Solid-State Deuterium NMR of Imidazole Ligands in Cytochrome *c* Peroxidase

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Received September 15, 1997

Abstract: We have measured hyperfine shifted NMR signals associated with deuterated imidazole bound to the high-spin Fe^{3+} state of cytochrome *c* peroxidase using deuterium magic angle spinning solid-state NMR. These experiments were performed on a mutant of cytochrome *c* peroxidase, CcP(H175G), for which replacement of the proximal histidine with glycine produced a cavity that can bind a variety of substituted imidazoles including imidazole or 2-methylimidazole. The mutant with imidazole bound is inactive; specifically its reaction with H_2O_2 is blocked. We observed deuterium NMR signals from the methyl-d_3 group of the perdeuterated 2-MeIm sample bound to H175G CcP. The signal displayed an upfield chemical shift and exhibited non-Curie temperature dependence, indicating the existence of low-lying excited electronic states. Upon introducing a nondeuterated competitive ligand, imidazole, a decrease in the intensity of this signal was detected, consistent with the assignment of the deuterium signal to the *bound* 2-methylimidazole in the solid state. The tensor of the deuterium static line shape indicates the methyl group on the untethered imidazole ring undergoes rather unhindered motion while the entire ring has relatively limited motion. No evidence for intermediate ring flipping exchange dynamics nor for large angular librations of the ring is observed. Thus the absence of enzymatic activity in the mutant is unlikely to result from excessive dynamic disorder of the untethered imidazole.

Introduction

Heme-containing proteins exist widely in plants, animals, and bacterial organisms typically functioning as oxygen carriers or electron-transfer mediators. Cytochrome *c* peroxidase (CcP) is in a smaller category of heme proteins that catalyze oxygen chemistry, along with cytochrome P-450 and catalase. The physiological function of CcP is to catalyze the hydrogen peroxide oxidation of ferrocytochrome *c*,[1] as described in Scheme 1.[2,3] CcP(Fe^{4+})$^{\bullet}$ or compound **I** contains two paramagnetic species, the oxy ferryl cation radical $(Fe{=}O)^{+\bullet}$ and the free radical, Tryptophan 191 (Trp$^{\bullet}$). The proximal ligands for these enzymes, CcP, catalase, and cytochrome P-450, are histidine, tyrosine, and cysteine, respectively. In each case, the proximal ligand is assumed to play a key role in the catalytic reaction as well as the binding of molecular oxygen.[4] One mechanism for heme enzymes that use hydrogen peroxide as an oxidant proposes that the proximal ligand "pushes" electron density into the hydrogen peroxide through iron(III), thereby weakening the O−O bond and facilitating its cleavage to give an oxyferryl porphyrin cation radical (compound **I**). This electron-donation effect has been observed in a model compound study on heterolytic and homolytic O−O bond cleavage steps of acylperoxoiron(III) porphyrin complexes.[5] A recent study on CcP shows that changing the proximal ligand has important effects on the activity of the enzyme.[6]

Scheme 1

I) $CcP(Fe^{3+}/H.S.) + H_2O_2 \dashrightarrow CcP(Fe^{4+}O)/Trp^{\bullet+}$ (compound I) $+ H_2O$

II) $CcP(Fe^{4+}O)/Trp^{\bullet+} + 2H^+$ 2 cyt c $(Fe^{2+}) \dashrightarrow CcP(Fe^{3+}/H.S.) + H_2O$ +2 cyt c(Fe^{3+})

A cavity was created in CcP by substituting the proximal histidine with glycine (H175G). Imidazole and substituted imidazoles could be introduced into this cavity of the binding pocket in place of the missing histidine by soaking protocols (Figure 1). The X-ray structure of CcP(H175)/Im complex (protein databank accession code, 1ccg) shows that the imidazole occupies the same position as the histidine ring in the wild type and that there was little deviation in the protein structure (ca. 0.14 Å in the backbone and heme, and 0.4 Å for all heavy atoms, and ca. 0.4 Å for the imidazole heavy atoms). Interestingly, the CcP mutant is bereft of activity even if imidazole is coordinated to the high-spin iron(III) at the proximal side. An oxyferryl species coupled to an organic radical similar to compound **I** has been generated, although more slowly than for the wild type, and has been studied spectroscopically.[7] The

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(1) Nicholls, P.; Mochan, E. *Biochem. J.* **1971**, *121*, 55−67.
(2) Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1980**, *255*, 8199−8205.
(3) Poulos, T. L.; Finzel, B. C. *Pept. Protein Rev.* **1984**, *4*, 115−171.
(4) Dawson, J. H.; Holm, R. H.; Trudell, J. R.; Barth, G.; Linder, R. E.; Bunnenberg, E.; Djerassi, C.; Tang, S. C. *J. Am. Chem. Soc.* **1976**, *98*, 3707−3709.
(5) Yamaguchi, K.; Watanabe, Y.; Morishima, I. *J. Am. Chem. Soc.* **1993**, *115*, 4058−4065.

