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NMR Studies of the Active Site of Isopenicillin N Synthase, a Non-Heme Iron(II) Enzyme†

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ABSTRACT: The active site structure of isopenicillin N synthase (IPNS) has been previously studied by the use of Mössbauer, EPR, electronic absorption, and NMR spectroscopies [Chen, V. J., Frolik, C. A., Orville, A. M., Harpel, M. R., Lipscomb, J. D., Surerus, K. K., & Münck, E. (1989) *J. Biol. Chem. 264*, 21677-21681; Ming, L.-J., Que, L., Jr., Kriauciunas, A., Frolik, C. A., & Chen, V. J. (1990) *Inorg. Chem. 26*, 1111-1112]. These studies have revealed three coordinated His residues along with three sites for substrate [δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine, ACV], NO, and water binding on the active Fe(II) of IPNS. We report here NMR studies of Fe(II)IPNS and its Co(II)-substituted derivative [Co(II)IPNS]. By the use of NOE techniques on the Co(II)IPNS-ACV complex, we have recognized a $-CH_2-CH<$ spin system at 14.6, 24.3, and 38.6 ppm that is assigned to the α and β protons of a coordinated Asp residue. Corresponding solvent nonexchangeable features are found near 40 ppm in Fe(II)IPNS and the Fe(II)IPNS-ACV complex, but the peaks are too broad for NOE effects to be observed. The binding of NO to the Fe(II) center results in a significant change in the configuration of the metal site: (a) The $C_\beta H_2$ resonances due to the coordinated Asp residue disappear. The loss of the signal may indicate a change of the carboxylate configuration from syn-like to anti-like or, less likely, its displacement by NO. (b) The imidazole NH resonance for one of the coordinated His residues in the Fe(II)IPNS-ACV complex also disappears, suggesting that this His residue is strongly perturbed and may be detached from the metal site. These results allow us to propose a scheme showing the effects of exogenous ligand binding on the active site of IPNS. To date, this is the first successful NMR study of the endogenous ligands of the Fe(II)-NO center in a non-heme Fe(II) protein.

The key steps in the biosynthesis of penicillin- and cephalosporin-related antibiotics in some microorganisms are the oxidative ring closure reactions of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) forming the β-lactam ring and the thiazolidine ring of isopenicillin N, the precursor of other penicillins and cephalosporins (Baldwin & Bradley, 1990; Baldwin, 1989; Baldwin & Abraham, 1988; Robinson 1988).

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The enzyme isopenicillin N synthase (IPNS) that aerobically catalyzes the four-electron oxidative process contains a single high-spin non-heme iron(II) center in its active site (Chen et al., 1989a,b). However, unlike in the oxidative cleavage reactions catalyzed by non-heme iron-containing dioxygenases in which oxygen atoms are incorporated into the substrates (Que, 1989), dioxygen is completely reduced to 2 equiv of H_2O in the oxidative ring closure reactions of ACV in the IPNS catalysis (Baldwin & Bradley, 1990; Baldwin, 1989; Baldwin & Abraham, 1988; Robinson, 1988).

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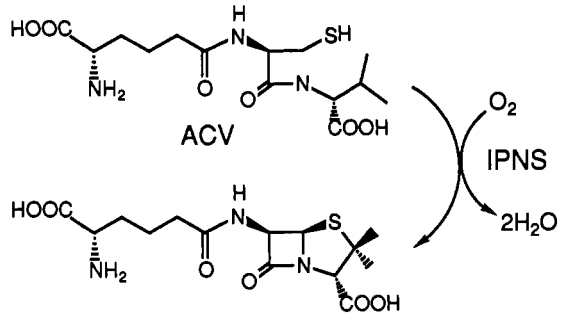

Isopenicillin N

Although the mechanism of IPNS catalysis has been extensively studied by the use of many different substrate analogues (Baldwin & Bradley, 1990; Baldwin, 1989; Baldwin & Abraham, 1988; Robinson 1988), much less is known about the coordination environment of the active site iron and the effects of substrate and O_2 binding on the iron active site.

Details of the coordination chemistry of the active site metal are beginning to emerge with the application of EPR, Mössbauer, electronic absorption, and NMR spectroscopies on Fe(II)IPNS and its Co(II) and Cu(II) derivatives [Co(II)IPNS and Cu(II)IPNS] (Chen et al., 1989a,b; Ming et al., 1990). Taken together, the spectroscopic studies have allowed a working model of the active site to be proposed consisting of three endogenous His ligands and available sites for exogenous ligands such as NO, solvent, and substrate bound via the Cys-S^- moiety. In this paper, we report further NMR studies of Fe(II)IPNS and Co(II)IPNS to refine this working model. We have applied NOE techniques to identify previously unassigned NMR features and provide evidence for Asp as the fourth endogenous ligand of the metal center of IPNS. We have also investigated the NMR spectra of Fe(II)IPNS–NO and Fe(II)IPNS–ACV–NO complexes, the latter as a model for the putative ternary ES–O_2 complex, and find that NO binding significantly affects the coordination of the endogenous ligands to the Fe(II) active site in IPNS. We thus demonstrate the utility of NO as an NMR probe of endogenous ligand binding, which complements its use in EPR as probe for exogenous ligand binding (Diner & Petrouleas, 1990; Arciero & Lipscomb, 1986; Arciero et al., 1985). These strategies may also be useful for investigating other Fe(II) enzymes.

