J|A|C|S
ARTICLES
Published on Web 08/12/2006

Evidence for Basic Ferryls in Cytochromes P450

Rachel K. Behan,[†] Lee M. Hoffart,[‡] Kari L. Stone,[†] Carsten Krebs,[†,‡] and Michael T. Green*[,†]

Department of Chemistry and Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received April 8, 2006; E-mail: mtg10@psu.edu

Abstract: Using a combination of Mössbauer spectroscopy and density functional calculations, we have determined that the ferryl forms of $P450_{BM3}$ and P450cam are protonated at physiological pH. Density functional calculations were performed on large active-site models of these enzymes to determine the theoretical Mössbauer parameters for the ferryl and protonated ferryl ($Fe^{IV}OH$) species. These calculations revealed a significant enlargement of the quadrupole splitting parameter upon protonation of the ferryl unit. The calculated quadrupole splittings for the protonated and unprotonated ferryl forms of $P450_{BM3}$ are $\Delta E_Q = 2.17$ mm/s and $\Delta E_Q = 1.05$ mm/s, respectively. For P450cam, they are $\Delta E_Q = 1.84$ mm/s and $\Delta E_Q = 0.66$ mm/s, respectively. The experimentally determined quadrupole splittings ($P450_{BM3}$, $\Delta E_Q = 2.16$ mm/s; P450cam, $\Delta E_Q = 2.06$ mm/s) are in good agreement with the values calculated for the protonated forms of the enzymes. Our results suggest that basic ferryls are a natural consequence of thiolate-ligated hemes.

Introduction

The role of the axial-thiolate ligand in cytochromes P450 has been a subject of debate. It is thought that the thiolate's main function lies in the activation of dioxygen for reaction.[1] In this role, the thiolate serves as a strong internal electron donor, facilitating cleavage of the dioxygen bond.[2] Evidence in support of this proposal comes from the enhanced basicity (relative to analogous myoglobin adducts) of ligands bound trans to the endogenous cysteinate of P450.[3−5] The inability of axial-ligand mutants of P450 to catalyze peroxide-driven hydroxylations, however, suggests that the thiolate's importance may extend beyond the generation of high-valent iron species.[6, 7]

X-ray absorption measurements indicate that the thiolate-ligated Fe(IV)oxo (ferryl) species in chloroperoxidase compound II (CPO-II) is basic.[8] The importance of this result lies in its connection to P450 chemistry. In the consensus P450 hydroxylation mechanism, compound I (a ferryl-radical species) abstracts hydrogen from substrate to form a protonated ferryl (similar to CPO-II), which rapidly recombines with substrate to yield hydroxylated product. Evidence suggests that the ability of metal oxos to abstract hydrogen scales with the strength of the O−H bond formed during H-atom abstraction.[9−11] In heme proteins, the strength of this O−H bond is determined by the one-electron reduction potential of compound I and the pK_a of compound II (eq 1).

$$\mathrm{D(O{-}H)} = 23.06E^0_{\mathrm{cmpd-I}} + 1.37\mathrm{p}K_{\mathrm{a\ cmpd-II}} + 57 \pm 2\ \mathrm{(kcal/mol)} \quad (1)$$

This equation highlights the importance of the ferryl pK_a and suggests an additional role for thiolate ligation in cytochromes P450, namely, to promote hydrogen abstraction at biologically viable compound I reduction potentials. This hypothesis rests upon two important assumptions: (1) that the rebound mechanism is operative in P450 hydroxylations, and (2) that basic ferryls are a general and unique feature of thiolate-ligated hemes. The first of these assumptions appears well-founded, as experimental and theoretical investigations continue to support the rebound mechanism.[12,13] The merit of the second assumption is not as clear, for high-valent ferryl species are generally thought to be electrophilic in nature.

