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Two-Dimensional ^1H NMR Studies of the Paramagnetic Metalloenzyme Copper–Nickel Superoxide Dismutase

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Two-dimensional ^1H NMR techniques are in widespread use for structure determination of diamagnetic macromolecules[1,2] but have only recently been applied to paramagnetic metalloproteins.[3-5] Normally, copper(II)-containing metalloproteins are unsuitable for ^1H NMR spectral studies because the relatively long electronic relaxation times result in broad signals.[6,7] However, for the copper(II)-containing protein copper–zinc superoxide dismutase[8-10] (Cu_2Zn_2SOD[11]), substitution of Zn^{2+} by either Co^{2+} or Ni^{2+} provides derivatives in which the electronic relaxation times are dramatically shorter[6,7] due to interaction between the Cu^{2+} and Co^{2+} [12] or Ni^{2+} [13] mediated by the imidazolate bridge that links them[8-10] (see Figure 1). We have found the Cu_2Ni_2SOD derivative to be particularly suitable for 2D ^1H NMR studies, and we report here such studies (NOESY[11]) of this derivative which have allowed us to assign almost all of the isotropically shifted resonances attributable to the metal-coordinated histidine residues. This report presents the first example of the application of 2D NMR techniques to the assignment of isotropically shifted signals due to metal-coordinated histidine residues.

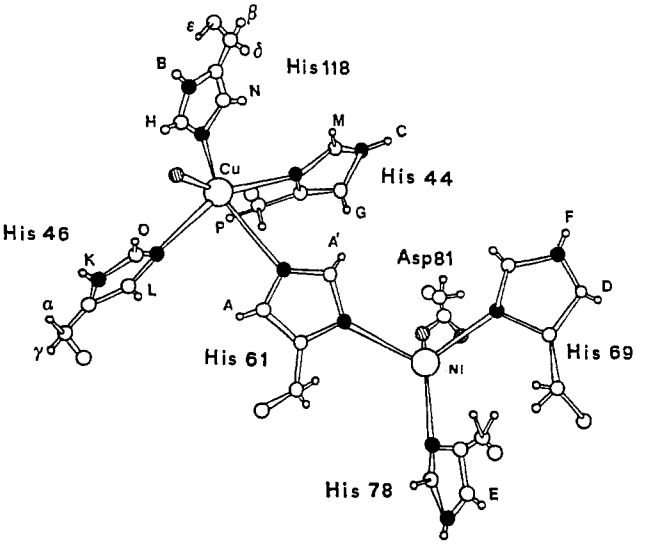

Figure 1. Schematic drawing of the active site of bovine Cu_2Ni_2SOD superoxide dismutase based on X-ray crystallographic studies.[10] The assignment of the isotropically shifted signals (Figure 2) to each individual proton of the coordinated His residues (as labeled) is based on NOESY and 1D NOE experiments.

The 1D and NOESY ^1H NMR spectra of bovine Cu_2Ni_2SOD are shown in Figure 2. We had previously determined that four resonances (B, C, K, F) disappeared when the 1D spectrum was recorded in D_2O buffer and had assigned them to four of the five exchangeable NH protons of the metal-coordinated histidine residues.[13] A fifth exchangeable NH proton was not detected, unlike the case of Cu_2Co_2SOD, in which all such resonances were observed.[12,14] The signals, B, C, and K were assigned to the NH protons of the three histidines coordinated to Cu(II) on the basis of the observation of shifts upon azide binding to Cu(II).[13] Signal F and the undetected NH resonance were therefore assigned to

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(11) Abbreviations: $M_2M'_2SOD$, M- and M'-substituted superoxide dismutase with M in the copper site and M' in the zinc site (both M and M' are in the 2+ oxidation state unless otherwise specified); NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy.
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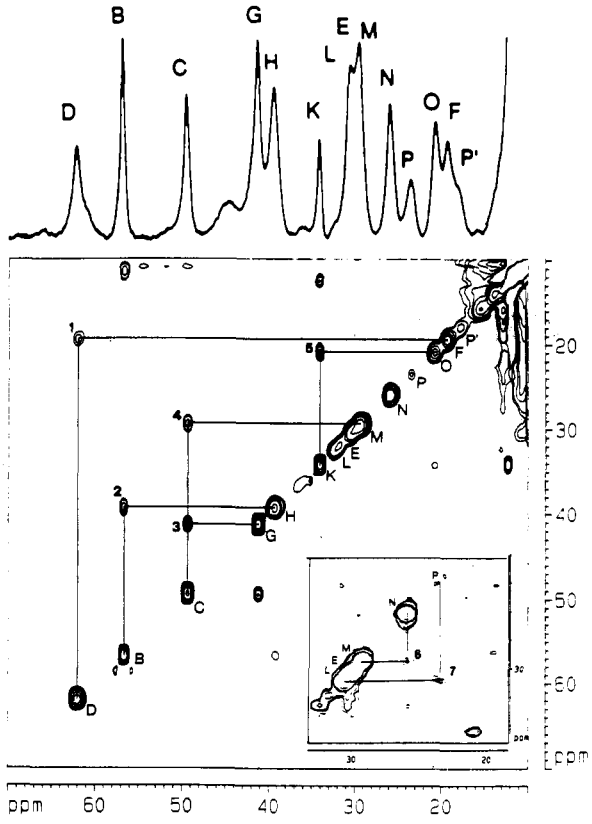

