}-substituted derivatives are threefold: (1) the metal binding affinities to the two metal binding sites are strongly affected by pH; (2) the phosphate binding affinity to the Co^{2+} in the copper site varies with pH and phosphate binding is associated with conformational changes of the active site; (3) the formation of the imidazolate bridge in the derivatives $Co_2M'_2SOD$ is pH dependent, i.e., his-61 residue is competing with phosphate for binding to Co^{2+} in the copper site in the derivatives $Co_2M'_2SOD$. This competition results in the formation of the bridging imidazolate in those derivatives at high pH but not at neutral pH. These influences of pH cause (1) Co^{2+} migration from the zinc site to the empty copper site in E_2Co_2SOD by change of the metal binding affinity, (2) a 4-coordinate (without the His-61 bridge) to 5-coordinate (with the His-61 bridge) conformational change for Co^{2+} in the copper site of $Co_2M'_2SOD$ due to changes of the phosphate binding affinity, and (3) the formation of the His-61 bridge in Ag_2Co_2SOD by deprotonation of the imidazole N_ϵ2-H of histidine-61 residue.

Acknowledgment. Financial support from the USPHS (GM 28222) is gratefully acknowledged. We also thank Dr. James A. Roe for many helpful discussions. Many discussions with Dr. Jane Strouse and Nazim Jaffer on NMR techniques are also acknowledged.

Registry No. SOD, 9054-89-1; Co, 7440-48-4; Cu, 7440-50-8; Zn, 7440-66-6; Ag, 7440-22-4; Pi, 14265-44-2.

(26) McDonald, C. C.; Phillips, W. D. *J. Am. Chem. Soc.* **1963**, *85*, 3736–3742.