To confirm the basic character of the thiolate-ligated ferryl in CPO, we recently examined CPO-II using a combination of density functional calculations and Mössbauer spectroscopy. The Mössbauer spectrum of CPO-II showed the presence of two distinct ferryl species in a 70:30 ratio. Calculated Mössbauer parameters and the previous EXAFS investigation allowed us to assign the major component of CPO-II as an iron(IV) hydroxide.[14] Importantly, our results suggest that density

[†] Department of Chemistry.
[‡] Department of Biochemistry and Molecular Biology.

(1) Dawson, J. H. *Science* **1988**, *240*, 433.
(2) 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.
(3) Sono, M.; Dawson, J. H. *J. Biol. Chem.* **1982**, *257*, 5496.
(4) Dawson, J. H.; Sono, M. *Chem. Rev.* **1987**, *87*, 1255.
(5) Liu, H. I.; Sono, M.; Kadkhodayan, S.; Hager, L. P.; Hedman, B.; Hodgson, K. O.; Dawson, J. H. *J. Biol. Chem.* **1995**, *270*, 10544.
(6) Vatsis, K. P.; Peng, H. M.; Coon, M. J. *J. Inorg. Biochem.* **2002**, *91*, 542.
(7) Auclair, K.; Moenne-Loccoz, P.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **2001**, *123*, 4877.
(8) Green, M. T.; Dawson, J. H.; Gray, H. B. *Science* **2004**, *304*, 1653.
(9) Mayer, J. M. *Acc. Chem. Res.* **1998**, *31*, 441.
(10) Gardner, K. A., Mayer, J. M. *Science* **1995**, *269*, 1849.
(11) Gardner, K. A., Kuehnert, L., Mayer, J. M. *Inorg. Chem.* **1997**, *36*, 2069.
(12) Hoffman, B. M. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3575.
(13) Meunier, B.; de Visser, S. P.; Shaik, S. *Chem. Rev.* **2004**, *104*, 3947.
(14) Stone, K. L.; Hoffart, L. M.; Behan, R. K.; Krebs, C.; Green, M. T. *J. Am. Chem. Soc.* **2006**, *128*, 6147.

functional theory (DFT) can be used in combination with Mössbauer spectroscopy to determine the protonation state of ferryl intermediates.

Our findings for CPO-II are suggestive, but CPO is not a P450. Chloroperoxidase cannot hydroxylate unactivated C−H bonds,[15,16] and P450 does not chlorinate.[17] P450 utilizes dioxygen,[17] while CPO cannot.[15,16,18] The ferryl forms of CPO and P450 show very different reactivities. Although CPO compound I can be prepared and spectroscopically characterized in high yield (90%),[19,20] a thorough spectroscopic characterization of P450 compound I is lacking.[21−24] Likewise, CPO-II is at least an order of magnitude more stable than P450-II.[24−26] Thus, it is clear that the properties of CPO do not always map onto P450, and it cannot be assumed that the ferryl form of P450 is basic.

Importantly, the UV/visible absorption spectra of CPO-II and P450-II have been suggested to be distinctly different. A stopped-flow spectrophotometric study of the reaction of P450 with peracids has assigned a 406 nm absorbance to the ferryl form of P450,[24] while the absorption spectrum of CPO-II contains a split Soret band with maxima at 367 nm and 438 nm.[26] If correct, the 32-nm shift in the Soret band would imply significant differences in the structures of CPO-II and P450-II. It has been suggested that the 406 nm absorption is indicative of an authentic iron(IV)oxo species (i.e., unprotonated P450-II).[24]

To determine if the ferryl form of P450 is best described as an iron(IV) hydroxide or an authentic iron(IV)-oxo species, we have performed density functional calculations and Mössbauer experiments on the ferryl forms of $P450_{BM3}$ and P450cam at physiological pH. Here we report the results of those experiments. We find that both $P450_{BM3}$-II and P450cam-II have Mössbauer parameters that are characteristic of protonated ferryl hemes. Our results suggest that basic ferryls are a natural consequence of thiolate-ligated hemes.