S0002-7863(97)03238-1 CCC: $15.00
Published on Web 09/16/1998

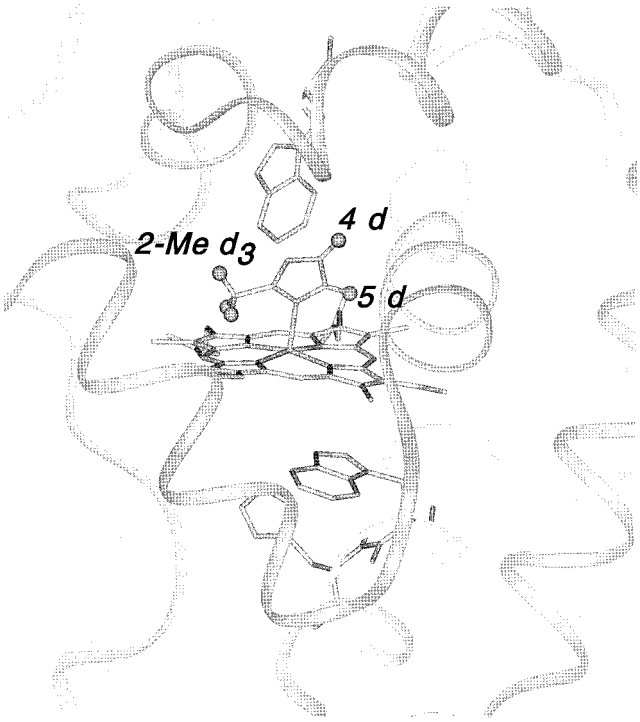

Figure 1. A proposed structure of CcP(H175G) cavity mutant and its 2-methylimidazole complex is based upon the structure of imidazole bound to this mutant, which is deposited with a protein databank accession code of 1ccg. Our figure depicts a structure that differs from that in the databank in that we built a 2-methyl group onto the imidazole. For 1ccg imidazole, ligation to the heme is very similar to that of the wild-type histidine ligand: the backbone RMS differences were 0.14 and the heme heavy atom differences were 0.4 Å. Direct ligation of the 2-MeIm to the heme was indicated by the perturbation of the UV/vis spectrum upon soaking of the ligand into the microcrystals.

absence of activity upon replacing the endogenous histidine ligand with an untethered imidazole presumably relates to altered energetics or conformation of these oxidized intermediates, which in turn alters the pathway or efficiency of its reaction with H_2O_2. The altered conformation or energetics in compound **I** could involve modified hydrogen bond strength to Asp-235, differences in the motional dynamics of the untethered ligand, or changes in electronic coupling due to the loss of covalent linkage between the heme and the protein. We address the hypothesis that the motion in the imidazole is different from the histidine using solid-state NMR methods, using CcP(H175G) mutant and isotopically labeled exogenous ligands.

Solution NMR has been widely used in the studies of both heme model compounds[8] and heme proteins.[9] The unpaired electrons of the iron can give rise to large paramagnetic chemical shifts of the nuclei in the porphyrin, making the peak assignments and determination of electron density distribution at the active site possible. In contrast to solution NMR, solid-state NMR studies of paramagnetic active sites of metalloproteins have been largely unexplored, mainly because of the assumption that signals would be difficult to detect. However, a recent solid-state NMR study of the ligands of paramagnetic small molecules shows that deuterium NMR signals at the vicinity of paramagnetic centers are generally detectable for a variety of metal electronic spin states, including some with long electron T_1 values.[10,15] The resolution of these signals was sufficient to reveal subtle distortions of the local structure (e.g., Jahn−Teller distortions undetected by X-ray crystallography). NMR signals from nuclei close to the paramagnetic ion were found to be either broad or undetectable in solution, and possible explanations have been addressed.[10] For example, the resting state of CcP contains a pentacoordinated high-spin iron(III) that is a relatively difficult species for solution NMR studies.[12] Line-broadening due to Curie relaxation becomes significant for species with molecular weight above 30 kDa, particularly for species with high magnetic moments; furthermore, species with relatively long electron T_1 relaxation times exhibit particularly broad lines.[13] On the other hand, these disadvantages can be less severe for solid-state NMR. According to our studies,[10] deuterium from paramagnetic species can generally display narrow lines; combining the advantages of narrow lines and short nuclear T_1 values, deuterium NMR should have reasonable sensitivity for the protein studies. In addition, solid-state NMR data can offer rich information about motional dynamics.[10,11,14] Here we report a solid-state NMR study of Im bound to the high-spin iron(III) and 2-MeIm, cavity bound in the CcP-(H175G) mutant. We hope not only to offer data on the motional dynamics of the untethered imidazole, but also to demonstrate that solid-state NMR has sufficient resolution and sensitivity to be used as a tool to study the structure and mechanisms of metalloproteins, including spin states that typically create difficulties for NMR spectroscopy (i.e. with large magnetic moments and slow electron spin−lattice relaxation rates).

