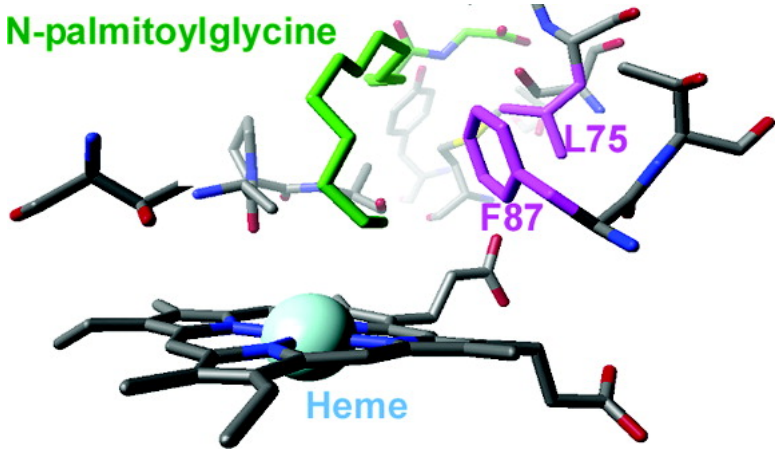
Article

Thermal Equilibrium of High- and Low-Spin Forms of Cytochrome P450 BM-3: Repositioning of the Substrate?

Tijana Jovanovic, Ramy Farid, Richard A. Friesner, and Ann E. McDermott

J. Am. Chem. Soc., **2005**, 127 (39), 13548-13552• DOI: 10.1021/ja0524604 • Publication Date (Web): 13 September 2005



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 6 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 09/13/2005

Thermal Equilibrium of High- and Low-Spin Forms of Cytochrome P450 BM-3: Repositioning of the Substrate?

Tijana Jovanovic, Ramy Farid, Richard A. Friesner,* and Ann E. McDermott*

Contribution from the Department of Chemistry, Columbia University, New York, New York 10027

Received April 15, 2005; E-mail: aem5@columbia.edu; rich@chem.columbia.edu

Abstract: We demonstrate that cytochrome P450 BM-3 in complex with N-palmitoylglycine undergoes a spin state change between room temperature, where optimal activity is seen, and low temperatures, where X-ray diffraction characterization has been carried out. On the basis of NMR measurements of the full-length protein, this spin state change is likely to be accompanied by a general structural rearrangement in the enzyme pocket. The substrate remains bound at all temperatures. We propose that the substrate may "slide" from a position directly atop the heme (thus displacing the ligating water) to the more distant position (thus restoring the ligating water) as the temperature is lowered. This proposal is evaluated on the basis of computational modeling of the protein−ligand complex, using a novel induced fit methodology. We thereby generate a structure with the ligand in close contact with the heme, similar in energy to the experimental structure. With this combination of theory and experiment we provide a specific proposal of how ligands may be positioned for chemistry for this enzyme.

Introduction

Given the importance of predicting the P450 reactivities of new pharmaceuticals, knowledge of conformation and location of ligands and inhibitors in the active sites of cytochrome P450 enzymes is of great importance. A number of enzyme−substrate complexes have been reported over the years which exhibit the substrate in a position presumably related to the conformation in which chemical catalysis takes place. Among them are mammalian isozymes P450 2C5,[1,2] 2C9,[3] and some bacterial isozymes such as P450cam,[4−7] P450eryF,[8,9] and a few others. On the other hand, among the enzyme−substrate complexes of cytochrome P450 enzymes deposited in the protein databank, a significant number involve substrates displaced by more than 6 Å from a closest approach to the heme. For example, the structure of P450 BM-3 with *N*-palmitoylglycine (NPG)[10] shows that positions where hydroxylation occurs are at least 7.5 Å away from Fe center, as seen in Figure 1. Mammalian CYP2C9 with warfarin,[11] CYP3A4 with progesterone,[12] and CYP2C8 with palmitic acid[13] also show the substrate distant from the heme. This difference in relative positions of substrates with respect to the heme is illustrated in Figure 1 for P450 BM-3 and P450cam. It is probably safe to say that these displaced configurations would be unproductive for chemistry without further rearrangement.