Figure 2. 600-MHz NOESY spectrum of bovine Cu_2Ni_2SOD in 50 mM phosphate buffer, at pH 6.2 and 298 K. Five cross peaks (signals 1–5) are observed due to dipolar connectivities involving the resonances of NH exchangeable protons of the metal-coordinated histidines. The inset shows a region of a NOESY spectrum recorded at 300 MHz. Additional connectivities 6 and 7 are observed due to interresidue connectivities. Conditions: mixing time, 15 ms; relaxation delay, 80 ms. Presaturation of solvent resonance was applied during both relaxation delay and mixing time. 512 experiments were collected in the F_1 dimension, over 1024 data points in the F_2 dimension. Data were collected using the phase-sensitive TPPI mode.[22] A 1024 × 512 data point matrix was always used, with zero filling applied in both dimensions to obtain a 2048 × 1024 data point matrix. A shifted squared sine weighting function was applied in both dimensions. Baseline correction was applied in both F_1 and F_2 dimensions. The standard Bruker software package was used. The inset shows a region of a NOESY spectrum recorded at 300 MHz using the same experimental conditions as above. The top trace is the 1D spectrum, recorded at 360 MHz, in which signals B, C, K, and F are solvent exchangeable signals.

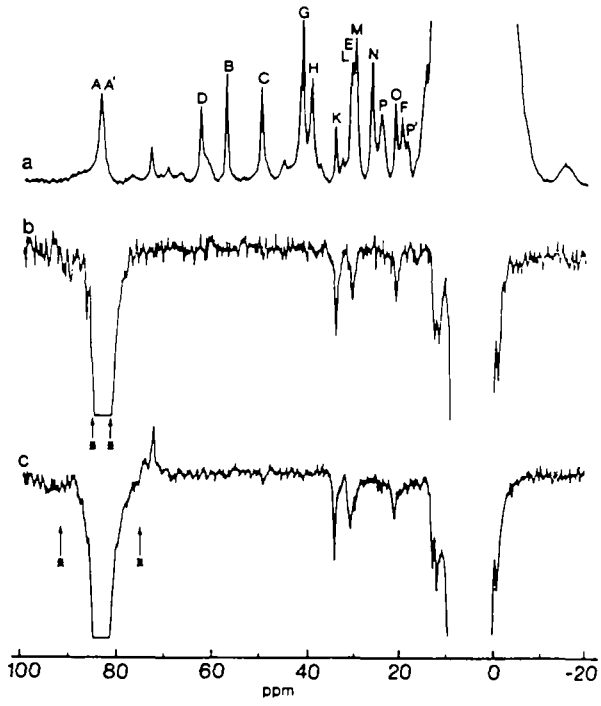

Figure 3. 200-MHz ^1H NMR spectrum of bovine Cu_2Ni_2SOD (a) and the NOE difference spectra obtained by the saturation of signal A (b) and signals A and A′ (c) for a period of 60 ms. The decoupler power is adjusted twice higher in (c) relative to that in (b) to saturate the broad signal A′ underneath signal A. The asterisks in (b) and (c) indicate the off-resonance positions.

the two histidines coordinated to the Ni(II). We also showed previously that the NH proton of the zinc site ligand His-69 exchanges with water much less rapidly then that of the other zinc site ligand His-78 in the reduced derivatives $Cu^I_2Co_2SOD$[15,16] and $Cu^I_2Zn_2SOD$.[17] We therefore assigned signal F to the His-69 residue on the assumption that the NH signal attributable to His-78 was undetected due to its greater lability. This assignment was supported by the deuterium-exchange rates of the NH signals assigned to His-69 and His-78 in $Cu^{II}_2Co_2SOD$.[12]

