One- and Two-Dimensional ^{1}H NMR Studies of the Active Site of Iron(II) Superoxide Dismutase from *Escherichia coli*

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Received July 8, 1993®

The iron(II) superoxide dismutase (FeSOD) from *Escherichia coli* exhibits relatively sharp well-resolved paramagnetically shifted NMR signals. These signals can be associated with the endogenous ligands characterized by X-ray crystallography for the oxidized form $Fe^{III}SOD$. Our results demonstrate that the active site remains intact upon reduction of the Fe(III) site, retaining the same coordination modes for the three N_ϵ-coordinated His residues (presumably His-26, His-75, and His-162) and the Asp residue (presumably Asp-158). The N–H resonances of the coordinated histidines are found at 88, 43, and 37 ppm, while the signals at 24.5 (E), 19 (G), and 15 (J) ppm are assigned to the Asp residue by the observation of nuclear Overhauser effects (NOE) and bond correlations among the resonances. Distances of 1.9 and 2.4 Å can be estimated between the protons E and G and the protons E and J, respectively, attributable to the $-C_\beta H_2-C_\alpha H<$ spin system of the Asp residue. The Asp assignments are corroborated by the observation of interresidue interactions between the $C_\alpha H$'s of Asp-158 and Trp-160. The resonances of the latter residue are identified by NOE and scalar connectivities, the most critical assignment being the $N_\alpha H$ at 22 ppm (F). According to the crystal structure of the corresponding enzyme from *Psedumonas ovalis* (Stoddard, B. L.; Howell, P. L.; Ringe, D.; Petsko, G. A. *Biochemistry* **1990**, *29*, 8885–8893), this proton is hydrogen-bonded to the free carboxylate oxygen of Asp-158. This interaction thus rationalizes the paramagnetic shift observed for signal F and its very slow exchange with solvent which occurs only upon long-term standing.

Carboxylate-containing amino acid residues, such as Asp and Glu, are frequently found as ligands for the metal ions in metalloproteins.[1] Such negatively charged carboxylate ligands in metalloenzymes can modulate the redox potential and the Lewis acidity of the metal center to impart properties appropriate for the different protein functions. X-ray crystallography has established the presence of endogenous carboxylate ligands in the active site of nonheme iron proteins, such as iron superoxide dismutase (FeSOD),[2] hemerythrin,[3] and the R2 subunit of ribonucleotide reductase.[4] However, it is very difficult to identify such ligands in metalloproteins by the use of physical methods other than crystallography.

Proton NMR spectroscopy has been useful for studying the metal-binding environments of many paramagnetic metalloproteins via the detection and assignment of the isotropically shifted resonances arising from the nuclei in close proximity to the metal, such as Fe(II/III) or Co(II).[5] Coordinated His, Tyr, and Cys residues are easily recognized by their characteristic isotropic shifts and relaxation properties.[6] However, the assignment of paramagnetically shifted features to coordinated carboxylate residues has been difficult because such residues do not have distinctive shifts; e.g., the carboxymethylene resonances in Fe(II) complexes can usually be found in the 20–50 ppm region[7] but can vary in position depending on whether the more basic *syn* or the *anti* lone pair coordinates to the metal center. Sometimes these features merge into the edge of the bulk diamagnetic protein resonances.

We have been interested in developing NMR strategies to alleviate such difficulties and have thus chosen the crystallographically characterized FeSOD to illustrate the use of NMR techniques (i.e., the nuclear Overhauser effect and bond correlated spectroscopy) to identify resonances arising from the coordinated carboxylate ligands in iron proteins. The Asp resonances have been identified by the NOESY and COSY connectivities in Fe^{II}-SOD. In addition, paramagnetically shifted features associated with the coordinated His residues are assigned.

Experimental Section

The FeSOD overproducing strain of *Escherichia coli* with a plasmid pJM19 was a kind gift from Dr. J. A. Fee. The cells

• Abstract published in *Advance ACS Abstracts*, December 1, 1993.

(1) (a) Carrell, C. J.; Carrell, H. L.; Erlebacher, J.; Glusker, J. P. *J. Am. Chem. Soc.* **1988**, *110*, 8651–8656. (b) Chakrabarti, P. *Protein Eng.* **1990**, *4*, 49–56.