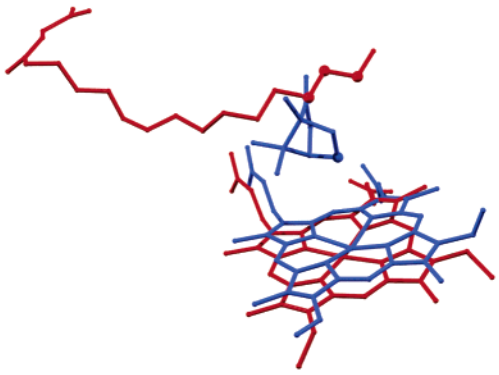


Figure 1. Positions of NPG bound to P450 BM-3 (red) and 1-R camphor bound to P450cam (blue) with hydroxylation sites presented with bold circles (8.3, 8.6, and 7.6 Å away from the heme iron for P450 BM-3 and only 4.21 Å away from the heme iron for P450cam). It is clear that the substrate is positioned close to the heme iron in P450cam, but far from the heme iron in P450 BM-3. PDB files 1JPZ[10] and 2CPP[4] were overlaid using 320 backbone atoms within conserved helices D, E, I, L, J, and K, employing Swiss PDB Viewer (SPDBV).[14] The RMS deviation for the backbone atoms was 1.57 Å. The figure was prepared with programs POV Ray (http://www.povray.org) and SPDBV.[14]

(1) Wester, M. R.; Johnson, E. F.; Marques-Soares, C.; Dijols, S.; Dansette, P. M.; Mansuy, D.; Stout, C. D. *Biochemistry* **2003**, *42*, 9335−9345.
(2) Wester, M. R.; Johnson, E. F.; Marques-Soares, C.; Dansette, P. M.; Mansuy, D.; Stout, C. D. *Biochemistry* **2003**, *42*, 6370−6379.
(3) Wester, M. R.; Yano, J. K.; Schoch, G. A.; Yang, C.; Griffin, K. J.; Stout, C. D.; Johnson, E. F. *J. Biol. Chem.* **2004**, *279*, 35630−35637.
(4) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *195*, 687−700.
(5) Schlichting, I.; Jung, C.; Schulze, H. *FEBS Lett.* **1997**, *415*, 253−257.
(6) Bell, S. G.; Chen, X. H.; Sowden, R. J.; Xu, F.; Williams, J. N.; Wong, L. L.; Rao, Z. H. *J. Am. Chem. Soc.* **2003**, *125*, 705−714.
(7) Raag, R.; Poulos, T. L. *Biochemistry* **1991**, *30*, 2674−2684.
(8) Cupp-Vickery, J. R.; Poulos, T. L. *Nat. Struct. Biol.* **1995**, *2*, 144−153.
(9) Cupp-Vickery, J.; Anderson, R.; Hatziris, Z. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3050−3055.
(10) Haines, D. C.; Tomchick, D. R.; Machius, M.; Peterson, J. A. *Biochemistry* **2001**, *40*, 13456−13465.
(11) Williams, P. A.; Cosme, J.; Ward, A.; Angova, H. C.; Vinkovic, D. M.; Jhoti, H. *Nature* **2003**, *424*, 464−468.
(12) Williams, P. A.; Cosme, J.; Vinkovic, D. M.; Ward, A.; Angove, H. C.; Day, P. J.; Vonrhein, C.; Tickle, I. J.; Jhoti, H. *Science* **2004**, *305*, 683−686.
(13) Schoch, G. A.; Yano, J. K.; Wester, M. R.; Griffin, K. J.; Stout, C. D.; Johnson, E. F. *J. Biol. Chem.* **2004**, *279*, 9497−9503.

10.1021/ja0524604 CCC: $30.25

The X-ray structures with substrates distant to the heme also raise questions about the origin and role of spin state changes for the cytochrome P450 enzymes. The dominant hypothesis has been that the heme changes from low-spin (LS) ($S = 1/2$, ferric) for the resting state, to high-spin (HS), ($S = 5/2$, ferric) for the substrate bound species due to the displacement of the sixth ligand to the iron, a water molecule.[15] More than a spectroscopic marker, this spin state change is thought to be crucial to the mechanism, in that the spin state modulates the redox potential of the heme. Because the reduction potential of the donor is intermediate between that of the HS ferric and that of the LS ferric, electron transfer to the heme can occur (and thereby initiate the chemical cycle) only if a substrate is present, potentially protecting the enzyme from auto-degradative chemistry. However, this simple hypothesis concerning the relation of water displacement to spin conversion has appeared somewhat muddy at times. Although for P450cam, essentially complete conversion from LS to HS is seen upon camphor binding at room temperature, it has been reported that both bound and unbound forms of P450cam show some degree of mixed spin states,[16] particularly at low temperature.[16,17] Curiously, the substrate dissociation constant and induced percent spin-state change have been reported to be independent of each other, at least in some cases,[18] and the extent of spin-state conversion is not correlated with the extent of reductant consumption or degree of coupling in certain cases.[19] For cytochrome P450 BM-3 in particular, the spin state change has been described as incomplete at room temperature for most of the substrates, which presents both a practical problem for characterization, and a conceptual problem for the mechanism of this highly efficient enzyme. Moreover, a general puzzle arises when substrates bind in nonproductive modes. Despite the distant siting, and the presence of a ligating water molecule seen in the X-ray structures, a substrate dependent spin state change is observed in solution UV−vis characterization. The issue of how a distantly located substrate displaces, or shifts, a water molecule has been discussed,[10] with the proposal of an important mediating role for the protein, whose pocket somehow stabilizes a sixth ligand vacancy.

