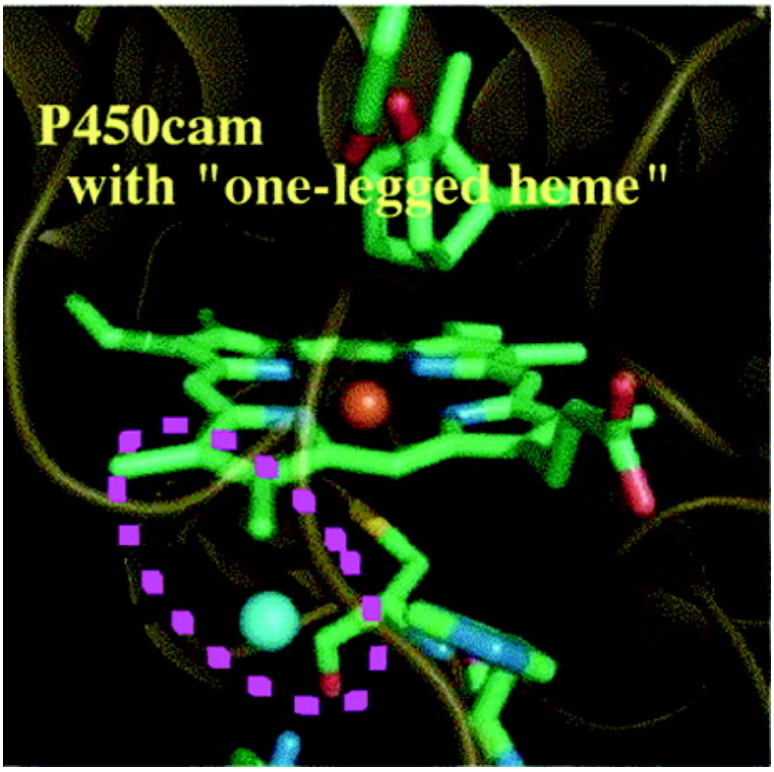
Communication

Evaluation of the Functional Role of the Heme-6-propionate Side Chain in Cytochrome P450cam

Katsuyoshi Harada, Keisuke Sakurai, Kenichiro Ikemura, Takashi Ogura, Shun Hirota, Hideo Shimada, and Takashi Hayashi

J. Am. Chem. Soc., **2008**, 130 (2), 432-433 • DOI: 10.1021/ja077902l



J|A|C|S
COMMUNICATIONS

Published on Web 12/19/2007

Evaluation of the Functional Role of the Heme-6-propionate Side Chain in Cytochrome P450cam

Katsuyoshi Harada,[†] Keisuke Sakurai,[‡] Kenichiro Ikemura,[§] Takashi Ogura,[§] Shun Hirota,[||] Hideo Shimada,*[,#] and Takashi Hayashi*[,†]

Department of Applied Chemistry, Graduate School of Engineering, Osaka University, Suita 565-0871, Japan, Institute for Protein Research, Osaka University, Suita 565-0871, Japan, Graduate School of Life Science, University of Hyogo, Ako 678-1297, Japan, Graduate School of Materials Science, Nara Institute of Science and Technology, Ikoma 630-0192, Japan, and Department of Biochemistry, School of Medicine, Keio University, Tokyo 160-8582, Japan

Received October 15, 2007; E-mail: thayashi@chem.eng.osaka-u.ac.jp

The heme *b* (protoheme IX) prosthetic group binds to the protein matrix of hemoproteins via multiple noncovalent interactions to form a holoprotein,[1] in which two peripheral heme-propionates have been generally regarded as functioning as anchors for the heme group by forming interactions with the generally polar protein matrix.[2] In the case of the cytochrome P450cam (P450cam), the monooxygenase responsible for *d*-camphor hydroxylation, the heme-6-propionate side chain forms hydrogen bonds with Thr101, Gln108, Arg112, and His355.[3] For example, the 6-propionate−Thr101 hydrogen bonding interaction is known to contribute to the thermostability of the protein.[4] The 6-propionate side chain has also been proposed[5] to participate in an electron-transfer pathway from reduced putidaredoxin (Pdx) to the heme via Arg112 located at the Pdx binding site.[6] Therefore, to understand the exact role of the 6-propionate side chain in P450cam, we prepared a novel P450cam reconstituted with a "one-legged heme" (Figure 1)[7,8] where the 6-propionate side chain of the heme is replaced with a methyl group. Here, we discuss the structural and functional roles of the 6-propionate side chain by spectroscopic and structural analyses of the reconstituted protein.