Computational Methods

Theoretical Mössbauer parameters were determined by performing DFT calculations on large active-site models of the protonated and unprotonated ferryl forms of P450cam and $P450_{BM3}$. In each case, the starting structures were taken from available crystal structures of ferric $P450_{BM3}$ and P450cam. The models employed contained a porphine, the appropriate distal ligand, and a portion of the proximal helix (86 and 80 atoms, respectively, for the ferryl species).[27,28] Inclusion of the proximal helix allows for the consideration of important hydrogen bonding between the helix and the axial-thiolate, while the geometry constraints allow us to examine structures that more closely resemble those found in the enzyme. The helix in the $P450_{BM3}$ model contained Cys400−Gln403. The P450cam model contained Cys357−Gln360. In both cases, all residues except Cys and Gly were converted to Ala.

Table 1. Iron-Ligand Distances and Spin Densities from DFT Calculations (B3LYP/6-311G)

			distances (Å)			spin densities		
protein	distal ligand	oxidation/ spin state	Fe–N (avg)	Fe–S	Fe–O(H)	Fe	O(H)	S
$P450_{BM3}$	OH^-	IV (S = 1)	2.01	2.39	1.80	1.99	0.23	−0.10
	O^{2-}	IV (S = 1)	2.01	2.56	1.65	1.25	0.88	−0.03
P450cam	OH^-	IV (S = 1)	2.03	2.38	1.80	2.01	0.25	−0.12
	O^{2-}	IV (S = 1)	2.03	2.51	1.66	1.23	0.90	−0.03
CPO	OH^-	IV (S = 1)	2.02	2.38	1.81	1.95	0.21	−0.03
	O^{2-}	IV (S = 1)	2.03	2.57	1.66	1.25	0.88	−0.02

Table 2. Mössbauer Parameters in mm/s

		theory [mm/s]		experiment [mm/s]	
protein	distal ligand	δ	ΔE_Q	δ	ΔE_Q
$P450_{BM3}$	OH^-	0.09	2.17	0.13	2.16
	O^{2-}	0.11	1.05		
P450cam	OH^-	0.11	1.84	0.14	2.06
	O^{2-}	0.14	0.66		
CPO[14]	OH^-	0.10	2.06	0.10	2.06
	O^{2-}	0.12	1.00	0.11	1.59

Mössbauer parameters were determined at optimized geometries. During optimizations, the positions of all atoms were constrained to their position in the ferric crystal structure except Fe, the distal ligand, the porphyrin nitrogens, alpha-carbons, meso-carbons, meso-hydrogens, and the proximal SCH_2CH. Geometry optimizations were performed at the B3LYP/6-311G level.[29] The iron-ligand bond distances and spin densities obtained for the optimized structures are listed in Table 1. Quadrupole splittings were determined at the B3LYP/6-311G level. Isomer shifts were determined using Neese's core properties (CP) basis set.[30−32] For this basis set, an integration grid containing 199 radial shells with 590 angular points per shell was used. The electron density at the Fe nucleus was determined using the atoms in molecules (AIM) option in Gaussian 03. Calculated Mössbauer parameters can be found in Table 2.

Experimental Procedures

Protein Preparation. ^{57}Fe-enriched cytochromes $P450_{BM3}$ and P450cam were obtained from overexpression in BL21 cells (Stratagene). These cultures were grown in M9 minimal media. At an O. D. of 0.8, protein expression was induced with 0.5 mM IPTG. At the time of expression, 1−2 mg/L of $^{57}FeCl_3$, 0.5 mM δ-aminolevulinic acid, and 1 mL/L of a solution of trace elements ($ZnCl_2 \cdot 4H_2O$, 1 g; $CoCl_2 \cdot 6H_2O$, 0.2 g; $Na_2MoO_4 \cdot 2H_2O$, 1 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $CuCl_2$, 1 g; and H_3BO_3, 0.2 g in 1 L of 10% HCl) were added to the cultures.