Experimental Section

Deuterated Im and Im Derivatives. Imidazole dissolved in D_2O with a catalytic amount of D_2SO_4 was placed in a sealed ace pressure tube and heated for 4 h at 200 °C.[16] After two such exchanges, 90% deuterium enrichment at Im position 2, 4, and 5 occurred. The deuteron at position 2 was exchanged for a proton in a large excess of water overnight. A similar procedure starting with 2-MeIm was used in making 2-MeIm-*2,2,2,4,5*-d_5. The deuterium content was measured by mass spectroscopy and shown to be better than 90% for all compounds. Im-$d_{4,5}$ was purified by repeated crystallization in benzene. 2-MeIm was purified by fractional distillation under reduced pressure.

CcP Cavity Mutant. The H175G mutant protein was initially purified and crystallized in the presence of 10 mM Im; the protein was more stable as the imidazole complex. CcP is largely insoluble at low ionic strength, and Im can be bound to the bis-aquo protein by soaking crystals in ligand-containing solutions.[7] Thus exchange of the isoto-

(6) Choudhury, K.; Sundaramoorthy, M.; Hickman, A.; Yonetani, T.; Woehl, E.; Dunn, M. F.; Poulos, T. L. *J. Biol. Chem.* **1994**, *269*, 20239−20249.

(7) McRee, D. E.; Jensen, G. M.; Fitzgerald, M. M.; Siegel, H. A.; Goodin, D. B. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12847−51.

(8) Walker, F. A.; Simonis, U. In *NMR of Paramagnetic Molecules*; Berliner, L. J., Reuben, J., Eds.; Plenum Press: New York, 1993; Vol. 12; pp 133−265.

(9) Satterlee, J. D.; Alam, S.; Yi, Q.; Erman, J. E.; Constantinidis, I.; Russell, D. J.; Moench, S. J. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum Press: New York, 1993; Vol. 12; pp 275−297.

(10) Liu, K.; Ryan, D.; Nakanishi, K.; McDermott, A. *J. Am. Chem. Soc.* **1995**, *117*, 6897−6906.

(11) Lin, T.-H.; DiNatale, J. A.; Vold, R. R. *J. Am. Chem. Soc.* **1994**, *116*, 2133-2134.

(12) Satterlee, J. D.; Erman, J. E.; LaMar, G. N.; Smith, K. M.; Langry. K. C. B.B.A. 743 246−255, 1983. Budd et al. *J. Am. Chem. Soc.* **1979**, *101*, 6091−6096.

(13) Banci, L.; Bertini, I.; Luchinat, C. *Nuclear and Electron Relaxation, the magnetic nucleus-unpaired electron coupling in solution*; VCH Publishers: New York, 1991.

(14) Wittebort, R. J.; Olejniczak, E. T.; Griffin, R. G. *J. Chem. Phys.* **1987**, *86*, 5411−5420.

(15) Prosser, R. S.; Hunt, S. A.; DiNatale, J. A.; Vold, R. R. *J. Am. Chem. Soc.* **1996**, *118*, 269−270.

(16) Harris, T. M.; Randall, J. C. *Chem. Ind.* **1965**, *October 9*, 1728−1729.

pically labeled ligands into the H175G/Im crystals was achieved by soaking the crystals in low ionic strength buffers containing the labeled Im.