Experimental Procedures

Cephalosporium acremonium isopenicillin N synthase (MW 38 400) was purified as metal-free protein from recombinant *Escherichia coli* according to published procedures (Kriauciunas et al., 1991) and stored in 0.1 M MOPS buffer at pH 7.1 in liquid nitrogen. The substrate ACV is a product of Incell (Milwaukee, WI), NO gas is from Matheson (Secaucus, NJ), and all other chemicals used are the highest grade commercially available. IPNS activity was determined by the initial rate of the consumption of dioxygen by a solution of Fe(II)-reconstituted enzyme in the presence of 5 mM ACV and ascorbate in 0.1 M MOPS at pH 7.1 as monitored by an oxygen-sensitive electrode; alternatively, the activity can be estimated by direct measurement of isopenicillin N produced under the above conditions using HPLC (Kriauciunas et al., 1991). The preparations with high specific IPNS activity of 4–7 units/mg were used in this study (1 unit of IPNS activity is defined as the consumption of 1 μmol of O_2 per minute under the above buffer conditions at 25 °C). These specific activity values are comparable to those of samples employed in previous spectroscopic investigations. The Fe(II)-reconstituted and the Co(II)-substituted enzymes were prepared by direct infusion of 1 equiv of metal ion into the apoenzyme in 0.1 M MOPS buffer at pH 7.1. The NO complexes of Fe(II)IPNS and Fe(II)IPNS–ACV were prepared by direct bubbling of NO gas into the protein solutions using a gas-tight syringe (Hamilton, Reno, NV). The formation of the NO complexes can be monitored by the appearance of the characteristic color of these complexes and can also be confirmed by both EPR and NMR spectroscopies.

EPR spectra were obtained on a Varian E-109 spectrometer with an Oxford Instruments ESR-10 liquid He cryostat. Electronic absorption spectra were taken at ambient temperature on a Hewlett Packard 8451A diode array spectrophotometer. Proton NMR spectra were obtained on an IBM AC-300 spectrometer at 300 MHz by the use of the modified DEFT pulse sequence (D1–90°–τ–180°–τ–90°–FID) (Hochmann & Kellerhals, 1980) to suppress the water signal and protein resonances in the diamagnetic region. The water signal at 4.8 ppm was used as the chemical shift reference for the isotropically shifted resonances in all the ^{1}H NMR spectra. The enzyme samples in H_2O buffer for NMR studies were concentrated to 2–3 mM by the use of a Centricon-10 microconcentrator (Amicon Corp., Danvers, MA), and those in D_2O buffer were prepared first by dialyzing the apo-IPNS solution against 0.1 M MOPS in D_2O at pD 7.1 to deuterate solvent-exchangeable protons in the protein, followed by metal reconstitution and concentration to 1–2 mM. The external standard used for quantitative determination of the intensities of the isotropically shifted resonances in the enzyme samples was a Co(II) complex of nitrilotriacetic acid (NTA) in D_2O. This complex, which was sealed in a capillary tube, showed a single resonance at 34 ppm at 10 °C, a feature that could be easily distinguished from the isotropically shifted resonances of the enzyme.

An NOE difference FID was obtained by computer addition and subtraction of the FID's with the decoupler pulse set alternately on the signal of interest and a reference position every 80 scans until a sufficient signal-to-noise ratio of the NOE difference spectrum was achieved. NOE's on paramagnetic species are difficult to observe owing to the large intrinsic longitudinal relaxation rate, ρ_i, as shown by (Neuhaus & Williamson, 1989)

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

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, $(I - I_o)/I_o$, when signal j is saturated] and σ_{ij} is the cross-relaxation between i and j which can be written as $-\hbar^2\gamma^4\tau_c/10r_{ij}^6$ with τ_c being the rotational correlation time and r_{ij} being the distance between the nuclei i and j. However, in some paramagnetic systems with larger τ_c values where there are pairs of nuclei in the close proximity of each other (i.e., having small r_{ij} values) such as those of the geminal or vicinal proton pairs, NOE has been demonstrated to be a useful tool for the assignment of isotropically shifted resonances such as in some heme-containing (La Mar et al., 1991; Thanabal et al., 1988; Satterlee & Moench, 1987), non-heme iron (Scarrow et al., 1990), iron-sulfur (Dugad et al., 1990), and Co(II)-substituted (Banci et al., 1989) metalloproteins.