After 18 h, the cells were harvested and lysed using a french press. $P450_{BM3}$ was purified using a Co^{2+}-affinity column (Talon, Clontech). The protein was loaded onto the column using buffer containing 0.1 M Kphos, pH 8, 300 mM NaCl, and 20 mM imidazole. $P450_{BM3}$ was eluted with the same buffer, except the concentration of imidazole was increased to 200mM. The bound imidazole was later removed using

(15) van Deurzen, M. P. J.; van Rantwijk, F.; Sheldon, R. A. *Tetrahedron* **1997**, *53*, 13183.
(16) van Rantwijk, F.; Sheldon, R. A. *Curr. Opin. Biotech.* **2000**, *11*, 554.
(17) Guengerich, F. P. *Chem. Res. Toxicol.* **2001**, *14*, 611.
(18) van de Velde, F.; van Rantwijk, F.; Sheldon, R. A. *J. Mol. Catal. B: Enzym.* **1999**, *6*, 453.
(19) Rutter, R.; Hager, L. P.; Dhonau, H.; Hendrich, M.; Valentine, M.; Debrunner, P. G. *Biochemistry* **1984**, *23*, 6809.
(20) Stone, K. L.; Behan, R. K.; Green, M. T. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 16563.
(21) The characterization of P450-I has been limited to that afforded by rapid-scan stopped-flow spectrophotometry. Several groups have reported the formation of P450-I in reactions of ferric P450 with *meta*-chloroperoxybenzoic acid.[22−24] In all cases, the putative intermediates were prepared in low yield and their representative spectra were obtained by singular value decomposition.
(22) Egawa, T.; Shimada, H.; Ishimura, Y. *Biochem. Biophys. Res. Comm.* **1994**, *201*, 1464.
(23) Kellner, D. G.; Hung, S. C.; Weiss, K. E.; Sligar, S. G. *J. Biol. Chem.* **2002**, *277*, 9641.
(24) Spolitak, T.; Dawson, J. H.; Ballou, D. P. *J. Biol. Chem.* **2005**, *280*, 20300.
(25) Lambeir, A. M.; Dunford, H. B.; Pickard, M. A. *FEBS* **1987**, *163*, 123.
(26) Egawa, T.; Proshlyakov, D. A.; Miki, H.; Makino, R.; Ogura, T.; Kitagawa, T.; Ishimura, Y. *J. Biol. Inorg. Chem.* **2001**, *6*, 46.
(27) Raag, R.; Poulos, T. *Biochemistry* **1991**, *30*, 2674.
(28) Ost, T. W. B.; Clark, J.; Mowat, C. G.; Miles, C. S.; Walkinshaw, M. D.; Reid, G. A.; Chapman, S. K.; Daff, S. *J. Am. Chem. Soc.* **2003**, *125*, 15010.
(29) Frisch J., et al. *Gaussian 03*, revision C.02; Gaussian, Inc., Wallingford, CT, 2004.
(30) Schöneboom, J. C.; Neese, F.; Thiel, W. *J. Am. Chem. Soc.* **2005**, *127*, 5840.
(31) Neese, F. *Inorg. Chim. Acta* **2002**, *337*, 181.
(32) Neese, F. *Curr. Opin. Chem. Biol.* **2003**, *7*, 125.

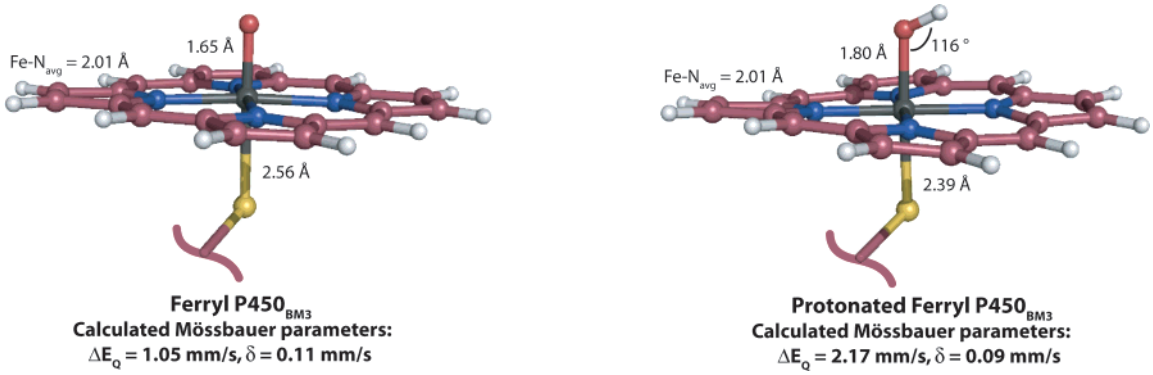

Figure 1. Theoretically determined metal−ligand bond distances and calculated Mössbauer parameters.