(2) (a) Ringe, D.; Petsko, G. A.; Yamakura, F.; Suzuki, K.; Ohmori, D. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3879–3883. (b) Stallings, W. C.; Powers, T. B.; Pattridge, K. A.; Fee, J. A.; Ludwig, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 3884–3888. (c) Stoddard, B. L.; Howell, P. L.; Ringe, D.; Petsko, G. A. *Biochemistry* **1990**, *29*, 8885–8893.

(3) (a) Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H.; McCallum, J. D.; Sanders-Loehr, J. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 713–716. (b) Holmes, M. A.; Le Trong, I.; Turley, S.; Sieker, L. C.; Stenkamp, R. E. *J. Mol. Biol.* **1991**, *218*, 583–593.

(4) Nordlund, P.; Sjöberg, B.-M.; Eklund. *Nature (London)* **1990**, *345*, 583–598.

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

(6) (a) Maroney, M. J.; Kurtz, D. M., Jr.; Nocek, J. M.; Pearce, L. L.; Que, L., Jr. *J. Am. Chem. Soc.* **1986**, *108*, 6871–6879. (b) Lauffer, R. B.; Antanaitis, B. C.; Aisen, P.; Que, L., Jr. *J. Biol. Chem.* **1983**, *258*, 14212–14218. (c) Wu, F.-J.; Kurtz, D. M., Jr. *J. Am. Chem. Soc.* **1989**, *111*, 6563–6572. (d) Pillai, R. P.; Lenkinski, R. E.; Sakai, T. T.; Geckle, J. M.; Krishna, N. R.; Glickson, J. D. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 341–349. (e) Balch, A. L.; Chan, Y.-W.; LaMar, G. N.; Latos-Grazynski, L.; Renner, M. W. *Inorg. Chem.* **1985**, *24*, 1437–1443. (f) Goff, H.; LaMar, G. N. *J. Am. Chem. Soc.* **1977**, *99*, 6599–6606. (g) Werth, M. T.; Kurtz, D. M., Jr.; Moura, I.; LeGall, J. *J. Am. Chem. Soc.* **1987**, *107*, 273–275.

(7) Borovik, A. S.; Hendrich, M. P.; Holman, T. R.; Münck, E.; Papaefthymiou, V.; Que, L., Jr. *J. Am. Chem. Soc.* **1990**, *112*, 6031–6038.

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were grown up from single-cell colonies at 37 °C in Luria broth medium (10 g of Bactotrypton, 5 g of yeast extract, and 5 g of NaCl in 1 L of H_2O at pH 7.5) with 50 μg of ampicillin/mL. FeSOD was purified to homogeneity as determined by electrophoresis by a slight modification of the method by Slykhouse and Fee.[8] The purified enzyme had specific activity of >6000 units/mg as assayed by the xanthine oxidase–cytochrome *c* method.[9] The purified enzyme exhibits an electronic absorption band centered around 350 nm (Hewlett Packard 8154A diode array spectrophotometer) and a rhombic EPR spectrum with features at g = 9.98, 4.99, 4.12, and 3.77 (Bruker ESP 300 EPR spectrometer) as previously reported.[8]

Samples for NMR studies were concentrated by ultrafiltration using a Centricon-10 microconcentrator (Amicon) and then transferred to NMR tubes and degassed. Sodium dithionite solution was added to reduce the protein, after which the NMR tubes were flame-sealed. Protein samples in D_2O buffer were prepared by passage down a PD-10 column equilibrated with deuterated buffer or by ultrafiltration with several changes of deuterated buffer. The protein samples were reduced with dithionite in the NMR tubes, and the tubes were flame-sealed.

Proton NMR spectra were recorded on a Nicolet NT300, an IBM NR/300, or a Bruker AMX360 spectrometer. A modified-DEFT multipulse sequence was used for the H_2O samples to suppress the water signal as well as the resonances in the diamagnetic region.[10] Spectra for samples in deuterated buffer simply required a 90° pulse. Chemical shifts are reported with respect to the water resonance at 4.8 ppm. Longitudinal relaxation times (T_1) for signals near the diamagnetic region (<30 ppm) were measured by the use of an inversion–recovery sequence with a short presaturation pulse to suppress the water resonance. T_1 values for the more downfield shifted signals were recorded using an inversion–recovery sequence in which a *1–3–3–1* selective excitation pulse sequence was incorporated. NOE difference spectra were obtained on the IBM spectrometer at 23 °C by computer manipulation of the FID's with the saturation pulse set alternately on the signal of interest and a reference position for a certain period of time (10–80 ms).