CcP(H175G)/2-MeIm-*2,2,2,4,5-d*$_5$ Complex. To reduce background NMR signals from natural abundance deuterium, deuterium depleted water was used in making the buffers. The mutant CcP protein microcrystal, which had an unlabeled Im bound to the high-spin iron(III), was soaked in deuterium-depleted water and allowed to stand for about 4 h at 4 °C to "wash out" exchangeable residual deuterium in the macromolecules; the process was repeated three times before deuterium-depleted water was replaced by 4 mM deuterated 2-MeIm solution. The suspension of protein crystals in the deuterated 2-MeIm solution was allowed to stand overnight, then was replaced with fresh solution three times to allow the exchange of deuterium-labeled 2-MeIm with nondeuterated bound imidazole to occur. All manipulations were carried out at 4 °C with solutions maintained at pH 6 adjusted by a small amount of phosphoric acid. Throughout the procedure, the protein crystals were kept at low salt concentration to prevent the protein crystals from dissolving.

Solid-State NMR Experiments. NMR spectra were recorded on a Chemagnetics 400 CMX spectrometer with a Larmor frequency for deuterium of 60.86 MHz. A Chemagnetics double resonance probe equipped with a 5 mm zirconium rotor was used. The spectra were collected by using a Block decay with a 0.1 s recycle delay. The use of such a short recycle delay was based on our previous solid-state NMR measurements of deuterium T_1 values in ligands of paramagnetic centers, which were typically several tens of milliseconds, and also on previous solution NMR measurements of spin-lattice relaxation time for protons in the high-spin ferric heme which are typically several milliseconds.[9] Use of such rapid relaxation delays helps to obtain spectra from proteins with sufficient signal-to-noise ratio within a reasonable period of time. The 90° pulse length was 3.2 μs and the data sampling rate was 1 MHz. Typically each spectrum was signal averaged for 12 to 24 h corresponding to 4×10^5 and 8×10^5 scans. The typical amount of protein used for NMR experiments was 50 mg.

To demonstrate the origin of the solid-state deuterium signal, we also introduced unlabeled imidazole as a competitive ligand into the same NMR sample rotor which contained the protein−deuterated imidazole complex. We compared the signal-to-noise ratios of the spectra from the two samples, which were signal averaged under the same instrumental conditions and over the same number of scans.

Data Processing. Before Fourier transformation, we applied a periodic filter, which reduces the amount of noise between the rotor echoes by changing the effective dwell value and audio-filter as a function of rotor position. A number of data points were deleted so that the free induction decay would start at the top of a rotor echo to avoid phase distortion after Fourier transformation. Due to a large background signal and probe ringing, our truncated free induction decay usually begins with the second or third rotor echo which occurs at approximately 200 μs. This causes a loss of signal amplitude but apparently no significant distortion of the line shape. The signal losses are significant, however. T_2 values were estimated according to the relation $T_2 = 1/\pi\Delta\sigma_{1/2}$ (where $\Delta\sigma_{1/2}$ is the full-width-half-height line width) and using the 14 ppm centerband line width: T_2 is 379 μs. If the intensities of the echoes can be estimated by using the expression $I_t = I_0 \cdot e^{(-n\tau_r/T_2)}$, where I_t and I_0 are the amplitudes of magnization at time t and time zero, n is the number of the rotor echo, and τ_r is the rotor period, then intensity is reduced to $0.68I_0$, $0.47I_0$, and $0.32I_0$ at the second, third, and fourth echoes, respectively. Thus it is crucial to reduce probe related artifacts so that the first rotor echo is distortionless.

Since the center peak of the solid-state deuterium signal is sometimes buried under the broad baseline of the free imidazole in solution, we extrapolated the isotropic chemical shift of the solid-state deuterium NMR signal from a linear fit of the spinning sideband positions, using data taken at two or more spinning speeds.

Results and Discussion

Deuterium solid-state NMR spectra of the perdeuterated 2-MeIm and Im-*4,5-d*$_2$ bound to CcP(H175G), occupying the site normally occupied by histidine 175 in the wild-type enzyme