Alternative binding modes of the substrate can be connected to other unusual phenomena that occur for the family of P450 enzymes. A prevalent hypothesis of substrate mobility in the pocket[20] is supported by an extensive body of otherwise surprising intramolecular competitive isotope effects, sometimes referred to as "metabolic switching". In fact, the observation that many of the mammalian enzymes are quite promiscuous in their scope of chemistry might be viewed as suggestive evidence for a plastic enzyme pocket, and possibly for substrate mobility in situ. Indeed, there are even documented cases of multiple substrates binding simultaneously to a single pocket![9,21] On the other hand, the observations by X-ray crystallography of distant and unproductive locations of substrates are unexpected.

In the face of these observations of nonproductive binding modes, it is clearly of interest to glean some evidence as to the nature of the productive binding mode, and some insight into the circumstances under which it arises.[22] In this report, we describe experimental evidence that the productive binding mode is well populated at higher temperatures, near physiological temperatures, and we utilize novel computational methods to predict its detailed conformation. This study provides an important, testable hypothesis with the potential of strong insights into the chemical mechanism and with insights into specificity observed in the products.

Materials and Methods

Cytochrome P450 BM-3 was prepared using modifications of prior procedures,[23−25] and its purity[26] and full activity[27] were confirmed. For UV−visible thermal equilibrium measurements, a 3.4 μM sample (0.6 mL) in 0.1 M MOPS pH 7.4, 5% ethylene glycol was monitored in the resting state; another sample of similar concentration was monitored in the presence of 30 equiv of NPG (i.e., well over the K_d value) at different temperatures. The substrate was directly added to the enzyme sample as 10 mM stock in 50 mM K_2CO_3. All UV−vis spectra were taken on a Shimadzu UV-1601 UV−visible spectrophotometer. The sample temperature was maintained with a water bath flow system, and was monitored with a thermocouple.

Two types of selectively isotopically enriched cytochrome P450 BM-3 enzyme were prepared for NMR measurements, modifications of an existing protocol[28] involving growth of the expression strain in the presence of defined media with the appropriate isotopically enriched amino acids, as described in detail elsewhere.[24] Sample "FAG" was enriched with ^{13}CO-Phe, ^{13}Cα-Ala, and ^{15}N-Gly and sample "LFG" was enriched with ^{13}CO-Leu, ^{15}N-Phe, and ^{15}N-Gly. ω-^{13}C enriched NPG and NPG including uniformly enriched ^{13}C palmitic acid were synthesized in two steps by the method described by Lapidot et al.[29] The products were recrystallized from ethanol and their formation and purity were confirmed using solution NMR and mass spectrometry.[24] Samples for NMR measurements were precipitated as follows: cytochrome P450 BM-3 in 50 mM MOPS pH 7.4, 20−30% glycerol was concentrated to 200 mg/mL and 1 equiv of NPG in 50 mM K_2CO_3 was directly added to the enzyme (20% was used for the LFG sample at 0 °C, and 30% for the others). Saturated binding was confirmed with UV−vis spectrometry at room temperature, distinguishing the HS ferric from the LS ferric state. The enzyme−substrate complex was precipitated using a batch method by addition of PEG in buffer to result in final concentrations as follows: 40% (w/v) PEG 8000 in 25 mM $MgSO_4$, 50 mM MOPS pH 7.4 (LFG labeling scheme at 0 °C) or 40% (w/v) PEG 8000 in 25 mM $MgSO_4$, 50 mM MOPS pH 7.4, 30% glycerol (all other samples). After NMR measurements the samples were redissolved and UV−vis spectra were again confirmed.

NMR spectra were recorded using a Varian/Chemagnetics Infinity Plus 600 MHz spectrometer, operating at Larmor frequencies of 599.3 MHz for proton, 150.7 MHz for carbon, and 60.7 MHz for nitrogen.