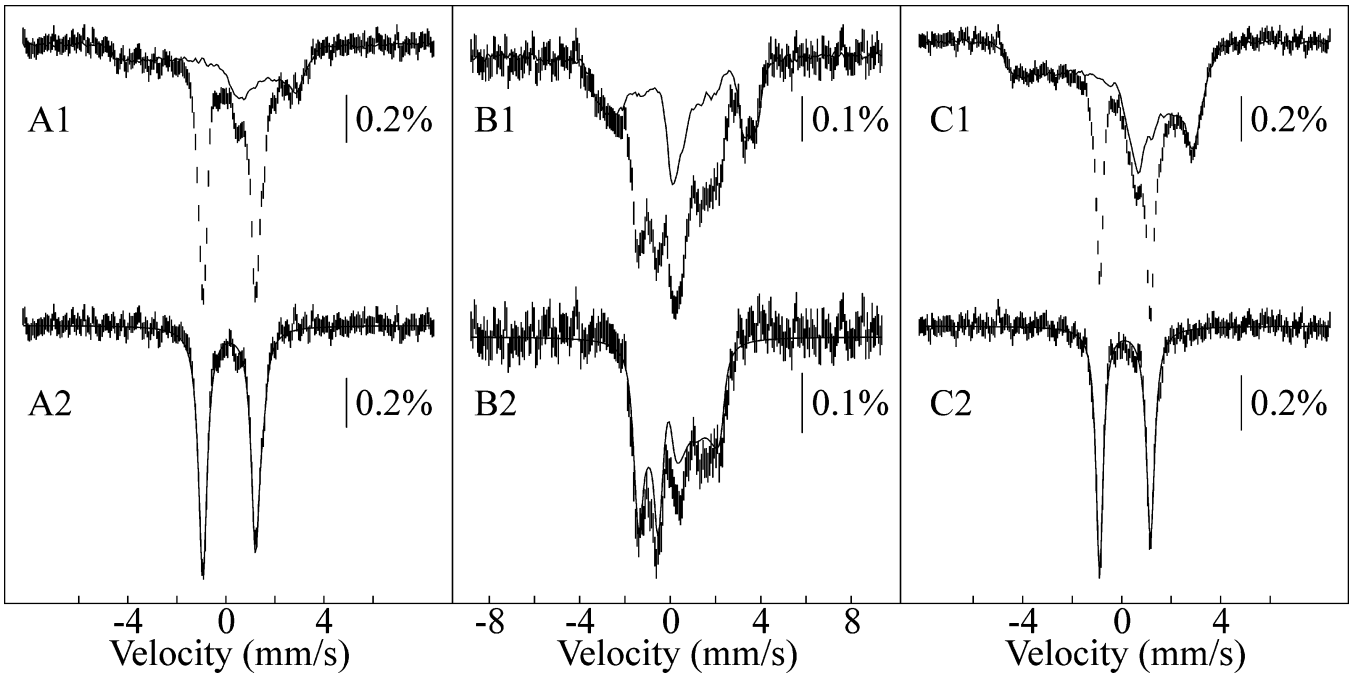

Figure 2. Mössbauer spectra (4.2 K) of samples of ferric P450 reacted with peracetic acid (hash marks). A1 and B1 are spectra of P450$_{BM3}$ recorded in external magnetic fields of 40 mT (A1) and 7 T (B1). C1 is the 40-mT spectrum of P450$_{cam}$. Spectra of ferric P450 recorded under identical conditions are overlaid as solid lines (44% for P450$_{BM3}$ 71% for P450$_{cam}$). Removal of ferric contributions yields spectra A2, B2, and C2. The low field spectra (A2, C2) were fit to quadrupole doublets with parameters described in the text (solid lines in A2 and C2). The solid line in B2 is a spin Hamiltonian simulation using the parameters quoted in the text.

size exclusion chromatography. The fractions of P450$_{BM3}$ with an Rz ($A_{418/280}$) greater than 1.4 were used for Mössbauer spectroscopy.

