yeast transcriptional activator are located in the major groove nine to ten base pairs apart, symmetrically displaced four to five base pairs from the central C of the recognition site.[15] This provides experimental validation of the Y-shaped model recently put forward for a class of DNA binding proteins important in the regulation of gene expression.[6k]

Summary. We have designed and synthesized two peptide reagents, BEG and TCE, for the introduction of the metal chelator EDTA at discrete amino acid residues of peptides and proteins by Merrifield solid-phase protein synthesis employing Boc amino acids. Due to the ease of synthesis, stability, coupling efficiency, and flexibility in choice of linker, TCE is likely the reagent for most applications. These reagents have been used to attach EDTA to three different DNA binding domains, which has allowed the tertiary structures of the protein-DNA complexes to be characterized by affinity cleaving.[14-16] This information will contribute to the body of knowledge necessary to define the principles for relating primary amino acid sequence to the tertiary structures of proteins bound to DNA and RNA. Moreover, because the reactive oxidant generated from EDTA·Fe can cause cleavage of proteins,[29] protein-EDTA·Fe molecules can be used for affinity cleaving studies of protein structure and protein-protein complexes.[30]

Acknowledgment. We are grateful for generous support from the DARPA University Research Initiative Program, Merck, an NSF predoctoral fellowship to J.H.G., and NIH traineeships to J.P.S. and D.P.M.

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NMR Studies of Nickel(II)-Substituted Derivatives of Bovine Copper-Zinc Superoxide Dismutase with Nickel(II) Bound in the Copper Site†

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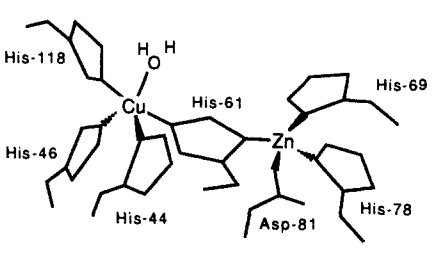
Abstract: Two new Ni^{2+}-substituted derivatives of bovine copper-zinc superoxide dismutase (Cu_2Zn_2SOD) with Ni^{2+} bound in the copper site have been prepared and studied by electronic and NMR spectroscopies. The Ni^{2+} binding environment of these derivatives is found to be very similar to that of Cu^{2+} in native SOD; i.e., the metal ion was coordinated to two histidines (presumably His-46 and His-118) through the $N_{\epsilon 2}$ nitrogen and to another histidine (presumably His-44) through the $N_{\delta 1}$ nitrogen as well as to the bridging His-61, which remained bridging even in the presence of anions. The anion binding properties of Ni^{2+} were also found to resemble those of Cu^{2+} in that site. Both azide and cyanide were found to bind to these Ni^{2+}-substituted derivatives forming an axially symmetric metal binding site. Azide binding to the derivatives caused all the isotropically shifted signals to shift. However, all of the signals were still isotopically shifted out of the diamagnetic region in the presence of a saturating amount of azide, indicating that none of the coordinated histidines was completely detached from the metal coordination sphere under these conditions. Cyanide binding to these derivatives caused Ni^{2+} to become diamagnetic, as shown by the disappearance of all the isotropically shifted ^1H NMR signals of the coordinated histidines. This observation leads us to the conclusion that the Ni^{2+} becomes square planar upon cyanide binding, a result similar to that observed for native SOD studied by several different spectroscopic methods, e.g., EPR and EXAFS. The change of the spin state of Ni^{2+} in these derivatives upon cyanide binding was also demonstrated by ^{13}C NMR spectroscopy using $^{13}CN^-$. Phosphate, however, did not perturb the Ni^{2+} in the copper site significantly, suggesting its possible binding to positively charged residues near the metal binding site similar to its interaction with the native enzyme. The similarities of the structure of the Ni^{2+} binding site in these derivatives and their anion adducts to those of the native SOD indicate that these derivatives can serve as good structural models for copper-zinc superoxide dismutase.

Copper-zinc superoxide dismutase (Cu_2Zn_2SOD)[1] isolated from bovine liver is a dimeric metalloprotein of molecular weight 31 200, containing a Cu^{2+} and a Zn^{2+} ion in each of its identical subunits bridged by the imidazole side chain of the histidine-61 residue.[2]



Much information concerning the nature of the metal binding sites, including geometries and configurations, anion interactions, and SOD activity, has been obtained from examination of the properties of derivatives in which spectroscopically interesting metal ions have been substituted for the native metal ions.[3] Ni^{2+} and Co^{2+} substitution for Zn^{2+} in bovine Cu_2Zn_2SOD has provided derivatives (i.e., Cu_2Ni_2SOD[4] and Cu_2Co_2SOD[5]) with high SOD activity, suggesting that these derivatives are good models for Cu_2Zn_2SOD. Because of the existence of magnetic coupling between Cu^{2+} in the copper site and Ni^{2+} or Co^{2+} in the zinc site,

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(1) Abbreviations: $M_2M'_2SOD$, M- and M'-substituted superoxide dismutase with M in the copper site and M' in the zinc site (an E in the above derivatives represents an empty site); EXAFS, extended X-ray absorption fine structure; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; DEFT, driven equilibrium Fourier transform; FID, free induction decay.
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the electronic relaxation rate of the unpaired electron of Cu^{2+} (which causes excessively broad ^1H NMR signals in uncoupled systems) is dramatically enhanced.[6-8] As a consequence, protons of the coordinated ligands in the copper site can be detected to be isotropically shifted with relatively narrow signal width.[4,6-8]

Although several metal ions, including Co^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+}, have been used to probe the zinc site, only three metal ions, Ag^+, Co^{2+}, and Zn^{2+}, have been successfully used to substitute the Cu^{2+} in the copper site.[3] In all the metal-substituted derivatives prepared, only when Cu^{2+} is in the copper site do the derivatives show significant activity.[3] While Ag^+ is an analogue of Cu^+ and has been used to probe the reduced form of the native enzyme, it is not a good probe for spectroscopic studies owing to its d^{10} electronic configuration.[4,8,9] Co^{2+} is not a good analogue for Cu^{2+} in Cu_2Zn_2SOD since the geometric and anion binding (especially phosphate binding) properties of these Co^{2+}-substituted derivatives have proved to be significantly different from those of the native enzyme.[10-15] Unlike Ag^+ and Co^{2+}, Ni^{2+} has geometric preferences very similar to those of Cu^{2+}.[16] Since the magnetic properties of Ni^{2+} and Cu^{2+} are very different,[16] the substitution of Ni^{2+} for the Cu^{2+} in copper proteins may be expected to cause minimal structural perturbations while providing a new spectroscopic probe of the native copper binding site.

The interaction of azide with the derivatives Cu_2Co_2SOD and Cu_2Ni_2SOD has been studied by ^1H NMR spectroscopy and detachment of a coordinated histidine was proposed.[6-8] Similarly, EXAFS studies on azide binding to the native enzyme showed a 0.27-Å increase of one Cu–N bond.[17] The azide binding to the enzyme has recently been shown by Banci et al. to correlate well with the SOD activity for a series of mutants of human Cu_2Zn_2SOD, in which Arg-143 (corresponding to Ag-141 in bovine enzyme, which has been proposed to be an important residue for SOD activity[14,15]) was mutated to Lys, Ile, and Glu.[18] Both azide binding affinity and SOD activity of these mutants showed the trend of Arg (i.e., wild type) > Lys > Ile > Glu.[18] Therefore, azide can be considered a superoxide analogue, at least in its binding to Cu^{2+}. Cyanide binding to the enzyme is unique due to its strong binding to the copper site of the enzyme, which not only inhibits the enzyme activity but also dramatically changes the geometry of the copper site as shown by several spectroscopic methods, including electronic,[19] EPR,[19,20] electron spin echo,[21] NMR,[22,23] and ENDOR[24] spectroscopies as well as EXAFS.[17] Cyanide binding to Cu^{2+} in the enzyme results in a dramatic change of the geometry of the Cu^{2+} from distorted 5-coordinate to more axially symmetric as shown by EPR[19,20] and EXAFS.[17] A replacement of one of the coordinated histidines from the copper site by cyanide has also been proposed on the basis of EXAFS and NMR studies.[17,22]

We report here the preparation and characterization of new Ni^{2+}-substituted derivatives of bovine Cu_2Zn_2SOD in which Ni^{2+} is bound in the native copper site. The binding of azide and cyanide to these Ni^{2+}-substituted derivatives of SOD was also investigated. Unlike those Co^{2+}-substituted derivatives with Co^{2+} bound in the copper site, which showed completely different structural and anion binding properties from native SOD as mentioned above,[10-15] the Ni^{2+} binding environment in these derivatives and their anion adducts is remarkably similar to that of the native Cu^{2+} binding site of the enzyme. The similarities of the geometry and anion binding properties of these Ni^{2+}-substituted derivatives and the native enzyme suggest that Ni^{2+} can be a good probe for study of the structural properties of the copper site of Cu_2Zn_2SOD.