(14) Guex, N.; Peitsch, M. C. *Electrophoresis* **1997**, *18*, 2714−2723.
(15) Poulos, T. L.; Raag, R. *FASEB J.* **1992**, *6*, 674−679.
(16) Sligar, S. G. *Biochemistry* **1976**, *15*, 5399−5406.
(17) Tsai, R.; Yu, C. A.; Gunsalus, I. C.; Peisach, J.; Blumberg, W.; Orme-Johnson W.; Beinert, H. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *66*, 1157−1163.
(18) Li, S. Y.; Wackett, L. P. *Biochemistry* **1996**, *32*, 9355−9361.
(19) Sibbesen, O.; Zhang, Z. P.; de Montellano, P. R. O. *Arch. Biochem. Biophys.* **1998**, *353*, 285−296.
(20) Smith, J. R. L.; Sleath, P. R. *J. Chem. Soc.-Perkin Trans. 2* **1983**, 621−628.
(21) Dabrowski, M. J.; Schrag, M. L.; Wienkers, L. C.; Atkins, W. M. *J. Am. Chem. Soc.* **2002**, *124*, 11866−11867.
(22) Modi, S.; Sutcliffe, M. J.; Primrose, W. U.; Lian, L. Y.; Roberts, G. C. K. *Nat. Struct. Biol.* **1996**, *3*, 414−417.
(23) Schwaneberg, U.; Sprauer, A.; Schmidt-Dannert, C.; Schmid, R. D. *J. Chromatogr. A* **1999**, *848*, 149−159.
(24) Jovanovic, T. Substrate binding to Cytochrome P450 BM-3: temperature and redox dependence. *Thesis*, Columbia University, New York, 2005.
(25) Sevrioukova, I.; Truan, G.; Peterson, J. A. *Biochemistry* **1996**, *35*, 7528−7535.
(26) Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370−2378.
(27) Schwaneberg, U.; Schmidt-Dannert, C.; Schmitt, J.; Schmid, R. D. *Anal. Biochem.* **1999**, *269*, 359−366.
(28) Williams, J. C.; McDermott, A. E. *Biochemistry* **1995**, *34*, 8309−8319.
(29) Lapidot, Y.; Rappopor, S.; Wolman, Y. *J. Lipid Res.* **1967**, *8*, 142−145.

A 4 mm triple resonance T3 magic angle spinning (MAS) probe (Varian Instruments) in $^{1}H^{13}C^{15}N$ mode was used, with a sample spinning frequency of 13 000 ± 5 Hz. Temperatures were corrected for heating caused by spinning and decoupling (by adding 20 °C to the temperature of the variable temperature flow gas, approximately calibrated for these conditions using external samples). Two-dimensional (2D) dipolar-assisted rotational resonance (DARR)[30] spectra of ^{13}CO-Phe, ^{13}Cα-Ala, ^{15}N-Gly labeled cytochrome P450 BM-3, with ligand bound, were acquired. $^{1}H-^{13}C$ cross polarization was accomplished with proton excitation at 6.7 ppm at a field strength of 47.8 kHz (at 15 °C) or 33.1 kHz (at 0 and −30 °C) and ramped carbon excitation at 114.5 ppm with an average field strength 32.6 kHz (at 15 °C) or 44.2 kHz (at 0 and −30 °C) and a linear downward-going ramp of amplitude approximately 10 kHz.[31] Contact times of 1.75 to 2 ms were used. During the mixing period, recoupling proton irradiation with a field strength of 13.4 kHz was applied. The spectral sweep widths were 78 kHz in the direct and 39 kHz in the indirect dimension. The number of points collected in the direct and indirect dimensions were 1024 and 512, respectively. A double cross polarization sequence[32] with a selective[33] $^{15}N-^{13}CO$ transfer (spectrally induced filtering in combination with cross polarization, or SPECIFIC CP) was used for acquiring the 2D $^{15}N^{13}CO$ spectrum of ^{13}CO-Leu, ^{15}N-Gly, ^{15}N-Phe labeled cytochrome P450 BM-3. $^{1}H-^{15}N$ cross polarization was achieved using a ramped[31] radio frequency field on ^{15}N, with an average field strength of 33.1 kHz (at −30 °C) or 35.2 kHz (at 0 °C), a linear downward-going ramp of amplitude approximately 7 kHz, and a contact time of 1.25 ms (at −30 °C) or 0.75 ms (at 0 °C). The $^{15}N-^{13}CO$ polarization transfer at −30 °C was achieved with a ^{15}N excitation at 116.4 ppm with a CP field strength of 32.4 kHz and a downward-going linear ramp for the carbon excitation at 188.1 ppm, with an average field strength of 44.8 kHz. The $^{15}N-^{13}CO$ polarization transfer at 0 °C was achieved with a ^{15}N excitation at 124.9 ppm with a CP field strength of 21.4 kHz and a downward-going linear ramp for the carbon excitation at 176.0, with an average field strength of 34.3 kHz. In both cases, the ramp amplitude of 2.5 kHz was used during a contact time of 3.5 ms. The number of points in the direct dimension was 1500, while the number of points in the indirect dimension was 64. The number of scans per t_1 point was 480 for spectra taken at 0 °C and 1152 for spectra collected at −30 °C. Using a recycle delay of 3 s, the total experiment time was between 26 and 61 h. Spectral widths of 78.125 kHz in ^{13}C dimension and 3.250 kHz in the ^{15}N dimension were used. These experiments are also described elsewhere.[24]

Two-dimensional spectra were processed with NMRPipe[34] and displayed with Sparky 3.100 (Goddard, T. D. and Kneller, D. G., University of California, San Francisco). For phase sensitive detection, the time proportional phase incrementation (TPPI) method[35] was utilized. For processing the $^{15}N^{13}CO$ spectra, cosine-bell and sine-bell (81° phase shift) apodization function were applied in the direct and indirect dimensions, respectively. Spectra were zero-filled to 8192 points in the carbon dimension, and exponential line broadening between 10 and 40 Hz was applied. The indirect dimension was zero-filled to 512 points.