NOESY experiments (1024 F2 × 512 F1) were carried out on a Bruker AMX360 spectrometer in the TPPI· phase sensitive mode[11] with a mixing time of 15 ms. A 5% random variation of the mixing time was used to eliminate zero-quantum coherence in the NOESY experiments. Presaturation was applied on the solvent resonance during both relaxation delay (~100 ms) and mixing time. A 10%-shifted Gaussian weighting function was applied in both dimensions prior to Fourier transformation, followed by a fifth-order polynomial baseline correction. 2D bond-correlated (COSY) experiments were carried out with a relaxation delay of 80 ms, during which the solvent signal was suppressed by a presaturation pulse. The time domain point for the F2 dimension was chosen to give different acquisition times of ~3–15 ms with F2 set to half the data points of F1 and a bandwidth of 25 kHz. Then the data were zero-filled to 1K × 1K data points and processed in magnitude mode with a 0°-shifted sine-square weighting function.

Results and Discussion

FeSOD contains a mononuclear high-spin nonheme Fe(III) active site. A comparison of the amino acid sequence of various SODs indicates an active site that is highly conserved.[2] The crystal structure of the enzyme from *Pseudomonas ovalis* has been determined by X-ray crystallography to 2.1 Å and shows the iron site to consist of three N_ϵ-coordinated His residues (His-26, His-74, and His-160) and one monodentate Asp residue (Asp-156). These residues correspond to the His-26, His-75, His-162, and Asp-158 residues in the *E. coli* enzyme (inset in Figure 1).[2]

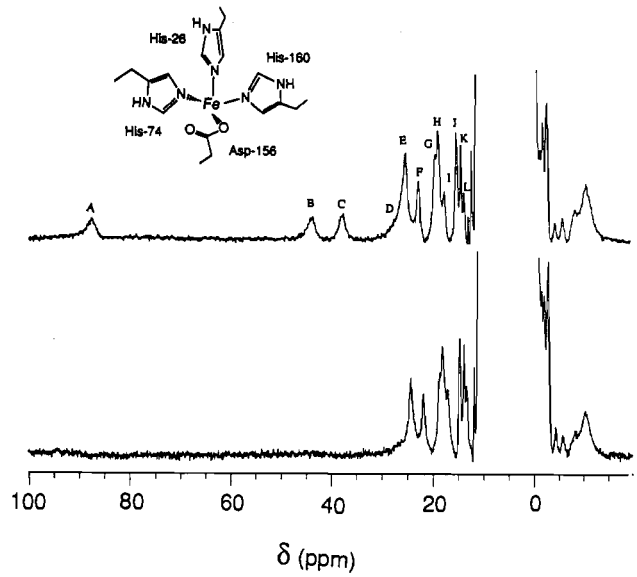

Figure 1. Proton NMR spectra of reduced FeSOD at 300 MHz and 300 K in 50 mM phosphate buffer: (top) in H_2O buffer, pH 7.4; (bottom) in D_2O buffer, pD 7.4. The inset is a schematic drawing of the active site of the enzyme based on the X-ray crystallographic studies of the *P. ovalis* enzyme,[2c] in which the conserved endogenous ligands His-26, His-74, His-160, and Asp-156 are replaced with the corresponding His-26, His-75, His-162, and Asp-158 of the *E. coli* enzyme.

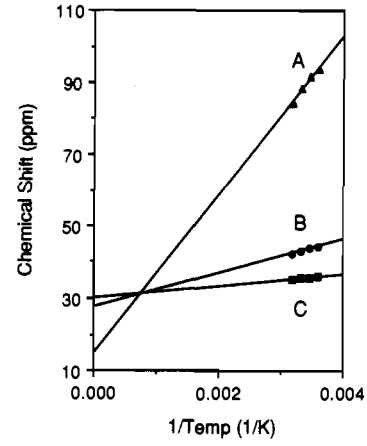

Figure 2. Plot of the chemical shifts of signals A–C versus inverse temperature.