Experimental Section

Bovine Cu_2Zn_2SOD purified to homogeneity was purchased from DDI Pharmaceuticals, Inc. (Mountain View, CA) as a lyophilized powder and was used without further purification. All other chemicals used are commercially available. The buffer solutions were prepared by using triply deionized distilled water (Millipore Co., Bedford, MA). Apo-SOD was prepared by dialyzing native Cu_2Zn_2SOD against ethylenediaminetetraacetic acid (EDTA) in acetate buffer at low pH, followed by exhaustive dialysis against 0.1 M NaCl in acetate buffer solution to removed protein-bound EDTA, which was followed by several changes of buffer to remove the salt.[25] The concentration of apo-SOD was determined by the Lowry method[26] or by direct measurement of the absorption at 258 nm with absorptivity of 2920 cm^{-1} M^{-1}.[27] The derivatives E_2Co_2SOD, E_2Zn_2SOD, and E_2Cd_2SOD were prepared by directly infusing 2 equiv of metal ions into apo-SOD in 50 mM phosphate at pH 6 and incubating in a cold room overnight. The protein solutions were then changed to pH 7.5 followed by direct infusion of 2 equiv of Ni^{2+} into the solutions. The formation of Ni_2Co_2SOD, Ni_2Zn_2SOD, and Ni_2Cd_2SOD could be monitored by electronic and NMR spectroscopies. The samples used for NMR studies were concentrated by ultrafiltration using a Centricon microconcentrator (Amicon Co., Danvers, MA) with a molecular weight cutoff of 10000.

The isotropically shifted ^1H NMR spectra were recorded on Bruker WP200 and IBM AF200 spectrometers at 200 MHz and a Bruker CXP90 at 90 MHz using the modified DEFT pulse sequence[28] to suppress H_2O and bulk diamagnetic protein signals. Typical spectra consisted of ~10000 scans with 8K data points over a sufficiently wide spectral width to cover all the signals. Chemical shifts were measured from the H_2O (HDO) signal, which was assumed to be at 4.8 ppm downfield from TMS. A 20-Hz additional line broadening was introduced to all the spectra by exponential multiplication of the FIDs in order to improve the signal-to-noise ratio. ^1H NMR spectra of the derivatives in H_2O were recorded on Bruker AM500 and IBM AF200 spectrometers using selective excitation with the $1\text{-}\bar{3}\text{-}3\text{-}\bar{1}$ hard pulse sequence.[29] The maximum excitation was centered around the signals of interest while the null was set on H_2O.

The spin-lattice relaxation times (T_1) of the isotropically shifted signals were measured by the modified DEFT sequence by varying the delay times between subsequent pulses,[28] and the values were determined by a nonlinear least-square fitting of the signal intensity as a function of the delay time. T_1 of the signals in the diamagnetic region at >10 ppm in H_2O solution were measured by the inversion-recovery method; however, selective excitation pulse sequences[29] were used for the 180° pulse (a

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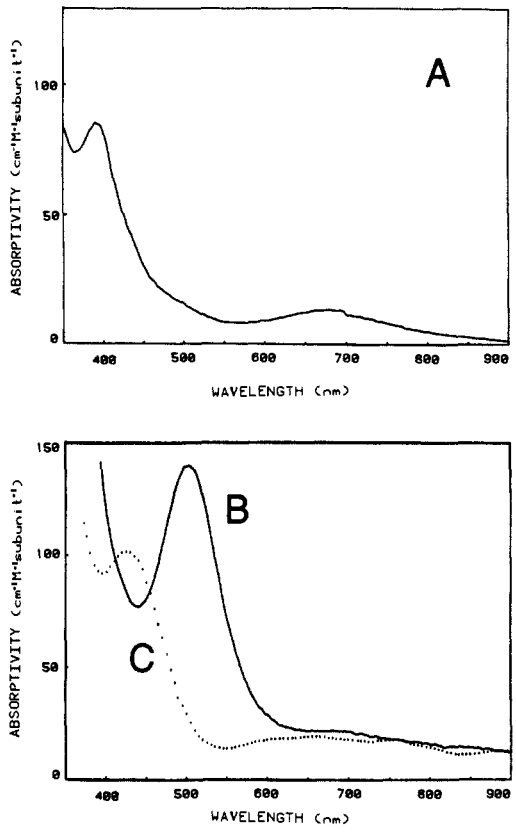

Figure 1. Electronic spectra of (A) Ni_2Zn_2SOD and derivatives in the presence of (B) a saturating amount of N_3^- and (C) 2 equiv of CN^- in 50 mM phosphate buffer at pH 7.5 and room temperature referenced against deionized water.

2-$\bar{6}$-6-$\bar{2}$ pulse sequence) and the 90° pulse (a 1-$\bar{3}$-3-$\bar{1}$ pulse sequence) separated by various delays between the two pulse sequences (eq 1).

$$D1\text{-}180°(2\text{-}\bar{6}\text{-}6\text{-}\bar{2})\text{-}\tau\text{-}90°(1\text{-}\bar{3}\text{-}3\text{-}\bar{1})\text{-}AQ(FID) \quad (1)$$

^{13}C NMR spectra of $^{13}CN^-$ (99% ^{13}C-enriched KCN, Aldrich Chemical Co., Milwaukee, WI) in different solutions of metal-substituted SODs were obtained on a Bruker AM360 spectrometer operated at 90.56 MHz at ambient temperature (~23 °C). The ^{13}C NMR chemical shift of cyanide is highly pH dependent owing to the fast equilibrium between its acid and base forms.[30] The base form, which has a chemical shift of 98.3 ppm downfield from dioxane,[30] is used as the reference (0 ppm) in this study. The electronic spectra were taken on a Beckman UV5270 spectrometer at room temperature referenced against deionized water. The pH of all the solutions were measured on a Corning Model 12 pH meter equipped with a combined microelectrode (Wilmad Glass Co., Inc., Buena, NJ).

Results

Nickel-Zinc Derivative (Ni_2Zn_2SOD). The introduction of 2 equiv of Ni^{2+} to the derivative E_2Zn_2SOD in 50 mM phosphate at pH 7.5 produced a new derivative, which we formulate as Ni_2Zn_2SOD, with Ni^{2+} bound to the empty site of E_2Zn_2SOD, on the basis of the evidence described below. This new derivative is yellowish green with absorption bands at 390 nm (85 cm^{-1} M^{-1} per subunit) and 675 nm (14 cm^{-1} M^{-1} per subunit) (Figure 1A). In the presence of azide, a new band at 500 nm appeared with a larger absorptivity of 140 cm^{-1} M^{-1} per subunit (Figure 1B). An absorption at 420 nm (~100 cm^{-1} M^{-1} per subunit) appeared when 2 equiv of cyanide was added (Figure 1C). The electronic absorption spectra of this derivative in the presence and absence of anions are completely different from those of the derivatives $Cu^I_2Ni_2SOD$ and Ag_2Ni_2SOD, with Ni^{2+} bound in the native zinc site, in which the presence of anions does not affect the spectral features due to Ni^{2+}, consistent with the absence of anion binding to metal ions in the zinc site.[4,8]

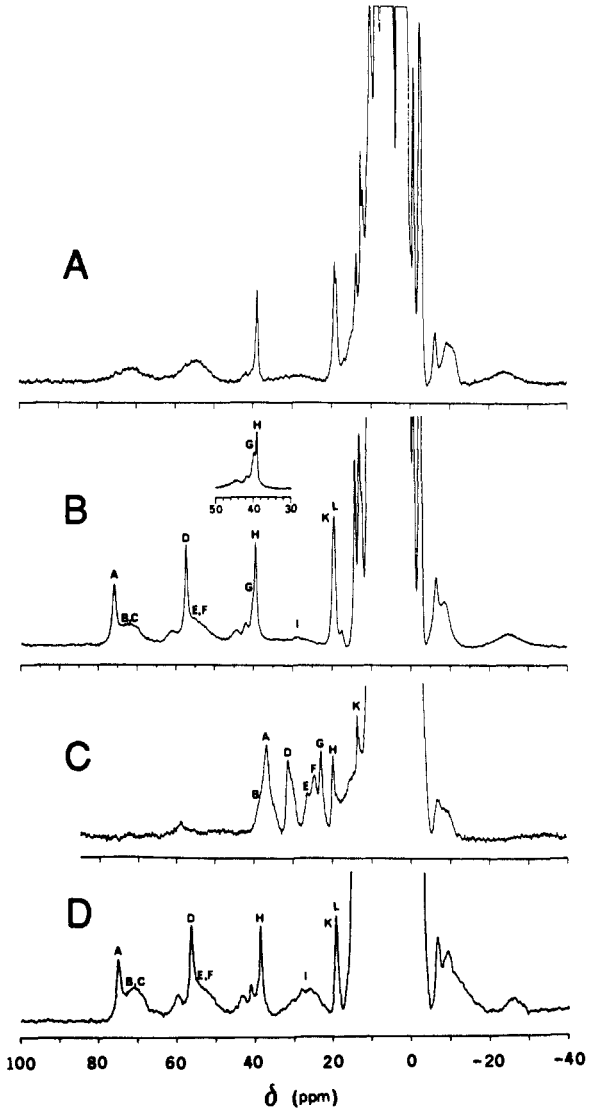

Figure 2. Isotropically shifted ^1H NMR spectra (200 MHz, ~23 °C) of Ni_2Zn_2SOD in 50 mM phosphate at pH 7.5 in (A) D_2O, (B) H_2O, (C) H_2O in the presence of a saturating amount of N_3^- and (D) H_2O at 90 MHz (25 °C) where the broad signal I was clearly detected. The inset in (B) was obtained by using a 1-$\bar{3}$-3-$\bar{1}$ selective excitation pulse centered around 40 ppm downfield from water in order to show the solvent-exchangeable signal G more clearly.