Insertion of the one-legged heme into apoP450cam was carried out according to the method of Wagner et al.[9] with minor modifications. The UV−vis spectrum of the purified reconstituted ferric protein has a Soret band wavelength at 391 nm characteristic of the high-spin state in the presence of 1 mM *d*-camphor with 100 mM KCl (Figure S1, Supporting Information).[10] The difference spectrum between CO-bound and reduced forms of the reconstituted P450cam indicates a Soret λ_{max} at 446 nm, indicating that the thiolate of Cys357 is ligated to the heme iron of the one-legged heme as seen in the wild-type protein. For the reconstituted ferric P450cam, the resonance Raman spectrum of the high-frequency region and ^{1}H NMR spectrum of the downfield region are comparable with the analogous spectra of the camphor-bound wild-type protein (Figure S2 and S3, Supporting Information). This suggests that removal of the 6-propionate side chain does not have a serious influence on the electronic and structural properties of the heme.

The rate constant of NADH oxidation by reconstituted P450cam with the one-legged heme was found to be 1150 μM min^{-1} (μM enzyme)$^{-1}$, while the rate for wild-type P450cam is 1350 μM min^{-1}(μM enzyme)$^{-1}$. The only product in the reaction with the reconstituted P450cam was 5-*exo*-hydroxycamphor, and the NADH consumption was found to be tightly coupled to total product formation as seen in wild-type P450cam. These findings indicate that the enzyme activity is maintained despite the removal of the 6-propionate side chain from the heme framework. In contrast, the Pdx affinity for the reconstituted protein is approximately 3.5-fold weaker than that observed for the wild-type protein, although the electron-transfer rate from reduced Pdx to ferric P450cam was found to be similar for both proteins (Figure S4, Supporting Information). These results indicate that the lack of the 6-propionate side chain has a slight influence on the Arg112−Pdx binding event.

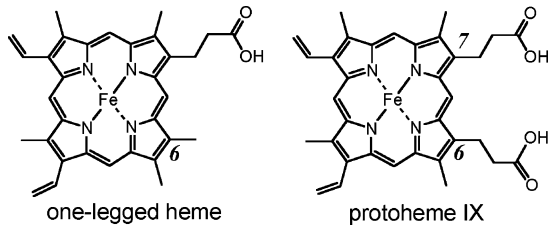


Figure 1. Structures of one-legged heme and protoheme IX.

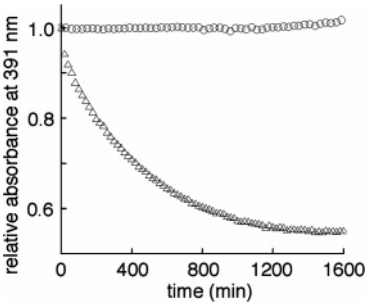


Figure 2. Time-dependent conversion of ferric P450 into an inactive P420 species for reconstituted (open triangles) and wild-type (open circles) P450cams, respectively, monitored at 391 nm in 50 mM potassium phosphate buffer at pH 7.4 containing 1 mM *d*-camphor and 100 mM KCl at 25 °C. [P450cam] = 2 μM.

Surprisingly, the reconstituted ferric P450cam was converted into an inactive species at 25 °C with a half-life of 300 min at a protein concentration of 2 μM (Figure 2) (no clear decay was observed for the wild-type ferric protein under these conditions). The final UV−vis spectrum which exhibits maxima at 369, 416, and 540 nm is similar to that obtained by pressure-induced ferric P420cam (Figure S5, Supporting Information).[11,12] This species, which is inactive toward the catalytic hydroxylation of *d*-camphor, is proposed to be the P420 structure derived from the protonation of the thiolate of Cys357 in P450cam.[12,13] The resonance Raman spectra of the reconstituted P450cam in the low-frequency region

[†] Department of Applied Chemistry, Osaka University.
[‡] Institute for Protein Research, Osaka University.
[§] University of Hyogo.
[||] Nara Institute for Science and Technology.
[#] Keio University.