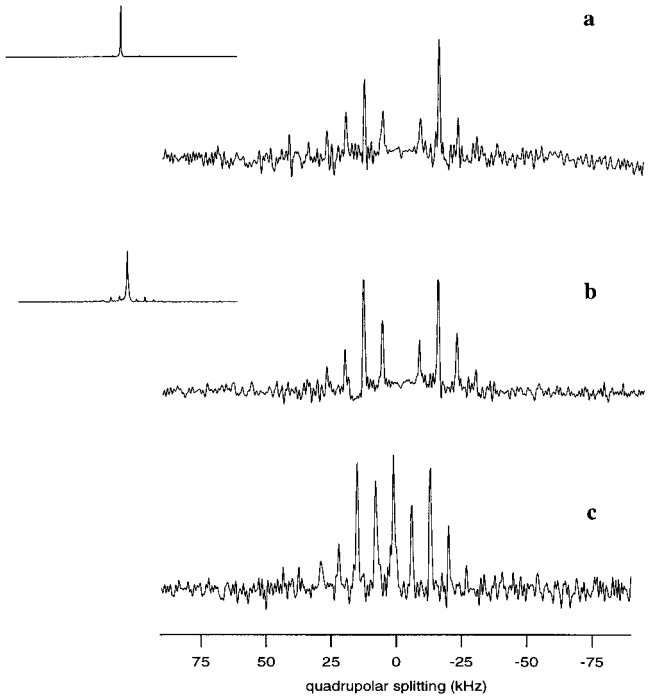

Figure 2. Deuterium solid-state magic angle spinning NMR spectra of predeuterated 2-MeIm bound to high-spin iron(III) CcP(H175G) mutant crystals. The spinning speed was 7000 Hz and the temperature was −10, −20, and −43 °C for panel a, b, and c, respectively. The number of scans for panel a, b, and c were 7.2×10^5, 1.0×10^6, and 5.6×10^5, respectively. The truncated free induction decay starts at the third rotor echo, which occurred at 284 μs. We applied a noise filtering function, deconvolution at the frequency of the solvent, and 100 Hz linebroadening before the Fourier transformation. The small insets for parts a and b are processed without deconvolution.

(see Figure 1), exhibit a spinning sideband intensity pattern typical for a methyl group, with a total line width of 40 kHz, for temperatures of −10, −20, and −43 °C (Figure 2, panels a, b, and c). We also see a single peak at −2 ppm that apparently arises from a liquid free species. The spectra recorded at different spinning speeds indicate that the center peak of the solid-state signal is buried under the broad base of the solvent peak. Thus, we determined the isotropic chemical shift of the solid state signal by a linear fit of the spinning sidebands. The chemical shifts from the linear fit are upfield shifted at all three temperatures, and show an appreciable non-Curie temperature dependence as shown in Figure 3, while the peak presumably arising from a solution species exhibits a temperature independent shift in the diamagnetic chemical shift region.

When we lowered the temperature from −10 °C to −20 °C and then to −43 °C, the solution signal from the unbound ligands exhibited a reduction in amplitude. At no temperature do we observe a resolved solid-state signal attributed to the unbound ligand in frozen solution. Instead we observe an appreciable increase in the line width of the solid state, bound, species. The chemical shift of the bound signal is around −2 ppm at −43 °C and the half-height line width is around 10 ppm, and so the deuterium signal from the frozen solution at −2.9 ppm partly overlaps with the signal from the bound state species (see Figure 2). The recycle delay we used was 0.1 s, presumably shorter than the T_1 values for the signal of the unbound form; correspondingly, any solid-state signal of diamagnetic species would presumably be saturated.

To demonstrate the origin of the upfield shifted solid-state signal, we also introduced 30 μL of a 4 mM solution of nondeuterated imidazole as a competitive ligand to the same

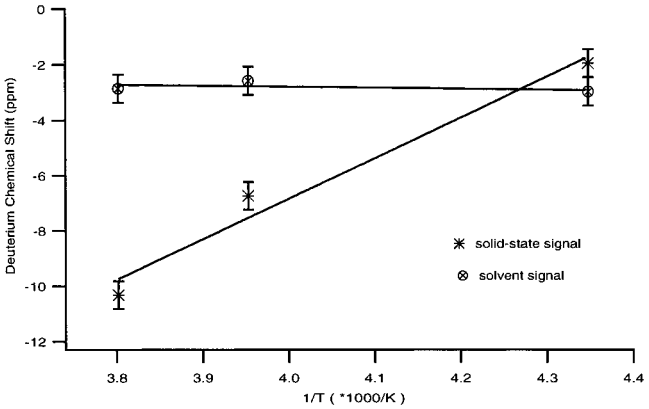

Figure 3. Chemical shift values of the center peak are plotted versus the inverse of the absolute temperatures. The centerband positions for the solid state MAS signal were obtained from the linear fit of spinning sideband frequencies; and that of the single peak we assume comes from the mother liquor was read directly from the spectra. The variation of the shift of the solid-state species (cavity bound ligand) exhibits anti-Curie behavior. The shift of the solution species is invariant over temperature, indicative of a diamagnetic environment.