The ^1H NMR spectrum of Ni_2Zn_2SOD shows at least 10 signals isotropically shifted in the region between 90 and -40 ppm (Figure 2B), of which, A, D, and H are relatively sharp signals. When the derivative was in a D_2O solution, the signals A, D, and G as well as a signal at the edge of the diamagnetic region disappeared (Figure 2A). The signal G has a low intensity due to fast exchange with the partially saturated solvent (caused by the modified DEFT pulse sequence). The signal could be clearly detected when a selective excitation 1-$\bar{3}$-3-$\bar{1}$ hard pulse[29] was applied (inset in Figure 2B). The phenomenon was also observed on the derivatives $M^I_2Ni_2SOD$ (M = Cu, Ag) in which solvent-exchangeable signals not clearly observed due to fast exchange with solvent could be unambiguously assigned by using the selective excitation pulse.[8,31] The signals at 72.4 (B + C), 55.0 (E + F), and 28 (I) ppm are broad at 200 MHz; however, they are relatively sharper at 90 MHz as shown in Figure 2D, where the signal at 28 ppm is clearly detected. The increase of the line width of the signals with magnetic field may arise from Curie relaxation and/or from field-dependent modulation of the zero-field splitting, which affects the electronic relaxation rate of the Ni^{2+} ion,[32] as observed in the

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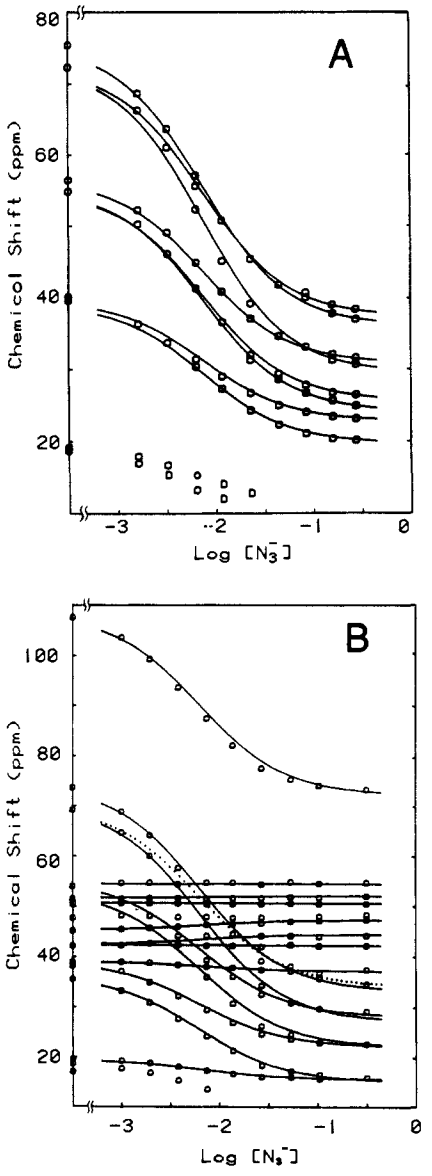

Figure 3. Plot of ^{1}H NMR chemical shifts (200 MHz, ~23 °C) of (A) Ni_2Zn_2SOD and (B) Ni_2Co_2SOD against $[N_3^-]$ in 50 mM phosphate buffer at pH 7.5. The numerical fittings (solid lines) were according to eq 2. Affinity constants of 135 and 160 M^{-1} were obtained for (A) and (B), respectively. The dotted line in (B) was obtained by assuming that a signal splitting might occur as that of the broad signal B + C in (A).

derivative Ag_2Ni_2SOD.[8,31] The chemical shifts and proton spin–lattice relaxation times (T_1) of this derivative are reported in Table I.

Azide binding to the derivative Ni_2Zn_2SOD could be detected by isotropically shifted ^{1}H NMR spectroscopy (Figure 2C). The bound azide exchanges rapidly with free azide in solution as compared to the NMR time scale; therefore, the chemical shift of each signal with respect to increasing amount of azide can be followed and is reported in Figure 3A. The two broad signals at 72.4 and 55.0 ppm are split by the addition of azide (Figure 3A and Table I). A fitting of the chemical shifts with respect to the concentration of azide to a simple equilibrium (eq 2),

$$\text{Ni,M'SOD} + N_3^- \overset{K}{\rightleftharpoons} \text{Ni,M'SOD–}N_3^- \qquad (2)$$

assuming a fast equilibrium with respect to the NMR time scale, gives an affinity constant K = 135 M^{-1}, coincident with those of native SOD (138 M^{-1} at pH 5.6),[6] Cu_2Ni_2SOD (111 M^{-1} at pH 6.5 in 50 mM phosphate),[8] and Cu_2Co_2SOD (150 M^{-1} at pH 5.5 in 10 mM acetate)[6,8] with Cu^{2+} in the copper site. Ni,M'SOD in eq 2 is a subunit of the derivative $Ni_2M'_2SOD$, and there is no attempt to take into account any cooperativity between the two subunits.

The intensity of the isotropically shifted signals of the derivative Ni_2Zn_2SOD decreased upon addition of cyanide, vanishing by the time 2 equiv of cyanide had been added. In the meantime, several signals could be detected in the region between 11 and 15 ppm with relatively long T_1's (40–60 ms) measured by the pulse se-

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Table I. ^{1}H NMR Chemical Shifts (200 MHz)[a] of the Protons in the Ni^{2+} Binding Site of (I) Ni_2Zn_2SOD, (II) Ni_2Co_2SOD in the Presence and Absence of Azide, and (III) Ni_2Cd_2SOD and T_1 of the Signals in (I) at 90 MHz

	chemical shift (T_1)[b]			chemical shift ($+N_3^-$)[c]	
signal	I	II	III	I	II
A	75.6 (11.5)	73	76.2	36.0	33.3
A′	*d*	107.6		*d*	72.4
B				37.3	34.1[e]
	72.4 (1.8)	69.2			
C				29.5	27.2
D	56.5 (17.8)	54.0	54.2	30.8	28.2
E				25.6	
	55 (<1.8)	52.6[f]			22.3
F				24.1	
G	39.7	39.0	39.8	22.8	22.4
H	39.0 (21.6)	35.0	35.6	19.7	14.8
I	28[g]	*g*			
K	19 (20.8)	19.6	20.3		

[a] In 50 mM phosphate buffer at pH 7.5. [b] Chemical shifts in ppm and T_1 in milliseconds. [c] From numerical fitting in Figure 3. [d] Not seen. [e] Signal splitting was not clearly observed due to serious signal overlap, but was obtained by the fitting assuming signal splitting similar to that in I would occur. [f] Revealed by azide titration and numerical fitting. [g] Detected at 90 MHz but not clearly observed at 200 MHz.

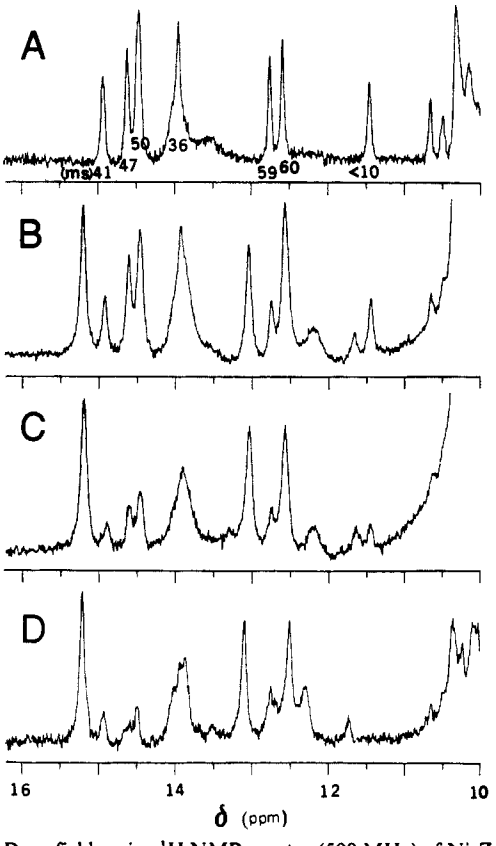

Figure 4. Downfield-region ^{1}H NMR spectra (500 MHz) of Ni_2Zn_2SOD in H_2O in the presence of (A) 2, (B) 8, and (C) ≫8 equiv of cyanide and (D) E_2Zn_2SOD in H_2O. All the solutions are in 50 mM phosphate at pH 7.5. The relaxation times are also reported in (A).

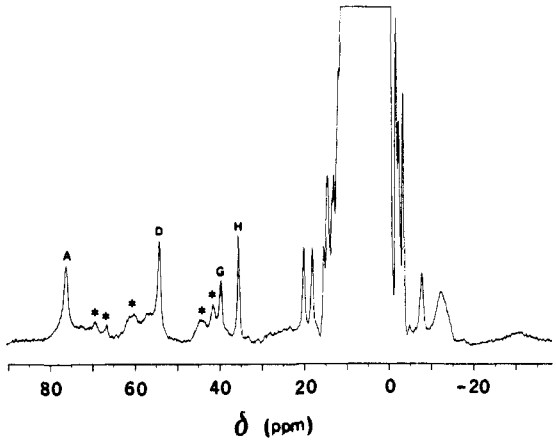

Figure 5. Isotropically shifted ^1H NMR spectrum of Ni_2Cd_2SOD at 200 MHz and ~23 °C in H_2O and 50 mM phosphate at pH 7.5. The signals A, D, and G are solvent exchangeable as judged by their disappearance in D_2O. The signals marked by asterisks are due to Ni^{2+} bound in the zinc site (see text).

quence as in eq 1 (Figure 4). In the presence of excess cyanide, the spectrum changed further and became very similar to that of the derivative E_2Zn_2SOD, suggesting that the loss of Ni^{2+} from the copper site had occurred (Figure 4C).