 10.1021/ja077902l CCC: $40.75

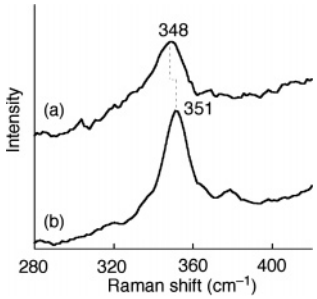


Figure 3. Resonance Raman spectra of 100 μM reconstituted (a) and wild-type (b) ferric P450cams in the 280−420 cm^{-1} region in 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM *d*-camphor and 100 mM KCl at room temperature. The excitation wavelength was 363.8 nm and the laser power was 6 mW.

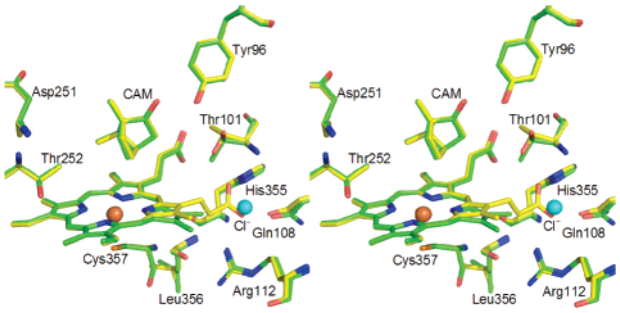


Figure 4. Superimposed structures of reconstituted (PDB ID: 2ZAW) and wild-type (PDB ID: 2ZAX) ferric P450cams. The carbon atoms of the reconstituted and the wild-type structures are shown as green and yellow sticks, respectively.

excited at 363.8 nm showed the Fe−S stretching mode at 348 cm^{-1}, which is shifted 3 cm^{-1} lower relative to the corresponding band of the wild-type protein (Figure 3).[14] Thus, the lower frequency data provide evidence that the lack of the 6-propionate side chain induces weakening of the Fe−Cys357 ligation. This in turn suggests that lengthening of the Fe−S bond facilitates the conversion to the thiolate-protonated P420 form.[15]

The X-ray structure of a tetragonal crystal of the reconstituted P450cam has been determined at a resolution of 1.55 Å at 100 K (Figure 4). Wild-type and reconstituted proteins are superimposable with a root-mean-square deviation (all Cα atoms) of 0.126 Å.[16] The one-legged heme is located in the same plane in the normal position of the heme pocket. The conformations of amino acid residues surrounding the heme pocket are generally similar to those of the wild-type protein, with the notable exception of the conformation of Thr101.[16,17] The hydroxyl group of Thr101, which interacts with the 6-propionate side chain in the wild-type protein, is found to form a hydrogen bond with Tyr96 with an intervening distance of 2.69 Å. Notably, a chloride anion occupies the position of the 6-propionate carboxylate as a counteranion. This chloride ion interacts with Gln108, Arg112, and His355 with intervening distances of 3.28, 3.27, and 2.95 Å, respectively. Although the decrease of the Pdx affinity for the reconstituted protein could originate from perturbation of the Arg112 residue and other residues in the general vicinity (vide supra), a clear difference in the geometry of Arg112 between both proteins was not seen. This is probably due to the presence of the chloride ion that replaces the 6-propionate side chain to retain the ion pair interactions and/or the formation of intermolecular tight salt-bridge with Asp202 in the crystals (Figure S6, Supporting Information). In contrast to wild-type P450cam, the thiolate of Cys357 is partially visible from the molecular surface of the crystal structure of the reconstituted P450cam. This suggests that bulk water could be accessible to Cys357 as a result of the removal of the 6-propionate side chain. Therefore, the reconstituted protein is readily converted into the inactive P420 species as a result of facile protonation of the Cys357 thiolate.