The ^{13}C spectra were referenced relative to DSS using the ^{13}C adamantane methylene peak at 40.26 ppm (from spectra run separately). The ^{15}N chemical shift axis was externally referenced to ammonium chloride using the value of 39.27 ppm relative to liquid ammonia at 25 °C.

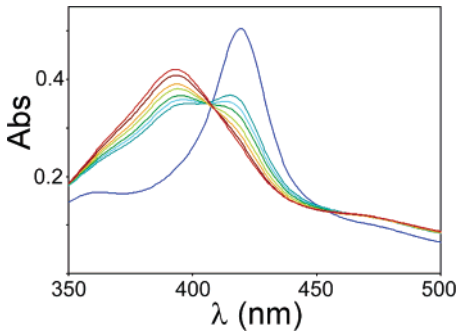


Figure 2. UV−vis spectra of 3.4 μM P450 BM-3 in 0.1 M MOPS pH 7.4, 5% ethylene glycol at −10 °C (dark blue) with the Soret maximum at 418 nm; a 1:30 mixture of P450 BM-3 (3.4 μmolar) and NPG was studied at the following temperatures: 37 °C (red); 20 °C (brown); 10 °C (orange); 5 °C (lime); 0 °C (green); −3 °C (light blue); −6 °C (teal). At physiological temperatures the Soret peak has an absorbance at 393 nm, while at lower temperatures, both 393 and 418 nm absorbance peaks are present. The populations of the spin states are clearly temperature-dependent in the temperature range from 40 to −10 °C.

Computational investigation of the enzyme−substrate structure was performed using a novel ligand docking method that accounts for both ligand and receptor flexibility (described in detail elsewhere).[36] Briefly, after standard protein structure preparation steps (e.g., adding hydrogens, assigning charge states, restrained minimization of the complex to remove steric clashes), Phe87 was mutated to alanine, the active site (ligand) water molecule was omitted, and the NPG substrate was redocked into the protein active site using the Glide program.[35,36] To ensure thorough sampling of ligand poses, a van der Waals scaling factor of 0.8 was used for both protein and substrate. The top 100 poses (as measured by the energy function in Glide) were saved, and protein structure prediction methods in the PLOP (Protein Local OPtimization) algorithm[37−39] embedded in the Prime package (Schrödinger, LLC; Portland, OR) were used to repredict the protein structure (with residue 87 now reverted to Phe) starting from each of the 100 poses. Reprediction was performed for side chains of a common set of residues derived from the union of all residues within 5 Å of the substrate for each of the 100 poses generated by Glide; a common set of residues is used in order to allow direct comparison of the total energies of the various complexes. After side-chain prediction, all atoms of the residues involved in the prediction (including backbone), as well as those of the substrate, were minimized. Prime employs an accurate protein force field (OPLS) and Surface Generalized Born (SGB) continuum solvation model, which has had great success in both side-chain and loop prediction on large test sets taken from the PDB, suggesting that ranking of the resulting structures using the Prime total energy is likely to yield reasonable results.

Results and Discussion

Using the heme optical signatures, we demonstrate that the enzyme−substrate complex exhibits a strong temperature dependence, such that the structure and spin state at low temperature, where X-ray crystallography is carried out, differ from those at room temperature, where chemistry is performed. Figure 2 shows the UV−vis spectrum for the unligated enzyme and for a 1:30 mixture of P450 BM-3 with NPG. The spectral band at 418 nm is characteristic of a LS ferric heme,[16,40] such as is