The phosphate buffer was replaced by a HEPES buffer at pH 7.5 by ultrafiltration with several changes of the HEPES buffer in order to study the influence of phosphate on the metal binding site of Ni_2Zn_2SOD. The isotropically shifted ^1H NMR spectrum of the derivative Ni_2Zn_2SOD was affected little by removal of the phosphate anion. The chemical shifts of the isotropically shifted signals changed by less than 3 ppm (out of a spectral width of ~120 ppm) in the presence of 50 mM phosphate. This result is contrary to the dramatic influence of phosphate on the geometry and spectra of the Co^{2+} derivative, Co_2Zn_2SOD,[10-13] in which phosphate is directly bound to the Co^{2+} ion in the copper site. The influence of pH on the isotropically shifted signals was not noticeable. The chemical shifts of most of the isotropically shifted signals of Ni_2Zn_2SOD at pH 10.2 in the presence or absence of phosphate were found to be almost identical with those in 50 mM HEPES solution at pH 7.5 except that the NH signals A and G had disappeared, due to fast exchange with solvent at high pH.

The derivative Ni_2Cd_2SOD was found to be similar to Ni_2Zn_2SOD with Ni^{2+} as the only chromophore. The isotropically shifted ^1H NMR spectrum of Ni_2Cd_2SOD showed three solvent exchangeable signals (A, D, and G in Figure 5) and one sharp CH signal H with chemical shifts similar to those of Ni_2Zn_2SOD (Table I). However, we found that Ni^{2+} was not bound exclusively to the copper site of E_2Cd_2SOD in our preparations as shown by the observation of signals due to Ni^{2+} binding to the zinc site,[31,33] presumably because Ni^{2+} competes with Cd^{2+} for the zinc site. Azide binding to this derivative was complicated by the lack of purity and consequently could not be studied.

Nickel-Cobalt Derivative (Ni_2Co_2SOD). The shape of the electronic spectrum of E_2Co_2SOD in 50 mM phosphate at pH 7.5 changed very slowly after the infusion of 2 equiv of Ni^{2+} into the solution. Eventually, a new band at 600 nm with a molar absorptivity of 420 cm^{-1} M^{-1} per subunit appeared (Figure 6A). The absorptions at 563 and 535 (shoulder) nm were essentially uninfluenced by the Ni^{2+} binding to the empty copper site of E_2Co_2SOD. An absorption at 393 nm with a lower absorptivity (100 cm^{-1} M^{-1} per subunit) was also detected, which was similar to that observed in Ni_2Zn_2SOD as described above and is therefore ascribed to nickel absorption (Figure 1A). A minor SOD activity (~1%) was detected for this derivative by using the cytochrome *c*-xanthine oxidase assay.[34] This trace activity may be due to residual Cu^{2+} in the copper site of the enzyme.

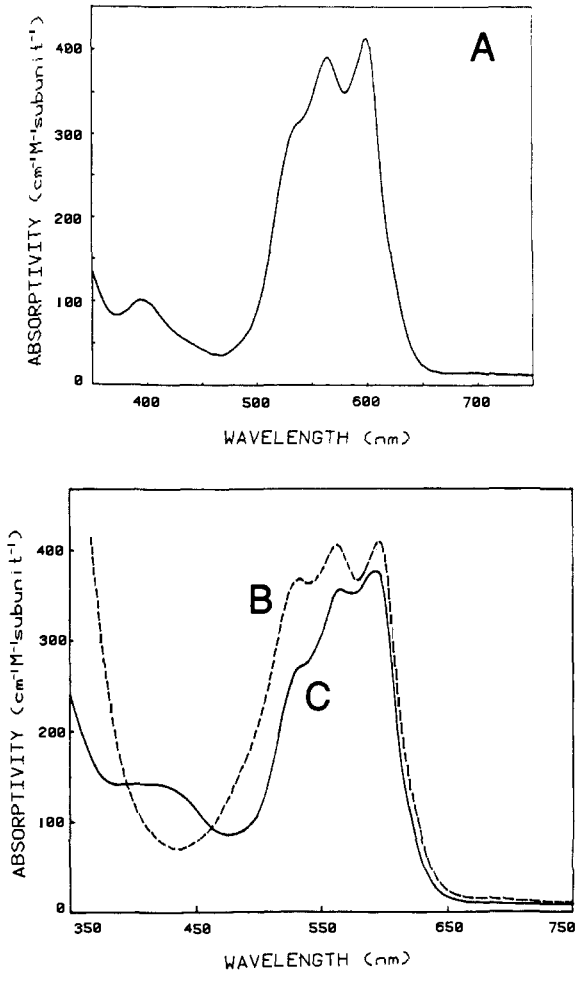

Figure 6. Electronic spectra of (A) Ni_2Co_2SOD and the derivative with (B) a saturating amount of N_3^- and (C) 2 equiv of CN^- in 50 mM phosphate at pH 7.5 and room temperature referenced against deionized water.

In the presence of azide, the absorption band due to the Ni^{2+} at 393 nm decreased and a new band appeared at 532 nm overlapping with the shoulder of the cobalt absorptions. However, the cobalt absorptions were not affected significantly by the presence of azide, consistent with the absence of anion binding to the Co^{2+} in the zinc site (Figure 6B). When 2 equiv of cyanide was added to this derivative, the band at 393 nm shifted to 415 nm (Figure 6C) in a fashion similar to that observed for CN^- binding to Ni^{2+} in Ni_2Zn_2SOD (Figure 1C). The changes in the Co^{2+} absorptions were not dramatic, the band at 600 nm shifted to 592 nm and became less distinct from the band at 563 nm.

This derivative, Ni_2Co_2SOD, has a particularly rich ^1H NMR spectrum consisting of isotropically shifted resonances from ligands bound to both Ni^{2+} and Co^{2+} ions spread over a range of ~150 ppm (Figure 7A). Some of the signals are nearly superimposed with those of Ni_2Zn_2SOD, indicating a very similar magnetic environment of these protons in both derivatives (Table I). When the derivative was prepared in D_2O solution, the ^1H NMR signals A, D, d, and G, as well as a signal near the diamagnetic region, L, disappeared (inset in Figure 7A), indicating fast exchange of these protons with solvent. The signals A, D, d, and G can be assigned to the imidazole NH protons of the histidines ligated to the paramagnetic Ni^2 and Co^{2+} ions. The signal L may be due to an amido NH proton in the proximity of the paramagnetic metal ions owing to its being only slightly isotropically shifted.

The preparation of this derivative was highly dependent upon the buffer used and the pH conditions. There was no detectable Ni_2Co_2SOD formed in phosphate solution at pH 5.5 as judged by electronic and NMR spectroscopies. The formation of Ni_2Co_2SOD at pH 6.5 was incomplete as compared to that at pH 7.5 in the same period of time (Figure 8). The formation of

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(34) McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1969**, *244*, 6049-6055.

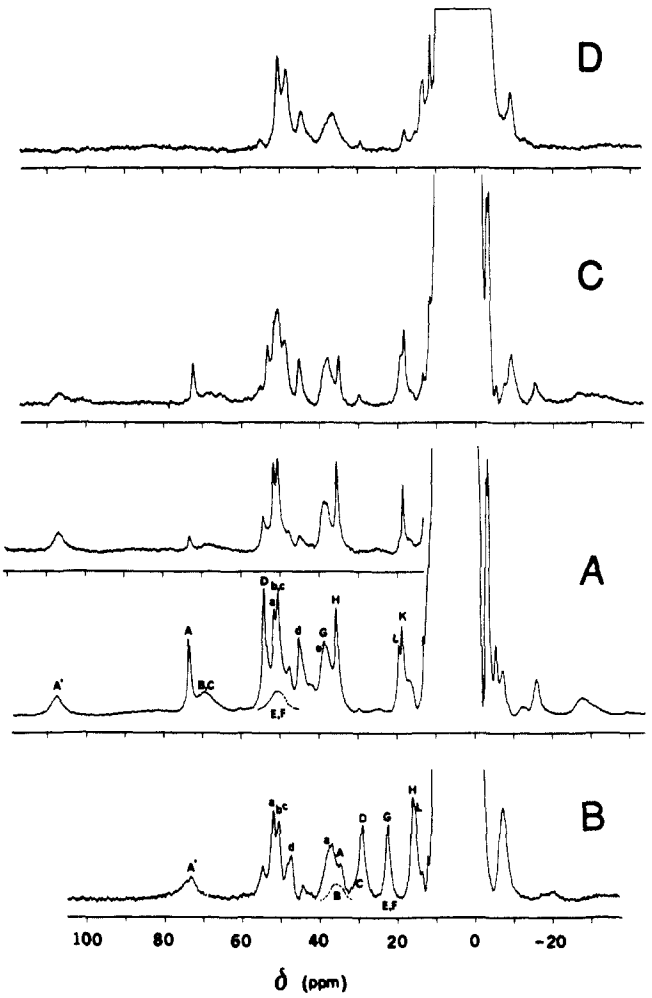

Figure 7. Isotropically shifted ^1H NMR spectra (200 MHz) of Ni_2Co_2SOD in (A) H_2O and D_2O (inset), (B) H_2O with a saturating amount of N_3^-, and in H_2O with (C) 1 equiv and (D) 2 equiv of CN^-. All the solutions above were in 50 mM phosphate at pH 7.5 and ~23 °C.