Reduction of the Fe(III) center with dithionite results in a high-spin Fe(II) center which provides favorably short electronic relaxation times; consequently, the ^1H NMR spectrum of Fe^{II}-SOD in 50 mM phosphate buffer at pH 7.4 exhibits many sharp, well-resolved isotropically shifted signals in the downfield region beyond 15 ppm and in the slightly upfield-shifted region (Figure 1, top). Signals A–C at 88, 43, and 37 ppm, respectively, disappear in D_2O buffer under the same conditions (Figure 1, bottom). These solvent-exchangeable resonances fall in the region expected for imidazole NH protons of coordinated histidine residues in Fe(II) proteins and Fe(II)–imidazole complexes.[6] The large range in chemical shifts for these resonances is typical for high-spin Fe(II) centers and likely arises from the magnetic anisotropy of the Fe(II) center. For comparison, deoxyhemerythrin azide exhibits His N–H shifts at 78.3, 66.8, and 47.3 ppm.[6a] The three solvent-exchangeable resonances in the ^1H NMR spectrum of Fe^{II}SOD are thus associated with the three endogenous His ligands on the metal center. Signal A has a T_1 value (5 ms) half as large as those for signals B (9 ms) and C (10 ms). The difference in

(8) Slykhouse, T. O.; Fee, J. A. *J. Biol. Chem.* **1976**, *251*, 5472–5477.
(9) McCord, J. M.; Fridovich, I. *J. Biol. Chem.* **1969**, *244*, 6049–6055.
(10) Hochmann, J.; Kellerhals, H. *J. Magn. Reson.* **1980**, *38*, 23–29.
(11) Marion, D.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1984**, *113*, 967.

T_1 values cannot be accounted for by differences in Fe–H distances and is likely the result of contributions to relaxation by a contact mechanism where extensive spin delocalization onto the ligand engenders a higher unpaired-electron density on the ligand.[5,12] Thus the most shifted resonance would have the shortest T_1 value. Indeed the three His NH signals in Co(II)-carbonic anhydrase (CA) and its anion-bound derivatives show very different T_1 values, with the most shifted signal being the shortest.[13] The temperature dependence of signal A also differs from those of signals B and C. That of signal A has a steeper slope and extrapolates to ca. 15 ppm at infinite T, while the temperature dependences of the other two extrapolate to ca. 30 ppm (Figure 2). This difference may also be attributed to the magnetic anisotropy of the high-spin ferrous center. On the basis of these observations, it is reasonable to assign the signal at 88 ppm to the unique axial His.

No solvent-nonexchangeable resonance is detected in the region of >30 ppm, the region expected for the C_δ–H protons of His residues N_δ-coordinated to Fe(II) centers;[6] thus the three His residues must be coordinated to the Fe(II) via N_ϵ, as found in the oxidized form of the enzyme. In such a binding mode, both the $C_\delta H$ and $C_\epsilon H$ His protons are α to the coordinated N_ϵ nitrogens and would afford isotropically shifted resonances too broad to be detected because of their short Fe–H distances.[6]

Several relatively sharp isotropically shifted resonances are observed in the 15–30 ppm region (Figure 1). Chemical shifts in this range are reasonable for carboxylate protons bound to iron(II) centers on the basis of the few available studies. For isopenicillin N synthase, features at ca. 42 ppm are ascribed to the β-CH_2 protons of a coordinated aspartate.[14] The propionate CH_2 protons of $[Fe^{II}M(BPMP)(O_2CC_2H_5)_2]^{n+}$ (BPMP = 2,6-bis[bis(2-pyridylmethyl)amino]-4-methylphenol; M = Fe(II), Zn(II), or Ga(III)) are observed in the 15–50 ppm region.[7] For example, the four propionate CH_2 protons of $[Fe^{II}Zn^{II}(BPMP)(O_2CC_2H_5)_2]^+$ are found at 36, 28, 18, and 15 ppm. Carboxymethylene isotropic shifts exhibit some variability due to two factors. There can be significant variations in the length of the Fe–O(carboxylate) bond which determine the extent of spin delocalization onto the ligand. Secondly, the delocalization of unpaired spin density onto a particular C–H bond depends on the cosine of the angle formed by the C–H bond and the p orbital on the carboxylate carbon.[15] However the isotropic shift values found for carboxymethylene protons are insufficiently distinct to allow their unequivocal assignment on the basis of the shifts alone. Thus, additional experiments are required to identify the resonances associated with the coordinated Asp residue in Fe^{II}-SOD.