Results

Apo-IPNS binds Fe(II) and Co(II) to afford Fe(II)IPNS and Co(II)IPNS, respectively, but only the Fe(II)-reconstituted enzyme is active. The two divalent metals appear to occupy the same site in the protein. The activity of Fe(II)-

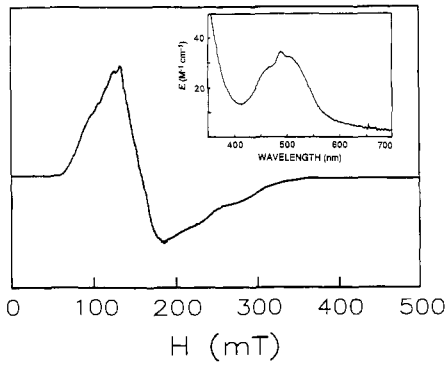

FIGURE 1: EPR spectrum of Co(II)IPNS in 0.1 M MOPS at pH 7.1 and 2.2 K showing the signal at $g \sim 4$ for the high-spin Co(II) site. EPR conditions: microwave frequency, 9.213 GHz; power, 0.2 mW; modulation amplitude, 1.25 G. The inset is the visible spectrum of Co(II)IPNS in 0.1 M MOPS at pH 7.1 and ambient temperature referenced against apo-IPNS. The relatively high energy of λ_{max} and its low absorptivity suggest a six-coordinate Co(II) in the metal-binding site of IPNS.

IPNS decreases systematically with the introduction of increasing amounts of Co(II) ion and is eliminated when a large excess (>100-fold) of Co(II) ion is present in the solution. Conversely, when catalytically inactive Co(II)IPNS is anaerobically incubated with 100-fold excess of Fe(II) ion, IPNS activity recovers with time. Metal ion binding to the active site is tight and stoichiometric, as indicated by their catalytic and spectroscopic properties (Chen et al., 1989a; Ming et al., 1990; Kriauciunas et al., 1991; Jiang et al., 1992). Fe(II)IPNS exhibits a single Mössbauer quadrupole doublet with parameters typical of a high-spin ferrous center and affords a sharp $S = {}^3/_2$ EPR signal when NO is introduced (Chen et al., 1989a,b). Co(II)IPNS exhibits an $S = {}^3/_2$ EPR signal with $g \sim 4$ and d–d transitions centered at 490 nm (Figure 1), both features similar to those of the Co(II)-substituted derivative of Fe–alcohol dehydrogenase (Bakshi et al., 1989). The electronic absorption maximum of 490 nm for Co(II)IPNS and its low absorptivity (35 M^{-1} cm^{-1}) are indicative of an octahedral metal-binding environment, as also found for Cu(II)IPNS (Ming et al., 1990).

We have taken advantage of the relatively favorable electronic relaxation properties of Fe(II) and Co(II) to observe paramagnetically shifted NMR resonances to gain insights into the nature of the active site residues of this enzyme. The proton NMR spectrum of Fe(II)IPNS in 0.1 M MOPS buffer at pH 7.1 shows a few isotropically shifted resonances, as reported previously (Figure 2A; Ming et al., 1990). Solvent-exchangeable resonances with an intensity associated with 3 H's are observed at ~65 ppm, falling in the chemical shift region for coordinated imidazole NH protons in Fe(II) complexes and proteins (Maroney et al., 1986; Lauffer et al., 1983; Pillai et al., 1980; Balch et al., 1985; Goff & La Mar, 1977). They have thus been assigned to the imidazole NH protons of three coordinated histidine residues (Ming et al., 1990). The resonance at 42 ppm integrating to 2 H's is not solvent exchangeable and may be associated on the basis of its isotropic shift to coordinated amino acid residues, such as the β-(or γ-)CH_2 of an Asp (or Glu) residue or the imidazole CδH protons of Nδ-coordinated His residues.

The ^{1}H NMR spectrum of the Fe(II)IPNS–ACV complex obtained under anaerobic conditions to prevent catalytic turnover differs from that of Fe(II)IPNS; the imidazole NH resonances split into two signals at 66 and 56 ppm with an intensity ratio of 2:1, and the CH resonances near 45 ppm also resolve into two signals (Figure 2C; Ming et al., 1990). One of the three coordinated histidines is clearly more affected by