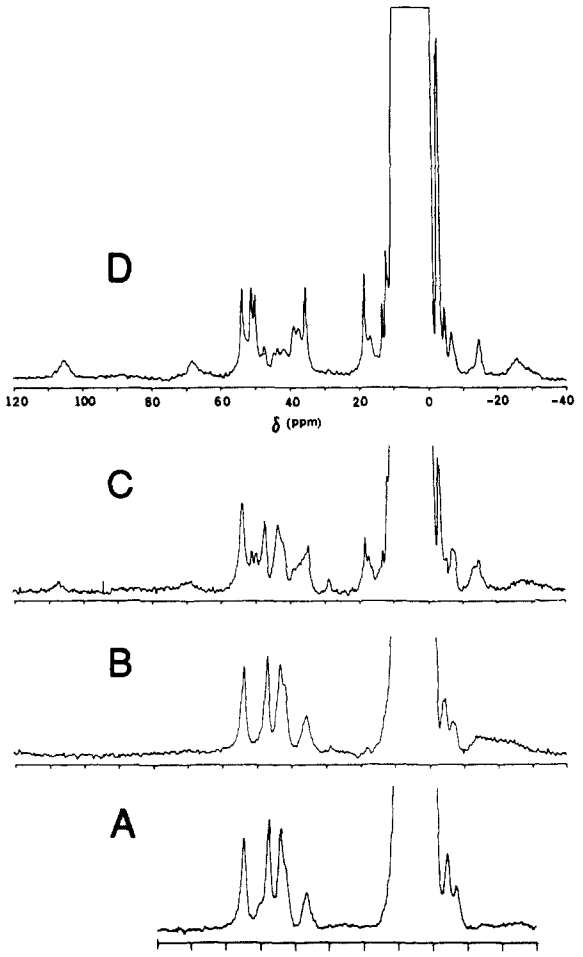

Figure 8. Isotropically shifted ^1H NMR spectra of E_2Co_2SOD (200 MHz, 25 °C) (A), in the presence of 2 equiv of Ni^{2+} at pH 5.5 (B), pH 6.5 (C), and pH 7.5 (D) in 50 mM phosphate solution. The solutions were prepared in H_2O under different conditions as above, allowed to stand for 2 weeks, and then lyophilized. NMR samples were prepared in D_2O from the lyophilized proteins.

Ni_2Co_2SOD reached a ≥95% completion at pH 7.5 in >2 weeks based on the residual NMR signals from E_2Co_2SOD. This observation is reminiscent of the pH- and buffer-dependent preparation of the Co^{2+} derivatives with Co^{2+} bound in the copper site, Co_2Zn_2SOD and Co_2Co_2SOD, which could be easily prepared only in the presence of phosphate or at pH >7.[10]

The isotropically shifted ^1H NMR spectrum of Ni_2Co_2SOD was also found to be significantly influenced by azide, as shown in Figure 7B. Similar to the case of Ni_2Zn_2SOD, the fast exchange rate of azide between its bound and free states with respect to the NMR time scale allowed the use of a titration technique to follow the changes of the isotropically shifted proton signals with increasing amount of azide (Figure 3B). The signals A', A, B, D, G, and H were influenced more significantly than others by azide, and their chemical shifts were also consistent with those of the signals in Ni_2Zn_2SOD (Table I). A fitting of the chemical shifts of the isotropically shifted ^1H NMR signals with respect to different amounts of azide to eq 2 gives an affinity constant K = 160 M^{-1}, which is close to those of Ni_2Zn_2SOD and other derivatives as mentioned in last section.

Some of the isotropically shifted ^1H NMR signals in Ni_2Co_2SOD are not perturbed by the presence of azide, including an NH signal at 45.1 ppm (signal d in Figure 7A), indicating that these signals are from the protons in the Co^{2+} binding site. Some of the signals from the Ni^{2+} binding site overlap with those from the Co^{2+} binding site in the region between 30 and 60 ppm. However, the proton signals in the Ni^{2+} binding site can be easily distinguished from those in the Co^{2+} binding site by azide titration in which the former signals are dramatically influenced by azide binding as compared to the latter signals. This result is an indication that Ni^{2+} in the copper site retains an open coordination site for anion binding while Co^{2+} in the zinc site has a closed coordination sphere similar to the metal coordination environment in the native protein.[2] The broad signals due to the proton in the Ni^{2+} binding site underneath the overlapping signals in the region of 40–60 ppm were also revealed by azide titration (Figure 3B).

The intensity of some of the isotropically shifted signals of Ni_2Co_2SOD decreased upon addition of 1 equiv of cyanide to the protein solution. In the presence of 2 equiv of cyanide, those signals disappeared and a new spectrum with less isotropically shifted signals was observed (Figure 7C and D). The spectrum of Ni_2Co_2SOD in the presence of 2 equiv of cyanide was different from that of E_2Co_2SOD (Figure 8A), indicating that the Co^{2+} binding geometries are different in these two species. It is interesting to note that the isotropically shifted signals of Ni_2Co_2SOD in the presence of 2 equiv of CN^- are very similar to those assigned to the Co^{2+} binding site of the derivative in the presence of a saturating amount of N_3^- (Figure 7B). This observation indicates that similar changes in the configuration of the Co^{2+} in the zinc site occur when CN^- or N_3^- bind to the Ni^{2+} in the copper site.

As in the case of Ni_2Zn_2SOD, the presence of 50 mM phosphate did not affect the isotropically shifted NMR spectrum significantly (<3 ppm). The isotropically shifted imidazole CH proton signals of this derivative were not dramatically influenced by high pH in the presence and absence of phosphate while the isotropically shifted NH signals A and G and the signal L disappeared due to faster exchange with solvent under alkaline conditions.

Cyanide Binding to the Derivatives Studied by ^{13}C NMR. The ^{13}C NMR signal of cyanide was not detected when 2 equiv of

Table II. ^{13}C NMR Chemical Shifts of Cyanide in the Solution of Different Metal-Substituted Derivatives of Copper–Zinc Superoxide Dismutase and Some Simple Complexes

solution	$^{13}CN^-$ [a]	pH	chemicl shift ($\Delta\nu_{1/2}$) [b]
Ni_2Zn_2SOD [c]	2	7.5	−37.1 (66 ± 5)
	4	7.8	−37.1, −49.7 (125)
	4	10.1	−37.0, −13 (430)
Ni_2Co_2SOD [c]	2	7.5	−47.6 (70 ± 4)
	4	7.8	−47.5, −49.6 (139)
	4	10.2	−47.4, *d*
	2 [e]	7.5	no signal detected
	4 [e]	7.9	−48.7
	4 [e]	10.2	*d*
Cu_2Zn_2SOD [f]	2	7.5	no signal detected
	4	7.7	−44.5 (~350)
	4	10.0	−14.9 (255)
Zn_2Zn_2SOD [g]	2	6.5	−51.1 (67)
	2	8.9	−20.1 (br)
E_2Zn_2SOD [g]	2	6.5	−51.1 (66)
	2	9.6	−16.3
E_2Co_2SOD [c]	2	7.2	−50.5 (~70)
	2	8.4	−34 (350)
BCAB [h]	1	8.9	−23.9
Co^{II}BCAB [h]	1 and 2		no signal detected
	14		br
HCAB [h]	1	7.6	−21.9
	1	8.9	−21.9
$ZnSO_4$ [h]	4	11.0	−19.7
	8		~−10.0
$NiSO_4$	4	10.2	−18.8 (21)
	8	10.2	−9.2
Ni^{2+}–His	1	12.5	−27.2 (153)
	2	12.5	−27.9 (31)

[a] Equivalents per protein dimer for SOD. [b] Chemical shift in ppm from 0.1 M KCN, $\Delta\nu_{1/2}$ in hertz. [c] In 50 mM phosphate solution. [d] The signal was beyond detection but appeared again when brought to lower pH. [e] Unenriched cyanide added for the first 2 equiv. [f] Unbuffered solution. [g] In 50 mM HEPES. [h] From ref 30; BCAB, bovine carbonic anhydrase B; HCAB, human CAB.

$^{13}CN^-$ was added to native SOD, reflecting the binding of cyanide to the Cu^{2+} site and broadening of the CN^- NMR signal by Cu^{2+}. The cyanide signal detected at the ratio $[CN^-]:[Cu^{2+}] = 2:1$ was broad and pH dependent. This observation indicates that free cyanide in solution may interact with the paramagnetic protein to some extent, causing a broadening of the signal. In contrast, a pH-dependent cyanide signal was detected when 2 equiv of cyanide was added to the solutions of E_2Zn_2SOD, Zn_2Zn_2SOD, and E_2Co_2SOD, indicating that there was no strong cyanide binding to these derivatives (Table II).

