Crystal Structure of Cytochrome $P450_{cam}$ Complexed with Its Catalytic Product, 5-*exo*-Hydroxycamphor

Huiying Li,[†] Shakunthala Narasimhulu,[‡] Lisa M. Havran,[§] Jeffery D. Winkler,[§] and Thomas L. Poulos*,[†]

Contribution from the Departments of Molecular Biology & Biochemistry and Physiology & Biophysics, University of California, Irvine, Irvine, California 92717, Harrison Department of Surgical Research, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received February 21, 1995[⊗]

Abstract: The crystal structure of the enzyme-product complex formed between cytochrome $P450_{cam}$ and 5-*exo*-hydroxycamphor has been refined to 2.2 Å and compared to the enzyme-substrate complex. The product occupies the same position as the substrate with the exception that the product 5-hydroxyl group forms a weak interaction with the heme iron atom as evidenced by continuous electron density between the product OH group and the iron atom. This interaction holds the heme iron in the low-spin configuration which prevents reduction of the heme iron by the physiological electron donor, putidaredoxin. This also prevents the wasteful transfer of reducing equivalents to the product complex.

Introduction

Cytochromes P450 catalyze the stereospecific monooxygenation of aliphatic or aromatic substrates using one O_2 derived oxygen atom. The most well understood of these enzymes is $P450_{cam}$, the camphor monooxygenase from *Pseudomonas putida*. $P450_{cam}$ catalyzes the 5-*exo* hydroxylation of camphor[1] (Figure 1). It would be useful for understanding of the entire catalytic cycle if crystal structures of each intermediate could be obtained. Fortunately, it has been possible to obtain the crystal structure of some of these intermediates.[2] The structures of the substrate free[3] and bound[4] forms are known as well as the Fe^{2+}–CO complex,[5] a model for the ferrous–oxy complex. The ferrous–oxy complex and the ferrous–deoxy complex are unstable and will require either cryogenic or rapid data collection techniques to obtain the crystal structures. The last intermediate that can be characterized using conventional crystallographic methods is the complex formed with the product 5-*exo*-hydroxycamphor. We now report the crystal structure of the product $P450_{cam}$ complex.

Experimental Section

5-*exo*-Hydroxycamphor was synthesized by reducing camphor with $LiAlH_4$ to form (−)-isobonyl acetate. Oxidation of (−)-isobornyl acetate[6] with chromium(VI) oxide[7] gave 5-ketocamphor and 4-ketocamphor in a 7:2 ratio. After separation of the isomers by column chromatography, deprotection and oxidation[7] of 5-ketocamphor gave the diketone which was reduced with LS-Selectride to give a 10:1 mixture of 5-*exo*-hydroxycamphor and 5-*exo*-hydroxyisoborenol.

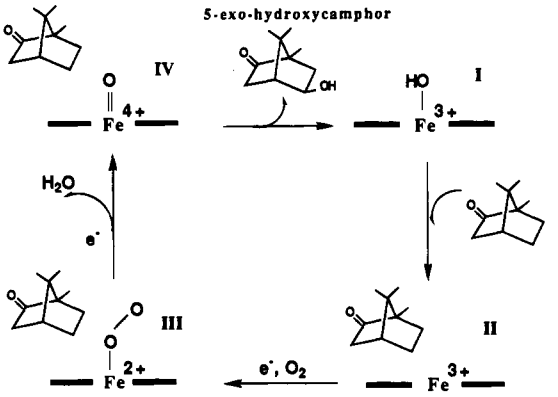

Figure 1. Abbreviated catalytical cycle for $P450_{cam}$. **I** is the ferric resting state in the low-spin form with an aqua ligand coordinated to the iron. The active site also contains a cluster of solvent molecules in the active site. Substrate binding **II** leads to the displacement of the aqua ligand and other active site water molecules to give a high-spin complex which allows for reduction and oxygenation to give the ferrous–oxy complex **III**. A second electron transfer step results in O–O bond cleavage and the hypothetical oxyferryl intermediate **IV**. In the final step, the iron-linked oxygen atom inserts into the C–H bond on C5 to give the product 5-*exo*-hydroxycamphor.

The substrate and product free cytochrome $P450_{cam}$ was crystallized in the presence of 50 mM dithiothreitol at 7 °C, utilizing the free interface diffusion method in capillaries that was previously described.[4] One week later, crystals were transferred to an artificial mother liquor consisting of 40% saturated ammonium sulfate in 50 mM potassium phosphate and 250 mM KCl, pH 7.0. A 20 mM stock solution of 5-*exo*-hydroxycamphor was made with the artificial mother liquor in the presence of 5% ethanol. Crystals were soaked with 1 mM 5-*exo*-hydroxycamphor for 24 h prior to data collection.

X-ray diffraction intensity data were collected at room temperature on a Siemens X-1000 area detector equipped with a rotating Cu Kα anode X-ray generator and a double-mirror focusing optic. Data reduction was carried out using XENGEN.[8]

* Author to whom correspondences should be addressed: FAX (714) 856-8540, E-mail poulos@uci.edu.
[†] University of California.
[‡] Harrison Department of Surgical Research, University of Pennsylvania.
[§] Department of Chemistry, University of Pennsylvania.
[⊗] Abstract published in *Advance ACS Abstracts*, June 1, 1995.