The nuclear Overhauser effect (NOE)[16,17] has been useful for identifying Asp and Glu protons in a metalloprotein NMR spectrum. Examples include Asp-81 of bovine E,Co-SOD (with the Cu depleted), Glu-72 of Ni(II)-substituted bovine carboxypeptidase A, and the Asp residues of isopenicillin N synthase[14] and uteroferrin.[18] NOE measurements[19] have been used effectively to estimate internuclear distances in many paramagnetic species using eq 1, where η_{ij} is the NOE on signal i when signal j is saturated for a period of time t (i.e. the fraction change in intensity of signal i, $(I - I_0)/I_0$, when signal j is saturated) and $\sigma_{ij} = -\hbar^2\gamma^4\tau_c/10r_{ij}^6$ is the cross-relaxation between i and j, with τ_c being the rotational correlation time of the molecule and r_{ij} being the distance between the nuclei i and j. Under the conditions of steady-state NOE, i.e. when a long saturation time is used ($t \rightarrow \infty$), eq 1 can be simplified to eq 2, and the internuclear distance

$$\eta_{ij}(t) = (\sigma_{ij}/\rho_i)[1 - \exp(-\rho_i t)] \quad (1)$$

$$\eta_{ij}(t \rightarrow \infty) = \sigma_{ij}/\rho_i \quad (2)$$

r_{ij} can be estimated on the basis of the experimental η_{ij} values in the steady state. When signal E at 24.5 ppm (T_1 = 18 ms) is saturated for 80 ms, significant NOE's are observed on signals G at 19 ppm (–48%) and J at 15 ppm (–26%), suggesting that they are in close proximity to each other. Signal G with a T_1 of 21 ms still shows clear NOE (–14%) when signal E is saturated for as short as 10 ms, while signal J, with a longer T_1 of 43 ms, shows only a small negative NOE (<5%). These results suggest that the protons corresponding to signals E and G are very close to one another, most likely a geminal pair, while the proton that gives rise to signal J is farther away from the geminal pair and likely to be a vicinal proton. When eq 2 is applied by using a τ_c value of 20 ns for FeSOD (a value comparable to proteins of similar size of ~40 kDa), the E–G and E–J internuclear distances can be estimated to be 1.9 and 2.4 Å, respectively. Although an estimation of internuclear distance can deviate significantly from the true situation for proton pairs that are not isolated two-spin systems,[19] our observations are consistent with the assignment of signals E and G to a geminal pair and of signal J to a vicinal proton associated with the geminal pair, i.e. an ABX system.

Irradiation experiments on signals G and J corroborate the results obtained for signal E. When signal G (as a shoulder at ~19 ppm) is irradiated for 80 ms, significant NOE's are observed on signals E and J. When the irradiation time is reduced to 10 ms, signal E still shows clear NOE, while signal J shows a much smaller effect. Since signal G is not resolved from signals H and I under the experimental conditions, signal G cannot be selectively saturated, so a quantitative analysis of the NOE's based on eqs 1 and 2 was not performed. When signal J is irradiated for 80 ms, NOE's are observed on both signals E and G, albeit to a smaller extent due to their shorter relaxation times (larger ρ_i values in eq 1). Furthermore, there is no clear NOE connectivity observed between signal J and other resonances in the diamagnetic region, indicating that it does not have a geminal partner. Thus signals E, G, and J arise from a $-CH_2-CH<$ moiety, and the presence of a $-CH_2-CH_2-CH<$ moiety on a ligand in the Fe(II) site can be ruled out.