(30) Takegoshi, K.; Nakamura, S.; Terao, T. *Chem. Phys. Lett.* **2001**, *344*, 631−637.
(31) Metz, G.; Wu, X. L.; Smith, S. O. *J. Magn. Reson. Ser. A* **1994**, *110*, 219−227.
(32) Schaefer, J.; McKay, R. A.; Stejskal, E. O. *J. Magn. Reson.* **1979**, *34*, 443−447.
(33) Baldus, M.; Petkova, A. T.; Herzfeld, J.; Griffin, R. G. *Mol. Phys.* **1998**, *95*, 1197−1207.
(34) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* **1995**, *6*, 277−293.
(35) Marion, D.; Wuthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967−974.
(36) Sherman, W.; Day, T.; Jacobson, M. P.; Friesner, R. A.; Farid, R., submitted to *J. Med. Chem.*
(37) Jacobson, M. P.; Friesner, R. A.; Xiang, Z. X.; Honig, B. *J. Mol. Biol.* **2002**, *320*, 597−608.
(38) Jacobson, M. P.; Kaminski, G. A.; Friesner, R. A.; Rapp, C. S. *J. Phys. Chem. B* **2002**, *106*, 11673−11680.
(39) Jacobson, M. P.; Pincus, D. L.; Rapp, C. S.; Day, T. J. F.; Honig, B.; Shaw, D. E.; Friesner, R. A. *Proteins* **2004**, *55*, 351−367.
(40) Jefcoate, C. R. *Methods Enzymol.* **1978**, *52*, 258−279.

expected for the resting state. The spectral band at 393 nm is characteristic of a HS ferric heme,[16,40] such as is expected when the sixth ligand water molecule is displaced from the heme. At temperatures above 20 °C, the enzyme is nearly completely in the HS form independent of buffer conditions, or protein concentration. The predominance of the HS form is consistent with the high specific activity of the enzyme at these temperatures. The LS species on the other hand is observed at lower temperatures. It should be mentioned that at a certain temperature the LS population is somewhat dependent on the buffer and cryoprotectant used, but the general phenomenon is observed in all cases that were studied. The substrate remains bound at temperatures from −10 to +40 °C, based on literature X-ray crystallography, our NMR data, and concentration dependent UV/VIS data. Similar results are obtained for a 1:5 stoichiometric mixture of P450 BM-3 with NPG, eliminating the possibility that the peak at 418 nm is due to free enzyme. With parameters used in this study, the enzyme is expected to be more than 99% saturated with respect to substrate binding. In addition, the X-ray data of Haines et al.[10] also argue conclusively that the ligand is indeed bound at temperatures below −20 °C, but that it is located distant from the heme. NMR data collected in our laboratory[24] also support the conclusion that the ligand is bound at these temperatures. Nonetheless, for the experimental conditions used in the study, at temperatures below 0 °C the enzyme exhibits a significant population of LS heme. Thus the UV−vis data are best interpreted in terms of a two-state system, where the temperature drives a transition between two positions of the ligand in the pocket.

These results could be interpreted as being consistent with the position of the substrate in the low-temperature X-ray crystal structure reported by Haines et al.,[10] in that the distant substrate position and the presence of a ligating water molecule (albeit apparently shifted) are more readily rationalized as a LS system. Although this interpretation differs from that originally put forward in the X-ray study, it is fully consistent with the data in hand.

We probed the hypothesis that the protein changes conformation between room temperature and low temperature using MAS solid-state NMR (SSNMR) studies. Solution NMR methods have been used to investigate structural perturbation in P450 family;[41] in this work, due to the large molecular weight of the system, we elected to apply solid state methods. Selectively-labeled cytochrome P450 BM-3 and NPG were studied as a function of temperature, pinpointing the thermally induced shift in both the resonances of the substrate and the protein backbone including atoms associated with the Phe81-Ala82 and the Leu86-Phe87 residues. Unambiguously, on all marker positions listed, as the temperature is lowered, a second form of the enzyme appears, distinct in chemical shift both from the higher temperature form and from the unligated form (not shown).[24] On the other hand, a number of control locations distant from the pocket show no shifts, or subtle shifts near the margin of error of the method. An example is shown in Figure 3; a solid-state NMR experiment in which the active site residue with one of the largest structural shifts upon binding,[10,42] Phe87, was probed selectively capitalizing on the fact that Leu86-Phe87 is the sole

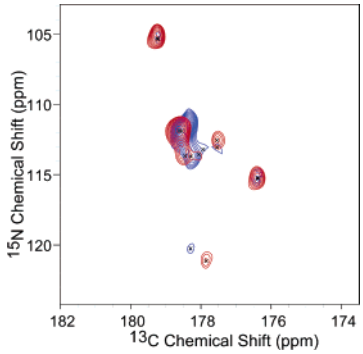


Figure 3. 2D solid-state NMR $^{15}N^{13}CO$ SPECIFIC CP spectrum of ^{13}CO-Leu; ^{15}N-Gly, ^{15}N-Phe labeled cytochrome P450 BM-3 bound with NPG at 0 (red) and −30 °C (blue). The $^{15}N^{13}C$ backbone pair associated with the only LF pair in the protein gives rise to the peaks with a ^{15}N chemical shift of approximately 120 ppm. Peaks with ^{15}N chemical shifts of 105−115 ppm are associated with the six LG pairs in the protein. The L86-F87 pair exhibits a pronounced shift as a function of temperature, whereas the six LG pairs exhibit more modest shifts. The L86-F87 pair is in the binding pocket, and in fact F87 plays a "gatekeeper" role in that its bulky side chain must be rotated in order to allow for substrate binding. The LG pairs are all much more distant from the binding pocket and from the heme.