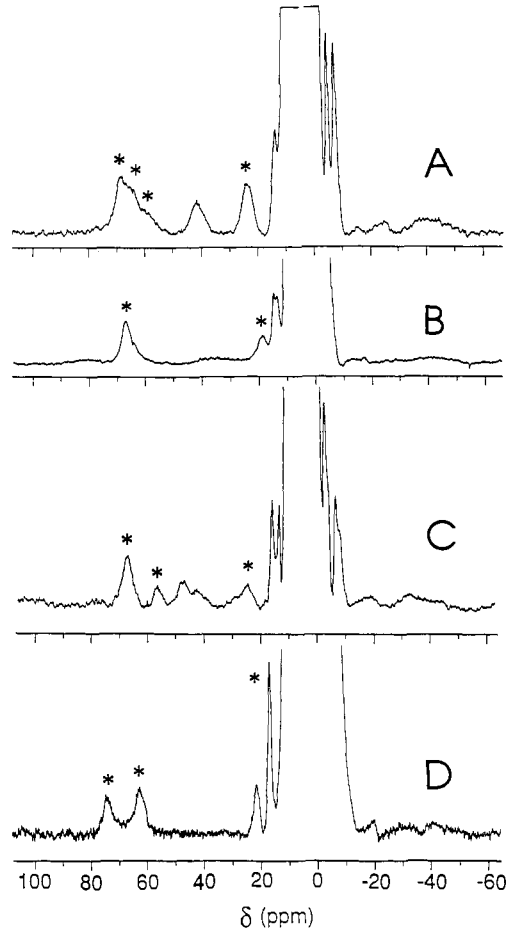

FIGURE 2: Proton NMR spectra (300 MHz and 10 °C) of (A) Fe(II)IPNS, (B) Fe(II)IPNS–NO complex, (C) Fe(II)IPNS–ACV complex, and (D) Fe(II)IPNS–ACV–NO ternary complex under argon in 0.1 M MOPS at pH 7.1. The signals marked by asterisks are solvent-exchangeable peaks that disappear in D_2O buffer. By the use of the complex $Co(NTA)^-$ as an external reference for the measurement of the integrated intensities of the isotropically shifted resonances, the solvent-exchangeable signals in the far-downfield region in panels A, B, and C are found to have similar integrated intensities (i.e., 3 H's). The NH resonances in the Fe(II)IPNS–ACV complex decrease in intensity (~2.2 H's) and resolve into two signals with about equal intensities upon NO binding to form the Fe(II)IPNS–ACV–NO complex (D).

the binding of ACV. The smaller downfield shift for the unique His residue suggests a weakening of the Fe–His interaction, perhaps arising from the coordination of the ACV thiolate trans to this histidine. Similarly, ACV binding to Cu(II)IPNS makes the two magnetically equivalent equatorial His residues distinct and diminishes the hyperfine interaction for one of these residues, as shown in our recent pulsed EPR studies (Jiang et al., 1991). The detection of the three NH resonances as well as the two CH resonances in the Fe(II)-IPNS–ACV complex suggests that all the endogenous ligands remain coordinated to the Fe(II) in IPNS upon ACV binding. Attempts to perform NOE experiments on the CH resonances of the Fe(II)IPNS–ACV complex were unsuccessful due to the broadness of the NMR features.

The six-coordinate environment associated with Co(II)IPNS raises the possibility of observing sharper isotropically shifted proton resonances for the coordinated ligands because of the favorably short electronic relaxation times associated with such a paramagnetic center. Such sharp features are indeed observed (Figure 3) and improve prospects for successful NOE experiments. The ^{1}H NMR spectrum of Co(II)IPNS shows only one solvent-exchangeable isotropically shifted signal at

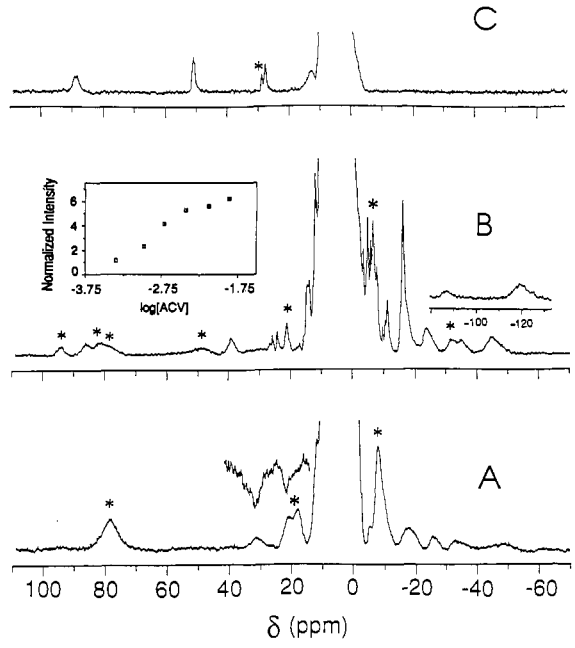

FIGURE 3: Proton NMR spectra (300 MHz and 10 °C) of (A) Co(II)IPNS, (B) Co(II)IPNS–ACV complex, and (C) Co(II) in the presence of ~5 equiv of ACV under argon in 0.1 M MOPS at pH 7.1. The signals marked by asterisks are solvent exchangeable. The inset in (A) is an NOE difference spectrum in which the signal at 31.7 ppm is saturated by the decoupler for 80 ms. The inset in panel B is a plot of the relative intensity of the signal at −16 ppm with respect to the increasing amounts of ACV (in terms of log [ACV]). An affinity constant of ACV binding to Co(II)IPNS of ~700 M^{-1} can be estimated from the plot. This titration course also shows that ACV is tightly bound to Co(II)IPNS and is in slow exchange between its bound and free states with respect to the NMR time scale.