2D NMR techniques have recently been successfully applied to paramagnetic metalloproteins;[17,20,21] they are particularly useful for the detection of interactions among overlapping resonances where selective irradiation becomes difficult. The NOESY spectrum of $Fe^{II}SOD$ in the region <30 ppm (Figure 3) reveals many cross signals. The 14–24 ppm sector of the spectrum reveals a set of cross signals relating peaks E, G, and J, which associates

(12) Navon, G.; Valensin, G. *Met. Ions Biol. Syst.* **1987**, *21*, 1–45.
(13) Banci, L.; Bertini, I.; Luchinat, C.; Donaire, A.; Martinez, M.-J.; Moratal Mascarell, J. M. *Comments Inorg. Chem.* **1990**, *9*, 245–261.
(14) Ming, L.-J.; Que, L., Jr.; Kriauciunas, A.; Frolik, C. A.; Chen, V. J. *Biochemistry* **1991**, *30*, 11653–11659.
(15) Scarrow, R. C.; Pyrz, J. W.; Que, L., Jr. *J. Am. Chem. Soc.* **1990**, *112*, 657–665.
(16) Banci, L.; Bertini, I.; Luchinat, C.; Viezzoli, M. S. *Inorg. Chem.* **1990**, *29*, 1438–1440.
(17) Moratal, J.-M.; Castells, J.; Donaire A.; Salgado, J.; Jiménez, H. *J. Chem. Soc., Dalton Trans.* **1992**, 3317–3324.
(18) Holz, R. C.; Que, L., Jr.; Ming, L.-J. *J. Am. Chem. Soc.* **1992**, *114*, 4434–4436.
(19) Neuhaus, D.; Williamson, M. P. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*; VCH: New York, 1989.
(20) (a) de Ropp, J. S.; La Mar, G. N.; Wariishi, H.; Gold, M. H. *J. Biol. Chem.* **1991**, *266*, 15001. (b) Satterlee, J. D.; Russell, D. J.; Erman, J. E. *Biochemistry* **1991**, *30*, 9072. (c) Banci, L.; Bertini, I.; Turano, P.; Ferrer, J. C.; Mauk, A. G. *Inorg. Chem.* **1991**, *30*, 4510. (d) Banci, L.; Bertini, I.; Turano, P.; Tien, M.; Kirk, T. K. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6956. (e) Emerson, S. D.; La Mar, G. N. *Biochemistry* **1990**, *29*, 1545. (f) Yamamoto, Y.; Osawa, A.; Inoue, Y.; Chûjô, R.; Suzuki, T. *FEBS Lett.* **1989**, *247*, 263.
(21) (a) Banci, L.; Bertini, I.; Briganti, F.; Luchinat, C.; Scozzafava, A.; Oliver, M. V. *Inorg. Chem.* **1991**, *30*, 4517. (b) Bertini, I.; Briganti, F.; Luchinat, C.; Messori, L.; Monnanni, R.; Scozzafava, A.; Vallini, G. *FEBS Lett.* **1991**, *289*, 253. (c) Busse, S. C.; La Mar, G. N.; Howard, J. B. *J. Biol. Chem.* **1991**, *266*, 23714. (d) Netteseim, D. G.; Harder, S. R.; Feinberg, B. A.; Otvos, J. D. *Biochemistry* **1992**, *32*, 1234. (e) Bertini, I.; Luchinat, C.; Ming, L.-J.; Piccioli, M.; Sola, M.; Valentine, J. S. *Inorg. Chem.* **1992**, *31*, 4433–4435.