As described above, the ^1H NMR signals of protons in the Ni^{2+} binding site of these two Ni^{2+}-substituted derivatives disappeared upon the addition of 2 equiv of CN^-. A single cyanide ^{13}C NMR signal was detected at −47.6 ppm (half-height line width, $\Delta\nu_{1/2} = 70 \pm 4$ Hz) and at −37.1 ppm ($\Delta\nu_{1/2} = 66 \pm 5$ Hz) referenced against 0.1 M KCN solution in Ni_2Co_2SOD and Ni_2Zn_2SOD solutions, respectively, at pH 7.5 when the ratio of $^{13}CN^-$ to Ni^{2+} was 1:1 (Figure 9 and Table II). An extra signal was detected with larger $\Delta\nu_{1/2}$ in both solutions when $[CN^-]:[Ni^{2+}] \geq 2$ at pH 7.8. The second signals were strongly pH dependent, moving downfield under high-pH conditions, and could be removed by ultrafiltration with several changes of buffer. However, the first signals were pH-independent and remained detectable after the second signals were wiped out by ultrafiltration. A similar observation of pH independence of a tightly bound cyanide in a metalloprotein was reported for cyanide binding to Zn^{2+} in carbonic anhydrase[30] (Table II).

^{13}C NMR spectra of cyanide in some simple complexes were also studied in our laboratory for comparison (Table II). Upon addition of Ni^{2+}, the cyanide signal of 0.1 M KCN solution moved to the upfield region without noticeable line broadening and eventually moved to −18.8 ppm when $[CN^-]:[Ni^{2+}] = 4:1$, i.e., when the complex $Ni(CN)_4^{2-}$ was formed. A 1:1 Ni^{2+}–histidine complex was paramagnetic with all the protons isotropically shifted;[35] however, it became diamagnetic when 2 equiv of cyanide was added, as shown by the disappearance of the isotropically shifted signals (Table II). The ^1H NMR spectrum of this diamagnetic complex in D_2O showed five signals with the two imidazole protons at 7.66 and 6.91 ppm and a three-spin system from the α-CH (3.49 ppm) and the β-CH_2 protons (2.95 and 2.80 ppm) with three coupling constants of 5.3, 7.7, and 14.7 Hz strongly suggesting a diamagnetic nature for this complex. When 1 equiv of CN^- was added to the 1:1 Ni–His complex, the cyanide ^{13}C NMR signal was detected at −27.2 ppm ($\Delta\nu_{1/2} = 153$ Hz). The observation of isotropically shifted ^1H NMR signals due to histidine also indicates that the complex remains paramagnetic upon binding of one CN^-. When 2 equiv of CN^- was added, the $^{13}CN^-$ NMR signal was detected at −27.9 ppm with $\Delta\nu_{1/2}$ of only 31 Hz and the complex became diamagnetic as judged from its high-resolution ^1H NMR spectrum. The larger $\Delta\nu_{1/2}$'s of the bound cyanide in the two new Ni^{2+}-substituted derivatives as compared to those in simple complexes may be accounted for by a significant increase in the rotational correlation time of the cyanide upon its binding to the proteins.

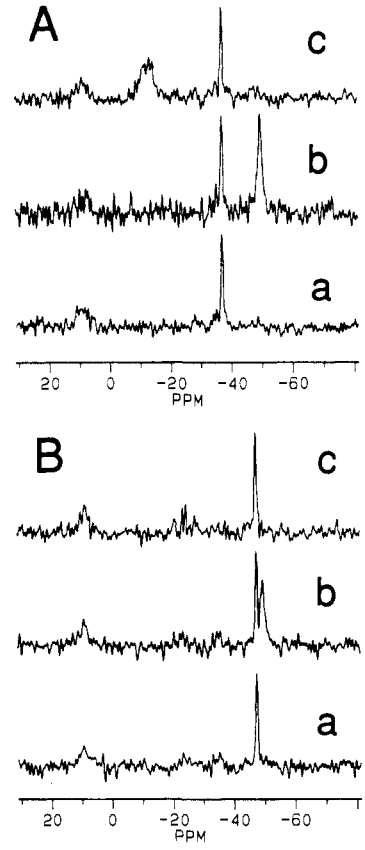

Figure 9. ^{13}C NMR spectra (referenced to 0.1 M KCN at 90.56 MHz and ~23 °C) of ^{13}C-enriched cyanide in 50 mM phosphate solution of (A) Ni_2Zn_2SOD with the ratio $[CN^-]:[Ni^{2+}] = 1$ at pH 7.5 (a) and with $[CN^-]:[Ni^{2+}] = 2$ at pH 7.8 (b) and pH 10.1 (c) and of (B) Ni_2Co_2SOD with $[CN^-]:[Ni^{2+}] = 1$ at pH 7.5 (a) and with $[CN^-]:[Ni^{2+}] = 2$ at pH 7.8 (b) and pH 10.2 (c). The signals at ~10 ppm downfield from the reference in all the spectra are the bulk protein carbonyl carbons; they are suppressed by the use of a short delay time.

Discussion

The electronic absorption due to Ni^{2+} in the new derivatives $Ni_2M'_2SOD$ ($M' = Zn^{2+}$, Co^{2+}) described here is different from that observed for M_2Ni_2SOD ($M = Cu^+$, Ag^+),[4] indicating that the Ni^{2+} binding environment of $Ni_2M'_2SOD$ is different from that of M_2Ni_2SOD. The electronic absorptions of some Ni^{2+}-substituted metalloproteins are listed in Table III, where a comparison can be made with respect to different geometries and ligands of the metal binding site. The absorption at ~390 nm (~25 600 cm^{-1}) in both derivatives is consistent with the ^3F–^3P

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Table III. Electronic Absorptions of Ni^{2+}-Substituted Metalloproteins[a]

protein	absorptions (ϵ)[b]	coordination	ref
St	590 (400), 550 (400)	S_2N_2, Td	c
Az	565 (320), 540 (350)	$S_2N_2(O)$	c, d
Mtn	750 (300), 560 (580)	S_4, Td	e
HLAD	680 (80), 570 (130)	S_2NO, Td	f
ATC	720 (330), 665 (250)	S?, Td	g
BSA	480, 420 (125), 340	N_4, Sq	h
PGM	1300 (5), 730 (7), 410 (23)	O_6?, Oh	i
M_2Ni_2SOD (M = Ag^+, Cu^+)	810 (sh), 755 (25), 525 (sh), 475 (70)	$N_3O(N_3O_2)$	4
CPA	1060 (3), 685 (7), 412 (24)	N_2O_3	37, 38
CA	640 (30), 390 (85)	N_3O_2	39
Ni_2Zn_2SOD	675 (14), 390 (85)	N_4O	j
Ni_2Co_2SOD	393 (100)	N_4O	j

[a] Abbreviations: St, stellacyanin; Az, azurin; Mtn, metallothionein; HLAD, horse liver alcohol dehydrogenase; ATC, aspartate transcarbamylase; BSA, bovine serum albumin; PGM, phosphoglucomutase; $M_2M'_2SOD$, M- and M'-substituted superoxide dismutase with M in the copper site and M' in the zinc site; CPA, carboxypeptidase A; CA, carbonic anhydrase; Td, tetrahedral-like; Sq, square planar; Oh, octahedral-like. [b] Absorption (nm); ϵ (cm^{-1} M^{-1} per nickel). [c] Lum, V.; Gray, H. B. *Isr. J. Chem.* **1981**, *21*, 23-25. Tennent, D. L.; McMillin, D. R. *J. Am. Chem. Soc.* **1979**, *101*, 2307-2311. [d] Norris, G. E.; Anderson, B. F.; Baker, E. N. *J. Am. Chem. Soc.* **1986**, *108*, 2784-2785. [e] Vasak, M.; Kagi, J. H. R.; Holmquist, B.; Vallee, B. L. *Biochemistry* **1981**, *20*, 6659. [f] Dietrich, H.; Maret, W.; Kozlowski, H.; Zeppezauer, M. *J. Inorg. Biochem.* **1981**, *14*, 297-311, where the band at 570 nm was assigned as S → Ni charge-transfer band. However, the bands at ~550 nm in Ni^{2+}-substituted stellacyanin and azurin have been assigned to d-d bands by Gray et al. (ref c) according to their MCD observations. [g] Johnson, R. S.; Schachman, H. K. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 1995-1999. [h] Laurie, S. H.; Pratt, D. E. *J. Inorg. Biochem.* **1986**, *28*, 431-439. [i] Ray, W. J., Jr.; Multani, J. S. *Biochemistry* **1972**, *11*, 2805-2812. [j] This report.

transition and the absorption at 675 nm (14815 cm^{-1}) in Ni_2Zn_2SOD is consistent with the highest ^3F-^3F transition of 5-coordinate high-spin Ni^{2+} complexes,[36] such as the Ni^{2+}-substituted derivatives of carboxypeptidase A[37,38] and carbonic anhydrase[39] (Table III).