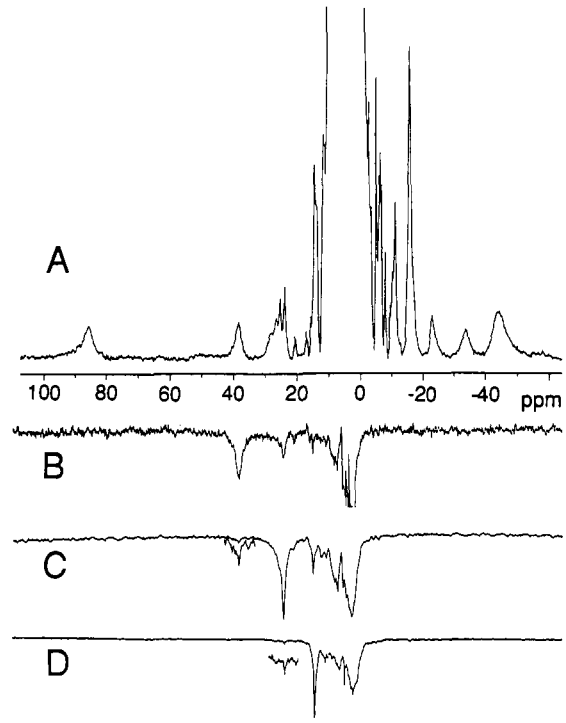

FIGURE 4: (A) Proton NMR spectrum (300 MHz and 14 °C) of Co(II)IPNS–ACV in 0.1 M MOPS at pD 7.1. (B–D) NOE difference spectra of Co(II)IPNS–ACV obtained by subtraction of the reference FID's from the FID's in which the resonances at 38.6 (B), 24.3 (C), and 14.6 ppm (C), respectively, are saturated by a decoupler pulse for 80 ms. The clear observation of the NOE's on the three resonances suggests the presence of a >CH–CH_2– moiety (of a coordinated Asp residue) in the metal site of IPNS.

78 ppm with an intensity of ~3 H relative to the resonance at 31.7 ppm (Figure 3A). The 78 ppm signal falls in the chemical shift range for the imidazole NH protons of coordinated His residues in Co(II) proteins with similar geometry, such as that in Co(II)-substituted concanavalin A (Bertini et al., 1987). The resonance at 31.7 ppm is a solvent-nonexchangeable signal which may correspond to the CH resonance at 42 ppm in the ^1H NMR spectrum of Fe(II)IPNS (Figure 1A). When the signal of 31.7 ppm in Co(II)IPNS is saturated for 80 ms by the use of the decoupler pulse, a strong NOE (−38%) is observed on the signal at 21 ppm (inset in Figure 3A). This NOE signal can still be clearly observed (~−25%) even when a 30-ms saturation pulse is applied. Similarly, saturation of the 21 ppm resonance clearly shows an NOE on the signal at 31.7 ppm (but to a smaller extent presumably due to the faster relaxation rate of the latter resonance). However, NOE's on other resonances near or in the diamagnetic envelope are not observed when either of the two resonances is saturated.

The substrate ACV binds to Co(II)IPNS very tightly and is in slow exchange between its bound and free forms with respect to the NMR time scale at 10° C. Addition of saturating amounts of ACV to Co(II)IPNS generates a new set of signals due to the Co(II)IPNS–ACV complex (Figure 3B). A plot of the relative intensity of the sharp new resonance at −16 ppm with respect to log [ACV] shows a sigmoidal curve (inset in Figure 3B), from which an affinity constant of ~700 M^{-1} for ACV binding to Co(II)IPNS can be obtained. In the course of the titration, a new signal appears at 38.6 ppm concomitant with the disappearance of the signal at 31.7 ppm. Three isotropically shifted solvent-exchangeable signals at 79, 82, and 94 ppm become clearly observed in the Co(II)-IPNS–ACV complex, along with a new CH signal at 86 ppm. We suspect the latter signal arises from one of the $C_\beta H_2$ protons of the Cys moiety of ACV, which has been demonstrated to directly coordinate to the active site metal in IPNS (Chen et al., 1989; Ming et al., 1990). When Co(II) ion is mixed with ACV or glutathione under the same conditions, a CH signal is detected at 86.8 or 92.5 ppm, respectively (Figure 3C). The chemical shifts of the $-CH_2-S^-$ proton resonances are also in the same general region for Co(II)-substituted stellacyanin (~90–120 ppm; Dahlin et al., 1989) and Co(II)-substituted rubredoxin (40–120 ppm; Moura et al., 1987). The large range in the chemical shifts of the coordinated $-CH_2-S^-$ proton resonances is likely due to (a) divergent S–Co(II) binding strengths which afford different unpaired electron–proton contact interactions and (b) different Co(II)–S–C–H dihedral angles in the various complexes (Bertini & Luchinat, 1986). The confirmation of these assignments awaits the preparation of ACV deuterated at the Cys moiety ongoing in our laboratories.

NOE experiments on the Co(II)IPNS–ACV complex have proven to be the most informative (Figure 4). When the signal at 38.6 ppm in the NMR spectrum of the Co(II)IPNS–ACV complex is saturated for 80 ms by the decoupler pulse, two NOE signals can be clearly detected at 24.3 ppm (−32%; −38% when a 200-ms saturation pulse is applied) and 14.6 ppm (~−15%) (Figure 4B). The signal at 24.3 ppm is still clearly observed in the NOE difference spectrum (−18%) even when the signal at 38.6 ppm is irradiated for as short as 15 ms, suggesting that the protons giving rise to the two resonances are in very close proximity of each other (see eq 1); however, there is no NOE clearly observed on the 14.6 ppm signal under such conditions, suggesting that the 14.6 ppm proton is more distant than the 24.3 ppm proton from the 38.6 ppm proton. Alternatively, when the signal at 24.3 ppm is irradiated for

80 ms, an NOE is detected on the signals at 38.6 and 14.6 ppm (Figure 4C). When the signal at 14.6 ppm is irradiated, an NOE is clearly observed only for the signal at 24.3 ppm (Figure 4D); the absence of an effect on the signal at 38.6 ppm may be due to the much faster relaxation rate of this signal as well as the larger internuclear distance (see eq 1). Furthermore, it is worth mentioning that there is no other NOE connectivity for the resonance at 14.6 ppm with any signal in the diamagnetic region when it is saturated for 15 and 80 ms, suggesting that it has no geminal counterpart which should have shown a strong NOE. These observations strongly implicate the presence of an isolated three-spin system, like an $X-C_\beta H_2-C_\alpha H<$ spin system, on an endogenous ligand to the metal center.