NMR rotor that contains the same sample. This addition resulted in a 30∼40% dilution of the deuterated 2-MeIm. With the same amount of protein and the same number of scans, we observed an ∼40% decrease in the intensity of the signal. This result, together with the upfield chemical shift and the temperature dependence of the chemical shifts, confirms the assignment of the NMR signal to the methyl groups of the *bound* deuterated 2-MeIm. If the signal were associated with weak-binding nonsaturable sites, then the singal intensity would have been invariant under the addition of unlabeled ligand.

The NMR signal of the protons on the porphyrin ring of heme with high-spin iron(III) has been observed with a downfield shift in solution.[17,18] It is very likely that the protons or deuteron on the proximal imidazole ring will also show downfield shifts, because the dominant mechanisms are probably through-bond contact shifts in both cases. The non-Curie temperature dependence of the chemical shift indicates the existence of low-lying excited electronic states with the energy difference from the ground state comparable to the electron Zeeman term. Non-Curie behavior has also been suggested in another solution NMR study of a CcP mutant.[19−21]

Although the 2-MeIm is also deuterated at the 4 and 5 positions on the imidazole ring, we did not observe additional signals associated with these sites in these spectra. Detection of these deuterons is compromised by the stoichiometry: two distinct deuterons for the ring versus three equivalent deuterons for the methyl group. The ring deuterons also have a larger effective quadrupolar coupling because they are presumably static, while the 3-site hop motion of the methyl deuterons averages the quadrupolar coupling to one-third of its static value, resulting in more signal in a few central spinning sidebands. We observed a signal from an imidazole-*4,5-d*$_2$ sample prepared in a similar manner, but containing more sample and with longer signal averaging. The intensity pattern was typical of the rigid methylene group (qcc $\sim$ 170 kHz). The center peak determined from the spinning speed dependence showed a downfield chemical shift around −1 ppm, with flat temperature dependence at −45 and −20 °C. We also attempted but failed to identify the deuterium signals associated with deuterated histidine incorporated into the protein. These deuterons would also have inherently low sensitivity. It is important to note that imidazole is known to have another binding mode to the protein at higher pH values, which differs from that exhibited by the 1ccg structure by much weaker ligation to the heme: 2.4 Å Fe−N distance as contrasted with the 2.1 Å distance in 1ccg. We presume that our structure is analogous to the 1ccg not only because of the pH at which it was prepared, but also because the UV spectrum resembled that of a bis imidazole coordinated heme (strong absorption in the blue).

In summary, the deuterium spinning sideband pattern of the bound 2-MeIm spectra at all three temperatures clearly indicates that the CD_3 group undergoes a 3-fold hop with a rate greater than 10^9 s^{-1} while the imidazole ring lacks other large angular motions. It is of interest to determine the protein interactions which inhibit free rotation of the imidazole, and how these compare to those of the wild type. We presume that the imidazole is fixed through the Fe−N coordination and the hydrogen bonding of the N-3 to the side chain of aspartic acid 235, which is very little displaced in the im-bound cavity mutant as compared with the his-containing wild type. In conclusion, these data rule out a disordered state for the "untethered" imidazole.

Acknowledgment. This work was supported by NIH Grants GM 49964 (to A.E.M.) and GM 48495 and GM 41049 (to D.B.G.). A.E.M. would like to acknowledge support from RC Cottrell CS 0046 and Kanagawa Academy of Science and Technology.

JA9732385

(17) La Mar, G. N.; de Ropp, J. S.; Smith, K. M.; Langry, K. C. *J. Biol. Chem.* **1980**, *255*, 6646−6652.

(18) Clark, K.; Dugad, L. B.; Bartsch, R. G.; Cusanovich, M. A.; La Mar, G. N. *J. Am. Chem. Soc.* **1996**, *118*, 4654−4664.

(19) de Ropp, J. S.; Mandal, P.; Brauer, S. L.; La Mar, G. N. *J. Am. Chem. Soc.* **1997**, *119*, 4732−4739.

(20) Schonbaum, G. R. *J. Biol. Chem.* **1973**, *248*, 502−511.

(21) La Mar, G. N.; Jackson, T.; Dugad, L. B.; Cusanovich, M. A.; Bartsch, R. G. *J. Biol. Chem.* **1990**, *265*, 16173−16180.