The binding of Co^{2+} to the zinc site of SOD has electronic absorption bands in the range between 600 and 500 nm and molar absorptivity of ~400 cm^{-1} M^{-1} per cobalt typical for Co^{2+} with tetrahedral coordination.[5,36] When the Co^{2+} ion in the zinc site is bridged to the metal ion in the copper site through the imidazolate of His-61, the shapes of the electronic absorption bands due to Co^{2+} in the zinc site are different from those of the derivatives without the bridge; specifically, the band at 583 nm decreases, and a new band at ~600 nm appears when the bridge is formed, similar to the phenomenon observed for the derivatives Cu_2Co_2SOD and Co_2Co_2SOD in the absence of phosphate.[13] On the contrary, the absorption due to Co^{2+} is not influenced by the metal binding in the copper site in the absence of the bridging histidine, as in the derivatives $Ag^I_2Co_2SOD$ and $Cu^I_2Co_2SOD$.[9,13] The changes in the electronic absorption spectrum of E_2Co_2SOD upon Ni^{2+} binding to the empty copper site are similar to those observed upon Cu^{2+} and Co^{2+} binding to the empty copper site of E_2Co_2SOD to form Cu_2Co_2SOD and Co_2Co_2SOD (in the absence of phosphate), respectively, in which the His-61 residue forms a bridge between two metal binding sites as mentioned above.[13] We therefore conclude that the imidazole bridge also exists in the derivative Ni_2Co_2SOD.

The changes in the electronic spectra of $Ni_2M'_2SOD$ upon the addition of azide and cyanide indicate that the anions are directly bound to Ni^{2+}. The energy and the absorptivity of the anion complexes of $Ni_2M'_2SOD$ are consistent with those of 5-coordinate Ni^{2+} complexes (vide ante).[36-39] The increase in the intensity of the ^3F-^3P transition upon azide binding to the Ni^{2+} was also observed in Ni^{2+}-substituted carbonic anhydrase,[40] although no detailed analysis was given. On the contrary, the Co^{2+} absorption bands in Ni_2Co_2SOD do not change dramatically upon anion binding to Ni^{2+}, indicating that the Co^{2+} coordination sphere is not significantly perturbed and therefore that the His-61 bridge remains intact. Owing to the extremely similar geometric and spectroscopic properties of the Co^{2+} in the copper site of both Co_2Co_2SOD and Co_2Zn_2SOD,[10-13] it is reasonable to conclude that the geometric configurations of Ni^{2+} in the copper site of Ni_2Co_2SOD and Ni_2Zn_2SOD are also very similar. Histidine-61, which is concluded to be a bridging ligand in Ni_2Co_2SOD, is therefore also expected to be a bridging ligand in Ni_2Zn_2SOD in the presence and absence of anions.

Isotropically shifted ^1H NMR spectroscopy has been successfully applied to the investigation of the metal binding sites of Ni^{2+}-substituted derivatives of SOD with Ni^{2+} bound in the zinc site.[4,8] The different protons from the coordinated histidine residues can be easily differentiated by spin-lattice relaxation time (T_1) measurements and half-height line width ($\Delta\nu_{1/2}$) of the isotropically shifted signals, where the protons ortho to the coordinated imidazole nitrogen (ortholike protons) have shorter T_1's and larger $\Delta\nu_{1/2}$'s than those of the protons meta to the coordinated imidazole nitrogen (metalike protons) owing to the fact that the former protons are closer to the Ni^{2+} ion than are the latter ones. The ^1H NMR signals of imidazole NH protons of coordinated histidine can be easily recognized by preparing derivatives in D_2O where the NH protons are deuterated and their ^1H NMR signals disappear.

The ^1H NMR spectrum of Ni_2Zn_2SOD shows three solvent-exchangeable signals isotropically shifted in the downfield region >30 ppm, reflecting that at least three histidines are coordinated to Ni^{2+} in addition to the bridging His-61, which does not provide an imidazole NH signal. The solvent-exchangeable signals are not assigned to coordinated amino or amido protons because such NH protons have been shown to give isotropically shifted signals in the far upfield region resulting from a spin-polarization mechanism[41] when coordinated to Ni^{2+} through the amino or amido nitrogens.[35] The NH protons of the protein backbone (not coordinated to Ni^{2+}) may also be isotropically shifted to the downfield region by the through-bond spin delocalization mechanism,[41] however, such shifts are expected to be much smaller because these protons are further away from Ni^{2+}. Assuming that the histidines are coordinated to Ni^{2+} in a manner similar to their binding to Cu^{2+} in the native protein, i.e., through the $N_{\epsilon 2}$ nitrogens of His-46, His-61, and His-118 and the $N_{\delta 1}$ nitrogen of His-44, we can assign the sharp signal H (due to a proton in the Ni^{2+} binding site) of the new Ni^{2+}-substituted derivatives to the $C_{\delta 2}H$ proton of His-44, the only histidine in the copper site ligated to the metal ion through the $N_{\delta 1}$ nitrogen (leaving the $C_{\delta 2}H$ proton as a metalike proton). Other histidines, including the bridging histidine, are ligated to Ni^{2+} through the $N_{\epsilon 2}$ nitrogens, leaving both the $C_{\epsilon 1}H$ and the $C_{\delta 2}H$ protons at the ortholike positions and thus giving broader signals.

There are four solvent exchangeable signals clearly detected in the derivative Ni_2Co_2SOD (Figure 7A). Of these, one is not influenced by the presence of azide and can be assigned to an imidazole NH proton in the Co^{2+} binding site. The other three solvent-exchangeable signals in Ni_2Co_2SOD have chemical shifts very similar to those of the imidazole NH signals in Ni_2Zn_2SOD in the absence and presence of azide and can therefore be assigned to the three imidazole NH protons in the Ni^{2+} binding site. If the conclusion proposed above that His-61 is a bridging ligand in these derivatives is correct, the Ni^{2+} binding site must be very similar to that of Cu^{2+} in the native protein, i.e., a 5-coordinate

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(41) La Mar, G. N. In *NMR of Paramagnetic Molecules, Principles and Applications*; La Mar, G. N., Horrocks, W. D., Jr.; Holm, R. H., Eds.; Academic: New York, 1973.

metal binding site with the metal ion ligated to His-118 and His-46 through the $N_{\epsilon 2}$ nitrogen and to His-44 through the $N_{\delta 1}$ nitrogen and bridged to the zinc site through the imidazolate of His-61. There are a total of seven ortholike imidazole CH protons in the copper site while only three broad signals were detected in the derivative Ni_2Zn_2SOD due to serious signal overlap. Azide titration allows us to resolve some but not all of the overlapping signals (Figure 3).

The ^1H NMR spectrum of Ni_2Co_2SOD is more complex than that of Ni_2Zn_2SOD due to the presence of a number of signals from the coordinated ligands in the Co^{2+} binding site. However, the good correlation between some of these signals and those of Ni_2Zn_2SOD allows us to assign the signals to the protons in the Ni^{2+} binding site (Table I). Moreover, azide titrations show that the signals influenced by the anion in Ni_2Co_2SOD have similar chemical shifts and the same characteristics as those of Ni_2Zn_2SOD, i.e., all the signals move upfield with increasing amounts of azide (Figure 3). Some of the isotropically shifted signals in Ni_2Co_2SOD are not significantly influenced by the presence of azide and therefore can be assigned to the protons in the Co^{2+} binding site (the zinc site), which is believed to have a closed coordination sphere when metal ions are bound to that site.[2,3]

In previous studies on $Cu_2M'_2SOD$ (M' = Co^{2+} or Ni^{2+}) by ^1H NMR spectroscopy, a group of signals was observed to shift into the diamagnetic region of the spectra upon azide binding to Cu^{2+} in the copper site.[4,6-8] This observation was attributed to bond breaking between Cu^{2+} and a coordinated histidine, which then moved to an axial position in the copper site. On the contrary, none of the isotropically shifted signals of $Ni_2M'_2SOD$ (M' = Co^{2+} or Zn^{2+}) moves into the diamagnetic region in the presence of a saturating amount of azide (Figure 3 and Table I), indicating that all the histidines in the copper site are still coordinated to the Ni^{2+} in these Ni^{2+}-substituted derivatives when azide is bound.

The studies of the azide and cyanide binding to native SOD by EPR[19,20] and EXAFS[17] have indicated that the binding of the anions to the protein moved the rhombic copper site toward a more nearly axial geometry. EXAFS studies further indicated that azide binding to Cu^{2+} caused displacement of an equatorial imidazole of the coordinated histidine from a Cu–N bond length of 2.00 Å to 2.27 Å (to the axial position) and the cyanide binding to Cu^{2+} at the equatorial position caused a complete detachment of a histidine from the Cu^{2+} coordination sphere.[17] The trend of the formation of an axially symmetric copper site in native SOD in the presence of different anions was found to follow the in-plane ligand field strength as $CN^- > N_3^- > NCO^- > NCS^- > F^-$.[42,43] Four-coordinate Ni^{2+} complexes have a strong preference for square-planar geometry,[16] especially in the presence of strong ligand fields such as that provided by cyanide anion. Such square-planar Ni^{2+} complexes are diamagnetic. We attribute the loss of the paramagnetism of Ni^{2+} in the derivatives $Ni_2M'_2SOD$ (which results in the disappearance of isotropically shifted ^1H NMR signals as shown in Figures 4 and 7) upon cyanide binding to formation of a square-planar configuration at the Ni^{2+} binding site with one coordinated histidine detached from the Ni^{2+} coordination sphere. Although there are some low-spin 5-coordinate Ni^{2+} complexes known, such complexes have only been observed for ligands of strong ligand field strength and π-bonding ability, such as phosphines and arsines. We believe that it is unlikely that the ligands to Ni^{2+} in our case provide a similar environment and we therefore favor the conclusion that Ni^{2+} becomes 4-coordinate upon cyanide binding in the protein. This conclusion is also supported by the ^{13}C NMR studies of $^{13}CN^-$ binding to these derivatives in which we found that a diamagnetic Ni^{2+} binding site was formed upon cyanide binding (Figure 9 and Table III). This property is similar to that observed for the native enzyme as studied by single-crystal EPR and EXAFS studies, in which an axially symmetric geometry (square-planar configuration with a coordinated histidine detached) in the copper site was also observed upon cyanide binding.[17,19,20]