We have also carried out ^1H NMR studies on the NO complexes of Fe(II)IPNS in the presence and absence of ACV. These studies can provide information about the influence of NO (and by inference, O_2) binding on the endogenous ligands in the Fe(II) site of the enzyme and complement that obtained from EPR studies on the nature of the exogenous ligands (Chen et al., 1989a,b). The ^1H NMR spectrum of Fe(II)IPNS is dramatically perturbed when NO is introduced to the enzyme; indeed, the solvent-nonexchangeable CH resonances at 42 ppm are completely eliminated (Figure 1B). The disappearance of the CH resonances may be due to either a decrease in (or even disappearance of) the hyperfine interaction between the ligand and the Fe(II) center or a dramatic increase in the relaxation rates (>5-fold) of the resonances. However, the isotropically shifted solvent-exchangeable resonances remain clearly observable. By the use of the complex $Co(NTA)^-$ as an external reference for the determination of the integrated intensities of the isotropically shifted resonances of the Fe(II)IPNS–NO complex, we find that the three coordinated His NH resonances have merged into one signal at 67 ppm with their integrated intensities unchanged upon NO binding [Fe(II)IPNS:Fe(II)IPNS–NO = 1:1.1 with respect to the external $Co(NTA)^-$ reference]. These results indicate that the proton relaxation behavior of the Fe(II)IPNS–NO complex is not dramatically different from that of Fe(II)IPNS and that the ligand bearing the CH group must experience a diminished hyperfine interaction with the Fe(II) because of a change in binding configuration or its displacement by NO.

The three NH signals due to the coordinated histidine residues and the CH resonances in Fe(II)IPNS are still clearly observed when ACV is bound to the enzyme (Figure 1C) (Ming et al., 1990). Introduction of NO to the Fe(II)IPNS–ACV complex results in the detection of only two isotropically shifted solvent-exchangeable resonances at 62.1 and 73.8 ppm with about equal intensity (Figure 1D). The integration ratio of the isotropically shifted solvent-exchangeable resonances of Fe(II)IPNS–NO:Fe(II)IPNS–ACV–NO is ~3:2.2 with respect to the external reference. The third solvent-exchangeable resonance, if present, is not clearly observed under the experimental conditions. Similar to the observations on the Fe(II)IPNS–NO complex, the CH resonances at 40–50 ppm in the Fe(II)IPNS–ACV complex also disappear upon the formation of the Fe(II)IPNS–ACV–NO complex.

Discussion

The active site metal in IPNS has been proposed on the basis of spectroscopic studies to have a distorted six-coordinate geometry with three His residues and three sites available for NO, ACV, and H_2O binding (Chen et al., 1989; Ming et al., 1990). Our present NMR results allow us to refine this working hypothesis further. The ^1H NMR spectra of Fe(II)IPNS and Co(II)IPNS show three solvent-exchangeable resonances at 60–90 ppm and two nonexchangeable features in the 30–50 ppm region. The three solvent-exchangeable features are associated with three endogenous His residues on the basis of their isotropic shifts. By comparison to model compounds, the two solvent-nonexchangeable features have isotropic shifts that may be associated with either (1) the $C_\delta H$ protons of two N_δ-coordinated His residues (Pillai et al., 1980; Lauffer et al., 1983; Wu & Kurtz, 1989; Balch et al., 1985; Goff & La Mar, 1977) or (2) the carboxymethylene protons of a Glu or an Asp residue (Borovik et al., 1990).

NOE techniques are useful for establishing proximal spatial relationships between observed resonances and allow us to distinguish between the two alternative assignments. If the 42 ppm resonances in Fe(II)IPNS were due to the $C_\delta H$ protons of N_δ-coordinated His imidazoles, the saturation of the resonances would produce NOE's on the $N_\epsilon H$ protons and also smaller NOE's, if observable, on the $C_\beta H_2$ and/or the $C_\alpha H$ protons near or in the diamagnetic region. However, if the resonances were due to the $C_\beta H_2$ (or $C_\gamma H_2$) protons of a coordinated Asp (or Glu) residue, the saturation of the resonances would produce NOE's on protons geminal and vicinal to the CH proton. In addition, a geminal pair of CH_2 protons, if resolved, would produce more sizable NOE's when one of the pair is saturated, compared to two protons in a vicinal relationship. The different NOE patterns for the different configurations would allow us to easily identify the coordinated amino acid residue giving rise to the CH resonances. The observation of significant NOE's between the two solvent-nonexchangeable resonances at 31.7 and 21 ppm in Co(II)IPNS and among the three features at 38.6, 24.3, and 14.6 ppm in the Co(II)IPNS–ACV complex eliminates the first alternative.