In summary, the present results clearly demonstrate two important functional roles of the heme-6-propionate side chain in cytochrome P450cam; fixation of the Pdx-binding site by the Arg112 residue, and stabilization of the Fe−S (thiolate) coordination to prevent the formation of the inactive P420 species. Particularly, the latter role indicates that the 6-propionate side chain−amino acid interaction is essential for P450cam to retain its enzyme activity.[18]

Acknowledgment. We gratefully acknowledge Prof. Tomitake Tsukihara for his valuable suggestions and help in carrying out the X-ray structure analysis. We also thank Prof. Teizo Kitagawa for the use of the resonance Raman equipment at the Okazaki Institute for Integrative Bioscience, Japan. This work is partially supported by MEXT, Japan, and JSPS.

Supporting Information Available: Experimental details, UV−vis, RR, and ^{1}H NMR spectra, kinetic data and crystal structures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Gunsalus, I. C.; Meeks, J. R.; Lipscomb, J. D.; Debrunner, P.; Munck, E. In *Molecular Mechanism of Oxygen Activation*; Hayaishi, O. Ed.; Academic Press: New York, 1974; pp 559−613.
(2) Schneider, S.; Marles-Wright, J.; Sharp, K. H.; Paoli, M. *Nat. Prod. Rep.* **2007**, *24*, 621−630.
(3) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *195*, 687−700.
(4) Manna, S. K.; Mazumdar, S. *Biochemistry* **2006**, *45*, 12715−12722.
(5) Roitberg, A. E.; Holden, M. J.; Mayhew, M. P.; Kurnikov, I. V.; Beratan, D. N.; Vilker, V. L. *J. Am. Chem. Soc.* **1998**, *120*, 8927−8932.
(6) Unno, M.; Shimada, H.; Toba, Y.; Makino, R.; Ishimura, Y. *J. Biol. Chem.* **1996**, *271*, 17869−17874.
(7) Smith, K. M.; Craig, G. W. *J. Org. Chem.* **1983**, *48*, 4302−4306.
(8) (a) Hayashi, T.; Nakagawa, T.; Harada, K.; Matsuo, T.; Hitomi, Y.; Hisaeda, Y. *Chem. Lett.* **2004**, *33*, 1512−1513. (b) Harada, K.; Makino, M.; Sugimoto, H.; Hirota, S.; Matsuo, T.; Shiro, Y.; Hisaeda, Y.; Hayashi, T. *Biochemistry* **2007**, *46*, 9406−9416.
(9) Wagner, G. C.; Perez, M.; Toscano, W. A., Jr.; Gunsalus, I. C. *J. Biol. Chem.* **1981**, *256*, 6262−6265.
(10) It is well known that under the present conditions, the wild-type protein is a *d*-camphor-bound high-spin speices.
(11) The conversion of P450 into P420 form was directly confirmed by monitoring the CO-form of the aliquots sampled from the protein solution using a UV−vis spectrophotometer.
(12) Martinis, S. A.; Blanke, S. R.; Hager, L. P.; Sligar, S. G.; Hui Bon Hoa, G.; Rux, J. J.; Dawson, J. H. *Biochemistry* **1996**, *35*, 14530−14536.
(13) Perera, R.; Sono, M.; Sigman, J. A.; Pfister, T. D.; Lu, Y.; Dawson, J. H. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3641−3646.
(14) Wells, A. V.; Li, P.; Champion, P. M.; Martinis, S. A.; Sligar, S. G. *Biochemistry* **1992**, *31*, 4384−4393.
(15) P420 can be produced by increased temperature or pressure, exposure to organic solvents, and extremes of pH. Ligand exchange to histidine from cysteine might occur to form P420 by these drastic procedures. However, the occurrence of histidine ligation is less likely for the present conversion observed at 25 °C, which includes a set of isosbestic points (Figure S5).
(16) The PDB codes of the wild-type and reconstituted proteins are 2ZAX and 2ZAW, respectively.
(17) Schlichting, I.; Berendzen, J.; Chu, K.; Stock, A. M.; Maves, S. A.; Benson, D. E.; Sweet, R. M.; Ringe, D.; Petsko, G. A.; Sligar, S. G. *Science* **2000**, *287*, 1615−1622.
(18) Recently, we also found that the functional role of the 7-propionate side chain is quite different from that of the 6-propionate side chain in P450cam. This observation will be discussed elsewhere in the near future.

JA077902L