The Ni^{2+} binding sites of the derivatives $Ni_2M'_2SOD$ are still paramagnetic upon azide binding, although to a lesser extent, as shown by the isotropically shifted ^1H NMR studies (Figures 2D and 7B) and the numerical fittings of the chemical shifts with respect to the amount of azide (Figure 3 and Table I). A decrease of $\geq$50% in the spin density (assuming a predominant contact interaction) on the protons of the coordinated ligands can be estimated based on the changes in the chemical shift upon azide binding to the Ni^{2+}.[41] This decrease may be due to significant decreases in ligand–metal orbital overlap caused by changes in metal coordination upon azide binding and/or the existence of an equilibrium between a paramagnetic 5-coordinate and a diamagnetic square-planar (with a histidine detached) azide-bound complex of $Ni_2M'_2SOD$ in which the latter does not contribute to the isotropic shift of the ligand nuclei. Whatever the reason is for the decrease in spin density on the coordinated ligands in the Ni^{2+} binding site upon azide binding, the conclusion made above that the azide complexes of $Ni_2M'_2SOD$ are still paramagnetic and that none of the coordinated histidine is completely detached remains appropriate. The two unpaired electrons in square-pyramidal Ni^{2+} complexes are in the two highest energy orbitals, $d_{x^2-y^2}$ and d_{z^2}. An axial histidine binding to Ni^{2+} in the copper site via the d_{z^2} orbital may result in transfer of spin density from Ni^{2+} to the axial histidine to a certain extent resulting in the observation of isotropically shifted proton signals of the histidine. This result is remarkably similar to that observed upon azide binding to native enzyme studied by EXAFS in which one Cu–N bond increased by 0.27 Å moving from the basal to the axial position; however, whether or not there is a direct bond between Cu^{2+} and the displaced histidine, and what the identity of the displaced histidine is, cannot be obtained by the available information in the EXAFS studies.[17]

In simple copper complexes, an increase in bond length from the equatorial position to the axial position is commonly observed owing to the Jahn–Teller distortion.[16] Therefore, it is not impossible that the axially coordinated histidine remains bound to Cu^{2+} in the copper site of native Cu_2Zn_2SOD in the presence of azide with a longer Cu–N bond length was concluded from the EXAFS studies.[17] The unpaired electron in Cu^{2+} complexes of square-planar and square-pyramidal coordinations is in the $d_{x^2-y^2}$ orbital, which points to the basal positions; thereby, the protons of the histidine displaced from an equatorial to an axial position by azide binding to Cu^{2+} in native SOD may feel little or no spin density at the axial position and show diamagnetic properties even though the histidine is still bound to the paramagnetic Cu^{2+} ion. The lack of contact interaction between ligands at the axial position and Cu^{2+} has also been shown by the disappearance of ^{17}O (of water) NMR paramagnetic relaxation, which occurred via a contact mechanism,[32] in Cu^{2+} aqueous solution in the presence of 2 equiv of ethylenediamine (en), i.e., forming the $Cu(en)_2(H_2O)_2^{2+}$ complex, where the water molecules occupied the axial protons.[44] The different changes in the isotropically shifted ^1H NMR signals of the displaced histidine between the Ni^{2+} derivatives $Ni_2M'_2SOD$ (M' = Zn^{2+}, Co^{2+}) reported here and the derivatives $Cu_2M'_2SOD$ (M' = Co^{2+}, Ni^{2+})[4,6-8] upon azide binding may therefore be explained by the different electronic configurations of Ni^{2+} and Cu^{2+}, which provide different mechanisms for obtaining isotropically shifted signals. The possibility of axially coordinated histidine with a weaker Cu–N bond originating from a basal histidine upon azide binding cannot be excluded by the results obtained with different methods. Thus, all of the results are consistent with the geometric change of a histidine moving from a basal position to an axial position and becoming more weakly bound to the Cu^{2+} or the Ni^{2+} in the copper site upon azide binding.

Phosphate interacts very weakly with Ni^{2+} in the copper site, as shown by the virtually identical NMR spectra (<3 ppm dif-

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ference) of these derivatives in the presence and absence of phosphate. This small influence indicates that phosphate is not directly interacting with Ni^{2+} in the inner coordination sphere but possibly with some positively charged residues in the proximity of the Ni^{2+} binding site. This result is similar to the studies of native SOD in which the Cu^{2+} site is not influenced by phosphate as observed by electronic absorption and EPR spectroscopies.[14,15] The influence of phosphate on the copper site of the derivative Cu_2Co_2SOD is also very small as shown by ^1H NMR spectroscopy.[45] However, this observation is completely different from the results observed for some Co^{2+}-substituted derivatives with Co^{2+} bound in the copper site, such as Co_2Zn_2SOD and Co_2Co_2SOD, for which phosphate causes dramatic changes in the electronic and NMR spectra, indicating that phosphate binds directly to Co^{2+} and causes changes in binding geometry.[13] The similar chemical shifts of the isotropically shifted signals of these two new derivatives at neutral and high pH indicate that the Ni^{2+} binding site in these derivatives is not perturbed significantly under different pH conditions. The similarity of the chemical shifts of the isotropically shifted signals at high pH in the presence of phosphate to those at pH 7.5 in the absence of phosphate indicates that phosphate does not interact with the derivatives significantly at high pH. The decrease of the phosphate interaction under highly alkaline conditions may be due to the deprotonation of a certain amino acid residue(s) in the active-site cavity (forming a less positively, or even a negatively, charged channel of the active site), which may result in decrease of the interaction of the anionic phosphate with the protein similar to that reported for native SOD[16] and Co^{2+}-substituted SOD.[46]

Conclusion

The configuration of the ligands about Ni^{2+} in these new derivatives Ni_2Zn_2SOD and Ni_2Co_2SOD is very similar to that of Cu^{2+} in native SOD, i.e., coordinated to one histidine through the $N_{\delta 1}$ nitrogen and to another two histidines through the $N_{\epsilon 2}$ nitrogen as well as to the bridging imidazolate of the His-61 residue (also through the $N_{\epsilon 2}$ nitrogen). Both azide and cyanide bind Ni^{2+} in these two derivatives leaving the bridging histidine intact as revealed by electronic spectroscopy. NMR studies further suggest that the geometric changes that occurred upon anion binding to the Ni^{2+} binding site are similar to those observed for the native protein studied by different spectroscopies; i.e., a rearrangement from a distorted 5-coordination with water and four histidines bound to an axially symmetric 4- or 5-coordination with the anion and three or four histidines bound. The phosphate binding properties of these derivatives, i.e., no direct binding to the metal in the copper site, is also similar to that of the native enzyme. The similarities of the metal binding environment of these two new Ni^{2+}-substituted SODs to those of native SOD in the presence and absence of anions suggest that these derivatives can serve as good structural models for copper-zinc superoxide dismutase.

The change in the electron spin state of Ni^{2+} from high spin to low spin in the presence of strong ligand fields, such as cyanide in this study, represents one of the classical properties of simple Ni^{2+} complexes[16] and can be well applied to studies of metalloproteins. The preference of Ni^{2+} to form square-planar coordination is relevant to that of Cu^{2+}, since Cu^{2+} normally also prefers a square-planar ligand field.[16] The similar geometric preferences of these two metal ions allow the use of Ni^{2+} as a good substitute for Cu^{2+} for studies of structural properties of copper proteins. As shown in this study, isotropically shifted ^1H NMR spectroscopy can be used successfully in the study of Ni^{2+}-substituted metalloproteins to determine the identity and configuration of the ligands, especially histidines coordinated through the $N_{\delta 1}$ or the $N_{\epsilon 2}$ nitrogen, in the metal binding site.

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Registry No. Ni, 7440-02-0; Cu, 7440-50-8; Co, 7440-48-4; azide, 14343-69-2; cyanide, 57-12-5; phosphate, 14265-44-2; histidine, 71-00-1.

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Communications to the Editor

Relationship between Amidic Distortion and Ease of Hydrolysis in Base. If Amidic Resonance Does Not Exist, Then What Accounts for the Accelerated Hydrolysis of Distorted Amides?

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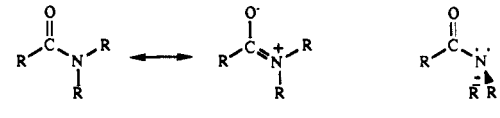
Chemists have invoked resonance theory[1] for amides (**1** ↔ **2**) to explain the short N-C(O) bond length,[2] planar geometry,[2] high N-C(O) rotational barrier,[3] infrared C=O stretching frequencies,[4] and kinetic stability toward nucleophilic attack/hydrolysis.[5] Wiberg and Laidig[6] have reported high-level ab initio calculations of planar **1a** and orthogonal **3a** along with Bader's[7] atomic population analyses for the various atoms in each conformer. In



1a R=H **2** **3a** R=H

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