The clear observation of the significant NOE between the CH signals at 31.7 and 21 ppm in Co(II)IPNS (inset of Figure 3A) even with a short saturation time suggests that these two signals arise from two protons in very close proximity to each other, such as the geminal carboxymethylene protons of a coordinated Asp or Glu residue. Since there is no NOE clearly detected on other protons, this observation cannot differentiate a coordinated Asp from a coordinated Glu in the metal site of IPNS.

For the Co(II)IPNS–ACV complex, three features exhibit NOE connectivities (Figure 4). A much larger NOE is observed between the signals at 38.6 and 24.3 ppm than between the signals at 38.6 and 14.6 ppm, suggesting that the 38.6 ppm proton is closer to the 24.3 ppm proton than to the one at 14.6 ppm. The absence of further NOE connectivities between the 14.6 ppm peak and features in the diamagnetic region indicated that this trio of peaks constitutes an isolated ABX spin system with the signals at 38.6 and 24.3 ppm being due to the geminal CH_2 protons and the 14.6 ppm signal being due to the vicinal CH proton. The chemical shifts of ~20–40 ppm of the CH_2 resonances in Co(II)IPNS and its ACV complex fall in the region of Co(II) coordinated $-CH_2-COO^-$ groups, such as in the Cu-depleted Co(II)-substituted (for Zn) derivative of bovine Cu,Zn-superoxide dismutase, in which the resonances at 37 and 43 ppm have been assigned to the $C_\beta H_2$ protons of the coordinated Asp-81 in the Co(II)-binding site from NOE studies (Banci et al., 1990). We thus assign the resonances at 38.6 and 24.3 ppm to the carboxymethylene protons of a coordinated carboxylate residue in the metal site of IPNS. In our NOE studies, the absence of a geminal partner for the signal at 14.6 ppm (which should show a negative NOE of $\gtrsim$50% to its geminal partner when it is

saturated for >80 ms on the basis of its relaxation time of ≳100 ms) rules out the presence of a $-CH_2-CH_2-CH<$ moiety (of a coordinated Glu residue) and suggests that the 14.6 ppm signal is due to the $C_\alpha H$ proton of a coordinated Asp residue in the active site of IPNS. IPNS would therefore appear to have four endogenous ligands (three His and one Asp) in its native and substrate-bound states, a ligand set similar to that found for Fe superoxide dismutase (FeSOD) (Stallings et al., 1983; Stoddard et al., 1990). Our recent NOE studies of Fe(II)SOD show similar connectivities for the $C_\alpha H$ and $C_\beta H_2$ protons of the coordinated Asp-156 residue associated with resonances at 15, 19, and 24.5 ppm.[1]

NO has traditionally been used as an O_2 surrogate for probing iron proteins that interact with O_2. The binding of NO to the Fe(II) center of IPNS is expected to alter the electronic relaxation time of the complex due to the change in electronic configuration. Nevertheless, the imidazole NH protons of the coordinated His residues show relatively sharp resonances in the NO complex of Fe(II)IPNS and Fe(II)-IPNS–ACV (Figure 2B,D) with isotropic shifts similar to those found in the enzyme and its ACV complex. These observations suggest that the iron center in the NO complexes of IPNS retains much of its Fe(II) character. The disappearance of the CH proton resonances near 40 ppm upon NO binding and the retention of the imidazole NH protons support the conclusion derived from NOE experiments that the CH resonances do not arise from the $C_\delta H$ protons of N_δ-coordinated histidine residues. The $N_\epsilon H$ and $C_\delta H$ resonances of such residues would be expected to be similarly affected by NO binding to Fe(II)IPNS because the $C_\delta H$ and the $N_\epsilon H$ protons (both beta to the N_δ nitrogen) would have very similar distances to the metal center and show very similar line widths as observed in many metalloproteins (Bertini & Luchinat, 1986). The effect of NO binding further demonstrates that the CH resonances near 40 ppm must derive from a residue distinct from the histidines.

The disappearance of the isotropically shifted CH resonances in the ^1H NMR spectrum of the Fe(II)IPNS–NO complex suggests that (a) the coordinated Asp residue is detached from the Fe(II) site of IPNS upon NO binding or (b) a change in the configuration of the Asp residue occurs that decreases spin delocalization onto the CH protons. It is difficult to rationalize the displacement of a carboxylate residue by NO in an active site where the carboxylate is the only negatively charged endogenous ligand, particularly when more readily displaced water ligands are available; so we favor the latter alternative. For a carboxylate, a change from syn to the less basic anti lone pair [$\sim 10^4$-fold difference in basicity (Gandour, 1981; Carrell et al., 1988; Li & Houk, 1989)] for binding to the metal center would weaken the Fe–O (carboxylate) bond and decrease the unpaired spin density delocalized onto the carboxylate moiety, resulting in significantly diminished isotropic shifts. A possible trigger for this putative syn-to-anti conversion is the displacement by NO of a water ligand that is hydrogen bonded to the syn-bound carboxylate (Figure 5A,C), since water molecules have been demonstrated to hydrogen bond to the carboxylate C=O moiety of the carboxylate-coordinated metal sites in proteins (Chakrabarti, 1990; Carrell et al., 1988). Alternatively, NO (or O_2) binding may induce a protein conformational change that causes the syn-to-anti conversion.