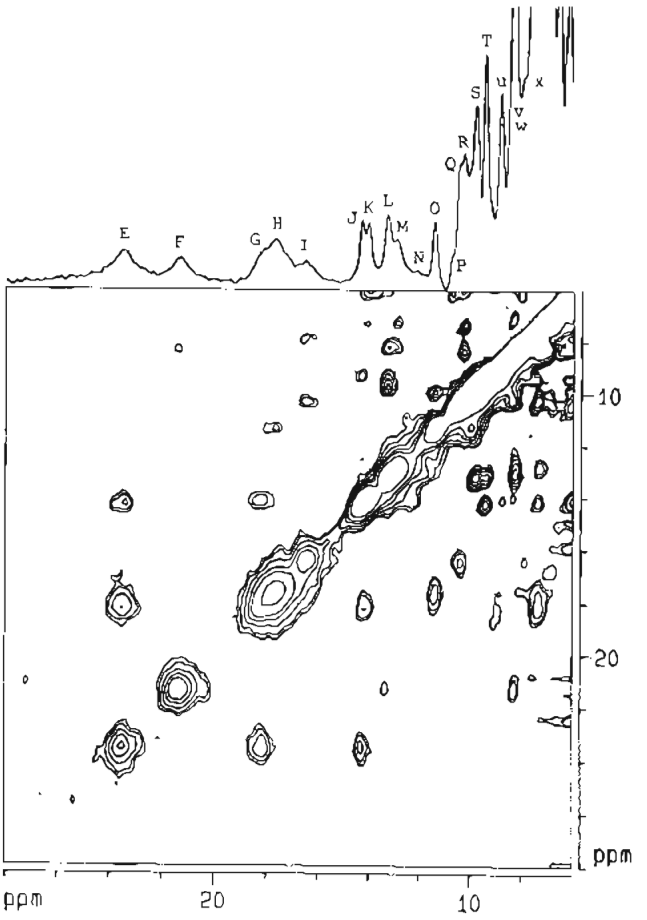

Figure 3. Proton NOESY spectrum (360.13 MHz) of $Fe^{II}SOD$ at 303 K in 50 mM phosphate buffer at pD 7.4. The top trace is a resolution-enhanced 1D spectrum using a shifted Gaussian weighting function. The spectrum was obtained as detailed in the Experimental Section. Many three-spin systems are revealed in this spectrum, where signals E, G, and J are assigned to the coordinated Asp-158 residue.

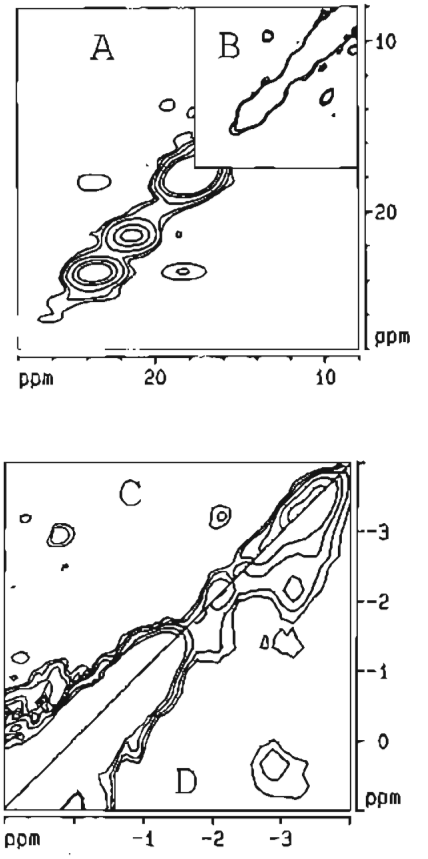

Figure 4. Proton COSY (A–C) and NOESY (D) spectra (360.13 MHz) of $Fe^{II}SOD$ at 303 K in 50 mM phosphate buffer at pD 7.4. Different acquisition times were used for clear detection of the cross signals (A, 2.58 ms; B, 8.3 ms; C, 16.12 ms) owing to the very different line widths of the isotropically shifted signals in the displayed spectral regions. Spectrum D is the upfield region of Figure 3. Both spectra were acquired and manipulated as described in the Experimental Section.

them with an ABX spin system. The cross signal between E and G is more intense (despite their shorter relaxation times) than those between E and J and between G and J, as expected for a geminal pair. In corroboration of the assignment, the corresponding COSY spectrum (Figure 4A) shows a correlation between signals E and G; cross signals between the vicinal protons are not observed because the relaxation properties of these protons only allow the observation of COSY cross peaks between protons with large scalar coupling. Furthermore there is no COSY cross signal associated with J, indicating that it does not have a geminal partner. The NOESY and COSY experiments thus corroborate the assignment of signals E, G, and J to a three-spin system, as deduced from the 1D NOE studies.

Among the amino acids that can act as metal ligands, those that have a $-CH_2-CH<$ moiety include Cys, Tyr, His, Ser, and Asp. All except Asp can be excluded on the basis of chemical shift arguments and their expected Fe–H distances. Cys $C_\beta H_2$ protons are typically found at ca. 80 ppm and have an Fe–H distance of 3.5 Å, Tyr $C_\beta H_2$ protons are expected to have shifts that average 30 ppm but are 7 Å away from the metal center, while His $C_\beta H_2$ protons are found at ca. 10 ppm for N_ϵ-coordinated residues.[6] On the basis of the chemical shift data and the connectivities demonstrated above, we thus associate the three isotropically shifted resonances, E, G, and J, with the coordinated Asp residue, with the signals E and G being due to the geminal $C_\beta H_2$ pair and the signal J being due to the $C_\alpha H$ proton.