LF sequential pair in the sequence. The ^{13}CO of Leu and ^{15}N of Phe were isotopically enriched by addition of these amino acids to the growth medium, and selective transfers for one-bond $^{13}C-^{15}N$ pairs was carried out in a 2D CP experiment as described in the materials and methods section. The cross-peak associated with the backbone amide $^{13}C-^{15}N$ pair for residues Leu86-Phe87 was recorded at 0 and −30 °C and clearly shows a large shift in position with this change in temperature. Neither position is identical to the spectrum of the unbound enzyme at the respective temperature, indicating that the substrate remains bound, as expected. In principle, changes in the pseudocontact shifts (PCS) due to proximity to the paramagnetic heme group could be responsible for these shifts.[43,44] The contribution of the PCS can be approximated using the expression

$$\delta_{PC} = \frac{1}{12\pi r^3}[\Delta\chi_{ax}(3\cos^2\theta - 1) + 1.5\Delta\chi_{rh}\sin^2\theta\cos 2\Omega]$$

The contribution of the PCS to the HS ferric P450 BM-3 was approximated using the magnetic susceptibility anisotropy values reported for metmyoglobin,[46] and the P450 crystal structure coordinates,[10] assuming that the *z* axis of the susceptibility tensor is perpendicular to the heme plane. The values predicted for the PCS for the carbonyl ^{13}C of Leu86 are between −0.1 and 1.6 ppm, while for the amidic ^{15}N of Phe87 they range from −0.3 to 1.7 ppm, depending on the azimuthal angle. However, the sign of the expected shift for the carbonyl and the nitrogen is generally the same; at no particular azimuthal angle do we find agreement with the data in that that the carbonyl ^{13}C chemical shift increases and ^{15}N chemical shift decreases on lowering the temperature. Our experimental result thus disagrees with the possibility that the observed shift of Leu86-Phe87 peak upon lowering the temperature is solely due to temperature effect

(41) Wei, J. Y.; Pochapsky, T. C.; Pochapsky, S. S. *J. Am. Chem. Soc.* **2005**, *127*, 6974−6976.
(42) Sevrioukova, I. F.; Li, H. Y.; Zhang, H.; Peterson, J. A.; Poulos, T. L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1863−1868.
(43) Bertini, I.; Turano, P.; Vila, A. J. *Chem. Rev.* **1993**, *93*, 2833−2932.
(44) Bertini, I. L. C., Parigi, G.; Eds. *Solution NMR of Paramagnetic Molecules: Applications to Metallobiomolecules and Models*, Revised Edition; Elsevier: Amsterdam, London, New York, Oxford, Paris, Shannon, Tokyo, 2001; Vol. 2, 376 pp.
(45) McKnight, J.; Cheesman, M. R.; Thomson, A. J.; Miles, J. S.; Munro, A. W. *Eur. J. Biochem.* **1993**, *213*, 683−687.
(46) Turano, P.; Battaini, G.; Casella, L. *Chem. Phys. Lett.* **2003**, *373*, 460−463.

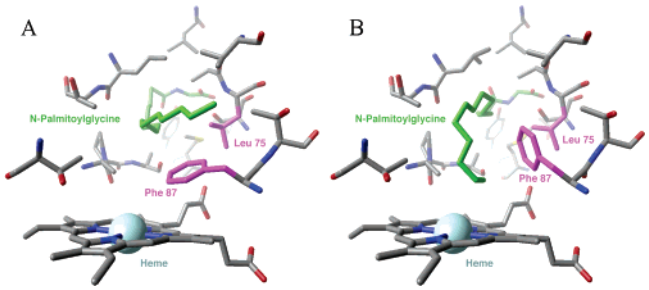


Figure 4. (A) X-ray structure of the active site of P450 BM-3 (1JPZ)[10] highlighting the side chains of Phe87 and Leu75 in magenta, the heme group in blue, and NPG substrate that is bound more than 7 Å from the heme in green. Some amino acid residues have been removed for clarity. (B) Induced-fit structure (IF7 in Table 1) showing the conformational changes that occur predominantly in the side chains of Phe87 and Leu75 upon binding of the substrate near the heme group.