Similar to the results for the Fe(II)IPNS–NO complex mentioned above, our NMR studies also suggest that the

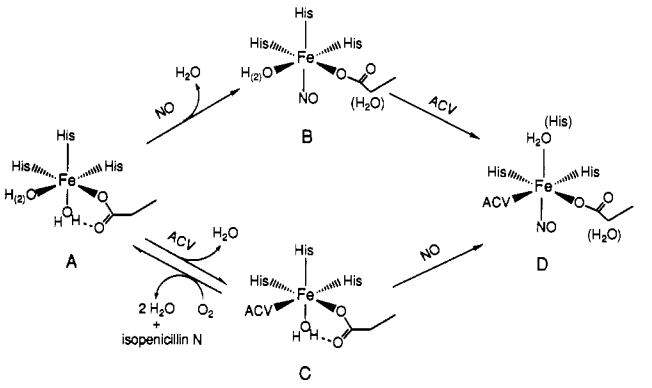

FIGURE 5: Changes in the active site of IPNS upon addition of substrate and NO on the basis of spectroscopic studies. (A) The presence of three endogenous His residues and one Asp residue is revealed by NMR studies. The Asp residue with a syn configuration may be further stabilized via a hydrogen bonding to a coordinated water molecule. The presence of another water molecule with a relatively low pK_a value can facilitate ACV binding by neutralizing the cysteinyl-SH proton. (B) The Asp residue may change its configuration from syn to anti or, less preferably, might be displaced upon NO binding. (C) The introduction of ACV to the enzyme causes a slight change in the ^1H NMR spectrum, suggesting that the endogenous ligands may retain their configuration in the Fe(II)-IPNS–ACV complex. (D) Similar to that in panel B, the configuration of the coordinated Asp may change upon NO binding to the Fe(II)IPNS–ACV complex with one of the coordinated His residues being detached. An alternative, but less preferable, situation would be that the Asp is displaced with all the three His residues remaining coordinated to the Fe(II) in the Fe(II)IPNS–ACV–NO complex.

coordinated Asp residue in the Fe(II)IPNS–ACV complex is dramatically perturbed by NO binding, resulting in the proposed configurational change of the coordinated Asp. Furthermore, the detection of only two isotropically shifted solvent-exchangeable resonances in the Fe(II)IPNS–ACV–NO complex suggests that one of the endogenous His residues in the Fe(II) site of IPNS is significantly affected upon the binding of both ACV and NO. Because of the NMR spectrum of Fe(II)IPNS is not as significantly affected by ACV binding as by NO binding (Figure 1), we conclude here that NO affords further significant perturbation on the configuration of the metal site in the Fe(II)IPNS–ACV–NO complex. Dramatic effects of NO binding have been observed previously on heme proteins, such as hemoglobin in which the binding of NO to the heme center of the T-state tetramer displaces the axially coordinated proximal His from the heme iron center (Magliozzo et al., 1987; Höhn et al., 1983; Hille et al., 1979). The displacement of an endogenous His ligand in the metal site of non-heme metalloproteins upon exogenous ligand binding is also precedented, as in Cu,Zn-superoxide dismutase upon anion (N_3^-, CN^-, etc.) binding (Bertini et al., 1985) and in protocatechuate 3,4-dioxygenase upon homoprotocatechuic acid binding (True et al., 1990). The disappearance of one NH resonance in the Fe(II)IPNS–ACV–NO complex can imply the displacement of a coordinated His residue upon NO binding, likely to be the one trans to NO; alternatively, a significant increase in the solvent-exchange rate (causing substantial line broadening) of this imidazole NH proton can also explain the observation (Bertini et al., 1985). However, EPR studies of the Fe(II)IPNS–ACV–NO complex in $^{17}OH_2$ buffer suggests the presence of coordinated water in addition to the two other exogenous ligands, ACV and NO (Chen et al., 1989a,b). Thus at least one of the four endogenous ligands must be displaced to maintain a coordination number of 6 or smaller. The NMR results on Fe(II)IPNS–ACV–NO suggest that the ligand displaced is either a His or the Asp residue. We favor His displacement on the basis of precedents in other

[1] L.-J. Ming, J. B. Lynch, and L. Que Jr., unpublished observations.

metalloprotein systems and on the argument that it is thermodynamically easier for NO to displace a neutral ligand than a negatively charged one. The presence of both the Asp carboxylate and the ACV thiolate in the Fe(II) site would be expected to lower the Fe(III/II) redox potential sufficiently as to allow O_2 binding and its subsequent activation. The scheme in Figure 5 developed on the basis of our spectroscopic studies represents but the first step in understanding the roles the iron center and its ligands play in the IPNS mechanism; further studies are necessary to substantiate this hypothesis.

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Registry No. IPNS, 78642-31-6; ACV, 32467-88-2; His, 71-00-1; Asp, 56-84-8; Fe, 7439-89-6; NO, 10102-43-9; H_2O, 7732-18-5.

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