The remaining resonances in the paramagnetically shifted region must be associated with other residues in the active site. Signal F disappears after long term (a few months) storage of

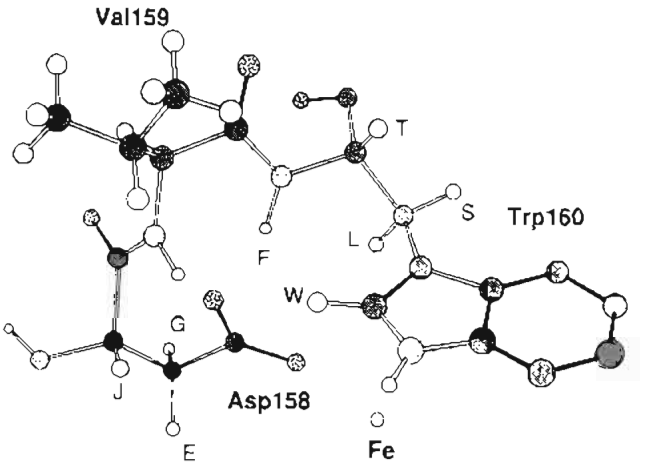

Figure 5. Chem3D representation of the active site in the vicinity of the coordinated aspartate based on the *P. ovalis* FeSOD structure (courtesy of D. Ringe and G. Petsko). However, the amino acids are numbered on the basis of the *E. coli* sequence. Letter labels on the residue protons designate the NMR signals to which the protons are assigned.

the enzyme in D_2O buffer. A signal that exchanges this slowly is likely to arise from a hydrogen-bonded NH proton in proteins.[22] Because of the paramagnetic shift observed, reasonable candidates for this signal are the $N_\alpha H$ protons of Val-159 and Trp-160, both of which are hydrogen-bonded to the carbonyl oxygen of the monodentate Asp ligand in the crystal structure of the *P. ovalis*

(22) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

enzyme;[2c] its downfield shift would then arise from unpaired spin density that is delocalized via the hydrogen-bonding interaction to the metal-coordinated carboxylate. Signal F exhibits NOESY cross signals with signals L and W, which also correlate with each other. Signal L in turn shows cross peaks with signals S and T. Furthermore signals L and S are strongly correlated in the COSY spectrum (Figure 4B), thereby assigning them to a geminal pair. The network of interactions observed for these signals and their paramagnetic shifts suggest that they arise from a single amino acid residue in close proximity to the iron center. On the basis of an examination of the active-site pocket found in the *P. ovalis* enzyme (Figure 5),[2c] our observations are most consistent with the assignment of the signals to Trp-160. The $N_\alpha H$ (signal F) which is hydrogen-bonded to the carbonyl oxygen of Asp-158 is in close proximity to the indole C2H (W) and one of the $C_\beta H_2$ protons (L); these latter two protons in turn are within 3 Å of each other. The other $C_\beta H_2$ proton is assigned to signal S, while the $C_\alpha H$ is associated with signal T.

Several of the remaining isotropically shifted features can be grouped into three-spin systems from the NOESY data in Figure 3: H, O, and R; Q, V, and X. From the COSY (Figure 4C) and NOESY (Figure 4D) data, we can discern two sets of signals: at −3.0 and 0.3 ppm and at −3.2, −2.1, and 0.7 ppm. Three of these ABX systems may be due to the α and β protons of the three coordinated His residues.

In summary, we have demonstrated the utility of 1D and 2D NMR techniques for identifying active-site resonances in paramagnetic nonheme iron proteins. In particular, the features arising from the Asp-158 metal ligand could not be assigned by the magnitude of their isotropic shifts alone and required through-bond and through-space connectivities to establish the identity of the $-C_\beta H_2-C_\alpha H-$ unit.

Acknowledgment. We thank Professors D. Ringe and G. Petsko for providing the coordinates to the *P. ovalis* FeSOD structure. This work was supported by the National Institutes of Health (Grant GM-33162, L.Q.). L.-J.M. is grateful for the funding provided by the University of South Florida. The Bruker AMX360 was purchased with funds provided by the National Science Foundation and the University of South Florida.