The relatively slow relaxation rates of the isotropically shifted signals in the ^1H NMR spectrum of Cu_2Ni_2SOD now allows usto complete the assignment of most of these signals by means of 2D NOESY spectroscopy (Figure 2). Five cross signals (1–5) that all involve solvent exchangeable peaks were detected. The NH protons of coordinated histidine residues are expected to give rise to relatively strong NOE connectivities to their vicinal CH ring protons, unless the latter are too broad to be detected because of the proximity of the paramagnetic metal center. Thus, each of the following sets of signals must be associated with an individual coordinated histidine residue: D, F (giving cross signal 1); B, H (2); C, G, M (3 and 4); K, O (5). Since the solvent exchangeable signal C exhibits NOE connectivities with two isotropically shifted CH signals, it can thus be assigned to one of the observable NH protons of the Nδ-coordinated histidine residues (i.e. His-44, His-69, or His-78) since each Nδ-coordinated histidine has an NϵH proton vicinal to both the CδH and the CϵH protons. We can eliminate His-69 and His-78 from consideration since those histidines are coordinated to Ni(II) and therefore their CϵH signals are expected to be too broad to produce appreciable NOE with the NϵH proton.[13] Thus signals C, G, and M can be assigned to the NϵH, CϵH, and CδH protons of His-44, due to the distinct coordination mode of that ligand relative to the other ligands in the copper site. This assignment is consistent with our earlier conclusion that C should be assigned to a copper site ligand (see above). Two of the other three pairs (B, H and K, O) can be assigned to the Nϵ-coordinated histidine residues in the Cu(II) site on the basis of our previous result that resonances B and K were shifted upon azide binding to Cu(II) in Cu_2Ni_2SOD.[13] The remaining pair (D, F) can then be assigned to the CδH and the NϵH proton, respectively, of the Ni(II)-coordinated His-69 residue, on the basis of the reasoning described above.

In the NOESY experiment at 300 MHz (Figure 2, inset) two additional cross peaks are observed, involving signals M–N and L–P. One-dimensional steady-state NOE experiments[14,18,19] have been carried out on the overlapped signals A and A′ (Figure 3a). At low decoupler power (Figure 3b), only the sharper resonance A is saturated, giving rise to the NOE's with signals K, L, and O. In Figure 3c, both signals A and A′ are saturated owing to the use of a higher decoupler power, which produces NOE's with

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additional peaks, C and M. We recall that signals C (exchangeable), G, and M are already assigned to His-44.

According to the X-ray crystal structure of the native enzyme, the nonexchangeable imidazole ring protons which are at less than 3.5 Å from His-44 imidazole ring protons are as follows: (1) His-118 CδH, which is 3.3 Å from His-44 CεH, (2) His-61 CεH, which is 2.7 Å from His-44 CεH and 3.1 Å from His-44 NεH, and (3) His-69 CεH, which is 2.5 Å from His-44 NεH. Because signal A′ has two NOE's with signals C and M, it is assigned as His-61 CεH. We can thus discriminate between signal G (His-44 CδH) and signal M (His-44 CεH) on the basis that the latter must be due to the proton that is closer to His-61 CεH. The M–N connectivity allows us then to assign signal N as His-118 CδH.

The connectivities observed in the 1D NOE difference spectra of Figure 3b discriminate between the pairs K, O and B, H. While no imidazole ring protons occur at less than 3.6 Å from both His 118 NδH and CεH, all three imidazole ring protons of His-46 are less than 3.5 Å from His 61 CδH. Hence signals K, O, and L, all experiencing NOE from signal A, are assigned to His-46 NδH, His-46 CεH, and His-46 CδH, respectively, and signal A is assigned as His-61 CδH. By exclusion, signals B and H are assigned to His-118 NδH and His-118 CεH, respectively. The assignment of signal L as His-46 CδH is further supported by the NOESY connectivity between L and P (cross peak 7, Figure 2), which allows the assignment of P as His-44 CβH, as was previously proposed on the basis of azide titrations[13] and similarly proposed in the case of Cu_2Co_2SOD.[14]

The proton resonances due to the coordinated His-44 (signals C, M, and G), His-46 (signals K, L, and O), and His-61 (signals A and A′) residues in $Cu^{II}_2Ni_2SOD$ have thus been fully assigned on the basis of NOESY and 1D NOE experiments. It had previously been observed that the isotropically shifted resonances K, L, and O shifted to the diamagnetic region in the presence of saturating amounts of azide.[13] The assignment of these resonances to the His-46 residue is consistent with the increase of the Cu(II)–NεH distance upon anion binding found also for the Cu_2Co_2SOD derivative[20] (but not the detachment of His-44 as proposed previously[13,21]).

The use of paramagnetic metal ions as NMR probes for the study of the metal-binding sites in metalloprotein has the advantages that resonances due to the amino acid residues in the coordination sphere of the metal ion are isotropically shifted out of the diamagnetic region and thus can be clearly observed and studied. However, assignment of the individual isotropically shifted signals due to coordinated histidines by correlation of their relaxation times with metal–proton distances has not been unambiguous owing to similarities in those relaxation times. In this report, we present for the first time the use of a 2D NOE technique (NOESY) for the assignments of the isotropically shifted resonances due to coordinated His residues in a nonheme metalloprotein. This study suggests that 2D NMR techniques will prove valuable in future studies of paramagnetic metalloproteins.

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