P450cam was expressed similarly to P450$_{BM3}$. Purification of P450cam was accomplished by first loading the protein onto a DEAE sepharose column. The column was washed with buffer containing 50 mM KPhos, pH 7.2, 500 μM camphor, and 2 mM DTT, and the protein was eluted with a 0−500 mM KCl gradient of the same buffer. P450cam should elute around 150−200 mM KCl. The fractions containing P450cam were pooled. The pooled fractions were then brought to 60% ammonium sulfate saturation. Following centrifugation, the resulting pellet was resuspended in buffer containing 50 mM KPhos, pH 7.2, 500 μM camphor, 2mM DTT, and 30% ammonium sulfate and loaded onto a Sephacryl 200 size exclusion column (GE Biosciences). P450cam was eluted with buffer containing 0.1 M KPhos, pH 7.0. P450cam used for Mössbauer experiments had an Rz ($A_{418/280}$) greater than 1.4.

Freeze-Quenched Samples. Freeze quench methods were used to generate the ferryl intermediates in P450$_{BM3}$ and P450cam. A four-syringe ram freeze-quench apparatus from Update Instruments (Madison, WI) was used for all freeze-quench experiments. Ferryl intermediates were generated in P450$_{BM3}$ and P450cam by reacting a solution of ferric protein (4 mM) with a 5-fold excess of peracetic acid in a 1:1 ratio, for a final protein concentration of 2 mM. All reagents were in 0.1 M Kphos, pH 7. Reactants were mixed through the shortest aging line (5.3 μL) at 4 °C and sprayed into a bath of cold isopentane, −145 °C, ∼4 ms after mixing. Samples were packed into a Mössbauer sample holder for analysis.

Mössbauer Spectroscopy. Mössbauer spectra were recorded on a spectrometer from WEB research (Edina, MN) operating in the constant acceleration mode in a transmission geometry. Spectra were recorded with the temperature of the sample maintained at 4.2 K. For low-field spectra, the sample was kept inside an SVT-400 dewar from Janis (Wilmington, MA), and a magnetic field of 40 mT was applied parallel to the γ-beam. For high-field spectra, the sample was kept inside a 12SVT dewar (Janis), which houses a superconducting magnet that allows for application of variable magnetic fields between 0 and 8 T parallel to the γ-beam. The quoted isomer shifts are relative to the centroid of the spectrum of a metallic foil of α-Fe at room temperature. Data analysis was performed using the program WMOSS from WEB research.

Results and Discussion

We have used Mössbauer spectroscopy in conjunction with density functional calculations to determine the protonation state

of the ferryl forms of P450cam and $P450_{BM3}$. Calculated bond distances and spin densities are listed in Table 1. Theoretically determined Mössbauer parameters are listed in Table 2. The results of our calculations are illustrated in Figure 1.

The 4.2-K/40-mT Mössbauer spectrum of a freeze-quenched sample of $P450_{BM3}$ is shown in Figure 2A (hash marks). An amount equal to 44% of the total intensity of this spectrum can be attributed to ferric $P450_{BM3}$. The 4.2-K/40-mT reference spectrum of ferric $P450_{BM3}$ is overlaid as a solid line. Removal of this component yields spectrum A2, which is dominated by a quadrupole doublet. In addition, there is a minor component, which is seen as a shoulder on the high-energy line ($\approx$1.5 mm/s). A2 can be analyzed with two symmetric quadrupole doublets with the following parameters: $\delta(1) = 0.13$ mm/s, $\Delta E_Q(1) = 2.16$ mm/s (48%) and $\delta(2) = 0.33$ mm/s, $\Delta E_Q(2) = 2.41$ mm/s (8%). The nature of the minor component is not well-understood.[33] The isomer shift of the major component is typical of a ferryl heme. Its quadrupole splitting parameter is nearly identical to that calculated for protonated $P450_{BM3}$-II (Table 2). This value, which is similar to that observed for protonated CPO-II, is significantly larger than the quadrupole splittings of other 6-coordinate ferryl intermediates (typically on the order of 1.4 mm/s).[14] Thus, the major quadrupole doublet is assigned as protonated $P450_{BM3}$-II.