Table 1. Predicted Low Lying Induced Fit Conformations Are Compared with the X-ray Structure[a]

structure	ligand RMSD (Å)	Phe87χ_1, χ_2	Leu75χ_1, χ_2	Δ, Prime energy	ω-n C to Fe distance (Å)		
					$n=1$	$n=2$	$n=3$
native	0.00	−148, −68	−70, −169		8.3	8.6	7.6
IF1	1.74	−145, −67	−71, 167	0.00	9.4	8.0	8.2
IF2	1.35	−158, −78	−84, 74	1.04	9.2	8.8	8.2
IF3	1.81	−145, −66	−70, 168	1.76	9.4	8.0	8.2
IF4	1.81	−145, −66	−70, 167	1.91	9.4	8.0	8.2
IF5	2.04	−146, −69	−74, 167	2.05	9.5	8.1	8.2
IF6	1.95	−146, −69	−74, 167	2.24	9.5	8.1	8.2
IF7	2.81	177, 40	−89, 69	5.82	3.6	4.4	5.8
IF8	2.01	−146, −69	−74, 167	6.78	9.5	8.1	8.2
IF9	2.09	−146, −69	−74, 167	7.62	10.	8.5	8.2
IF10	5.01	−146, −65	−69, 168	8.89	7.6	8.4	7.7

[a] The X-ray structure and computed IF7 structure are shown in Figure 4.

on PCS or due to the spin state change. It is therefore likely that the shift we observed must be attributed in part to conformational change. Moreover, other marker positions also show temperature dependent shifts for the bound form of the enzyme; some are more distant from the heme and therefore are unlikely to be due to PCS.

The foregoing results have prompted us to carry out a computational investigation of the BM-3/NPG complex, beginning from the low-temperature crystal structure available in the PDB (1JPZ). The low-temperature X-ray structure is shown in Figure 4A; the closest approach of the ligand to the heme is 7.5 Å, which is clearly too distant to enable reactive chemistry to occur, as noted above. However, the picture is immediately suggestive of a mechanism that might enable closer approach of the ligand. Phe87 is interposed between the ligand and the heme; if it were to adopt at different rotamer state, as suggested by the NMR shifts described above, the hydrocarbon tail of the ligand might be able to approach close to the catalytic iron center.

Table 1 summarizes the results for the top 10 induced-fit predictions of active site conformation as ranked by total energy of the complex (force field plus continuum solvation terms). The lowest energy structures are very close in root-mean-square deviation (RMSD) to the native structure, confirming that the induced fit protocol described in the Materials and Methods section is capable of recovering the native structure. However, there is also one structure (IF7) in which a concerted motion of the Phe87 and Leu75 side chains yields dramatically different distances of the ligand tail to the Fe atom of the heme. For this structure, the ω-1, ω-2, and ω-3 carbons of NPG are within 3.6−5.8 Å of the iron, which may be consistent with a conformation from which to perform catalytic chemistry; these carbons have been experimentally found to serve as principal hydroxylation sites for the enzyme.[10] Furthermore, the total energy of this structure is only 5.8 kcal/mol above that of the nativelike predictions. The structure of the new complex is shown in Figure 4B; in addition to the substrate, the deviations of the Phe87 and Leu75 residues from the native positions are highlighted.

The structure and energy gap presented above should not be taken as possessing a high degree of precision. The simulations were done using a molecular mechanics model for the heme, which undoubtedly is lacking in sophistication and associated accuracy. Also, we have not carried out the extensive sampling needed for computation of free energy differences within an accuracy of a few kcal/mole, which ideally would include molecular dynamics simulations in explicit solvent. On the other hand, the results indicate that the energetics of the proposed structure are clearly in the ballpark of thermal accessibility, and, given the remarkable correspondence with the requirements for activation of the heme to the HS form (i.e., displacement of the water ligand of the Fe, which the tail region shown in Figure 4B would clearly accomplish) and subsequent catalytic chemistry, it seems highly likely that the actual conformational change detected in the experiments described above is close to what is shown in Figure 4B. As noted above, neither the experiments nor the calculations are at present of sufficient precision to address the issue of the thermodynamics of this problem in a quantitative fashion. More accurate simulations, to complement additionally detailed experiments, are feasible and represent the next logical step in our investigation.

This work resolves a puzzle about the distant binding of substrates in a simple way, through a combination of theory and experiment. For many of the mammalian enzymes, plasticity of the pockets and mobility of the substrates remain central observations, crucial for understanding the scope of chemistry. The low temperature structures, with ligands bound in pockets distant from the heme, are presumably important indications of the range of binding surfaces accessed by ligands at all temperatures. On the other hand, this work demonstrates that the distant pockets documented by low temperature crystallography are not likely to be the major site of binding under physiological conditions. With the substrate more likely sited directly atop the heme in a HS ferric room temperature species, the mechanism, would appear to be much simplified, for this enzyme, and probably for many others in the superfamily.

Acknowledgment. The authors express their appreciation to Professor Julian Peterson for the generous gift of P450 BM-3 plasmid, and Amano Enzyme USA Co., Ltd. for the kind gift of immobilized Pseudomanas cepacia Lipases PS-C and PS-D. We acknowledge support from NIH Grant Nos. GM61251 and GM40526.

JA0524604