$P450_{BM3}$-II was further characterized by recording Mössbauer spectra in a 7-T external magnetic field (Figure 2B). Removal of the contribution of ferric $P450_{BM3}$ (solid line in B1) from the raw data (hash marks) yields the reference spectrum of $P450_{BM3}$-II (B2, hash marks). It can be simulated using a spin Hamiltonian formalism in the slow relaxation limit. While the experimental data are insufficient to uniquely determine the spin Hamiltonian parameters, the following parameters, which are typical of ferryl heme species, were assumed: zero-field splitting parameters, $D = +23$ cm^{-1} and $E/D = 0$, $\mathbf{g} = (2.1, 2.1, 2.0)$, asymmetry parameter $\eta = 0$, $\mathbf{A}/g_N\beta_N = (-19, -19, -7)$ T.[34,35] Mössbauer parameters (δ and ΔE_Q) were taken from the 40 mT data. Simulations reveal the sign of ΔE_Q is positive, in agreement with DFT calculations. The data are consistent with an $S = 1$ ground state for $P450_{BM3}$-II. The difference between data and simulation at $\sim$0 mm/s is most likely due to contributions from the minor (8%) component (see above).

Figure 2C shows the Mössbauer spectrum of a freeze-quenched sample of P450cam (hash marks). The majority of the spectrum (71%) can be attributed to ferric P450cam (solid line in C1). Removal of its contribution yields spectrum C2 (hash marks), which can be analyzed with one quadrupole doublet (solid line in C2). The experimentally determined parameters ($\delta = 0.14$ mm/s and $\Delta E_Q = 2.06$ mm/s) are in good agreement with those calculated for protonated P450cam-II (Table 2). Our experimental result is also in agreement with Schünemann and co-workers, who observed an intermediate (13% yield; $\Delta E_Q = 1.94$ mm/s and $\delta = 0.13$ mm/s) during the reaction of P450cam and peracetic acid.[36,37]

Conclusion

We have provided theoretical and experimental evidence for protonated ferryl intermediates in cytochromes P450. Our findings suggest that basic ferryls are a natural consequence of thiolate-ligated hemes. These results appear to lend credence to the theory that nature is using thiolate ligation to promote hydrogen abstraction (and subsequent hydroxylation) in P450s; however, it remains to be determined if basic ferryls are a unique property of thiolate-ligated hemes.

Acknowledgment. This work was supported by the National Science Foundation (M.T.G), the Petroleum Research Fund (M.T.G.), and the Arnold and Mabel Beckman Foundation (M.T.G.). R.K.B. is supported by a grant from the Herman Frasch Foundation. M.T.G. is an Alfred P. Sloan Fellow.

Supporting Information Available: Full reference citation for ref 29. This material is available free of charge via the Internet at http://pubs.acs.org.

JA062428P

(33) A species with similar parameters was found as a minor component in an annealed sample of cryo-reduced CPO-I.[14]

(34) Debrunner, P. G. *Phys. Bioinorg. Chem. Ser. v4 1989* **1989**, *4*, 137.
(35) Oosterhuis, W. T., Lang, G. *J. Chem. Phys.* **1973**, *58*, 4757.
(36) Schünemann, V., Jung, C., Trautwein, A. X., Mandon, D., Weiss, R. *FEBS* **2000**, *479*, 149.
(37) Schünemann, V.; Jung, C.; Terner, J.; Trautwein, A. X.; Weiss, R. *J. Inorg. Biochem.* **2002**, *91*, 586.