(1) *Cytochrome P450: Structure, Mechanism, and Biochemistry*; Oritz de Montellano, P., Ed.; Plenum Press: New York, 1986.
(2) Raag, R.; Poulos, T. L. *Frontiers in Biotransformations* **1992**, *7*, 1.
(3) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *Biochemistry* **1986**, *25*, 5314.
(4) Poulos, T. L.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *127*, 309.
(5) Raag, R.; Poulos, T. L. *Biochemistry* **1989**, *28*, 7586.
(6) Darby, N.; Lamb, N.; Money, T. *Can. J. Chem.* **1978**, *57*, 742.
(7) Allen, M. S.; Darby, N.; Salisbury, P.; Sigurdson, E. R.; Money, T. *Can. J. Chem.* **1978**, *57*, 733.
(8) Howard, A. L.; Gilliland, G. L.; Binzel, B. C.; Poulos, T. L.; Ohlendorf, D. H.; Salemme, F. R. *J. Appl. Crystallogr.* **1987**, *20*, 383.

0002-7863/95/1517-6297$09.00/0

Table 1. Statistics of Data Collection and Refinement of Cytochrome $P450_{cam}$ Complexed with 5-*exo*-Hydroxycamphor

cell parameters	
a (Å)	109.0
b (Å)	104.3
c (Å)	36.4
space group	$P2_12_12_1$
no. of observations	92902
no. of independent reflcns	22937
R_{merge} (%)	9.75
data completeness (%) at	
30.0−4.3 Å	99.6
4.3−3.0 Å	99.0
3.0−2.6 Å	96.0
2.6−2.3 Å	91.0
2.3−2.2 Å	79.5
no. of reflcns used in refinement with $F > 2\sigma(F)$	18162
resolution range of refinement (Å)	10.0−2.2
$R = \Sigma\|\|F_o\| - \|F_c\|\|/\Sigma\|F_o\|$ (%)	17.1
no. of protein atoms	3208
no. of water molecules	214
rms in bond lengths (Å)	0.007
rms in bond angles (deg)	1.44
coordinate error (Luzzati plot) (Å)	0.2

The initial difference Fourier $F_o - F_c$ electron density map was calculated using the observed structure factors of the 5-*exo*-hydroxycamphor complex data and calculated structure factor and phases from the refined camphor-bound $P450_{cam}$ model except the camphor was removed from the model prior to all calculations. The 5-*exo*-hydroxycamphor model was manually fit to both $2F_o - F_c$ and $F_o - F_c$ electron density maps. The complex structure then was refined by conventional positional refinement using XPLOR.[9] The *R* factor dropped from the initial 21% to 18%. The side chain conformations of some surface residues as well as solvent structure were adjusted according to the new $2F_o - F_c$ map with TOM.[10] The partial occupancies for the side chains of several surface residues and the majority of water molecules in the 1.7 Å camphor-bound $P450_{cam}$ model which was refined with PROLSQ[11] were assigned to 1.0 followed by individual temperature factor refinement. A summary of the data collection statistics and the results of crystallographic refinement are given in Table 1.

Results and Discussion

The $P450_{cam}$-product complex exhibited a UV−visible absorption spectrum (Figure 2) similar to that of the low-spin complex. The characteristic peak near 417 nm and the absence of a peak near 390 nm is indicative of a primarily low-spin complex. This is consistent with previous studies where both visible[12] and electron paramagnetic spectroscopy[13] show that product binding to $P450_{cam}$ gives a predominantly low-spin complex with a dissociation constant of ≈ 10 μM.[14]

The overall structure of the 5-*exo*-hydroxycamphor-bound $P450_{cam}$ is identical to that of the camphor-bound enzyme. The root-mean-square (rms) difference in main chain atoms between the two structures is less than 0.2 Å except for both termini of the protein and a few surface residues. The rms shift of the 10 common atoms between the product and substrate is 0.13 Å, with 5-*exo*-hydroxycamphor being closer toward the heme iron.

The initial $F_o - F_c$ difference map calculated at 2.3 Å showed excellent electron density for the 5-*exo*-hydroxycamphor molecule (Figure 3). The electron densities for each of the substiuents, the carbonyl group, the 5-hydroxy group, and the three methyl groups are well-resolved. The well-defined electron density indicates that the product is held in one unique orientation. As with the substrate, camphor, the 2-carbonyl group makes a strong H-bond (2.72 Å) to the Tyr96 side chain. The 5-hydroxyl group interacts with the heme iron atom as indicated by the extended electron density connecting the iron and oxygen atoms. The Fe−O distance initially refined to above 2.7 Å, which is between the typical ligation interaction (1.9−2.2 Å for Fe−O in P450s) and nonbonded van der Waals interaction (>3.0 Å). This generated some problems in how to model the interaction during the refinement with XPLOR.[9] The default parameters treat the Fe−O interaction as a nonbonded contact and tend to push the oxygen farther from the iron during crystallographic refinement leaving residual positive difference electron density, clearly indicating that the O and Fe atoms are closer than the van der Waals distance. Instead of using nonbonded contact restraints, trial-and-error bonded restraints were imposed until the electron density difference maps exhibited no residual difference density around the Fe−O bond. The final refined Fe−O distance is 2.68 Å using a restraint of 2.65 ± 0.05 Å.

Despite the relatively long Fe−O distance, the interaction apparently is strong enough to pull the iron atom into the porphyrin plane, achieving a hexacoordinated low spin state indicated by the spectroscopic results (Figure 2). In the substrate complex, the iron is about 0.4 Å out of the plane giving a high-spin pentacoordinated heme probably because the close juxtaposition of the camphor to the heme iron precludes the binding of an aqua ligand. It thus appears that once the substrate is hydroxylated by the iron-linked oxygen atom, the product remains in exactly the same place as the substrate enabling the formation of a weak Fe−O bond. Given these apparently specific interactions which are identical to those in the substrate complex, it is interesting that the product exhibits a 5−10-fold weaker affinity for the enzyme.[14,15] The simplest explanation lies in the difference in polar properties of the product and substrate. The product is a more polar molecule and hence might be expected to interact less favorably in the hydrophobic active site than the substrate. The relatively long product

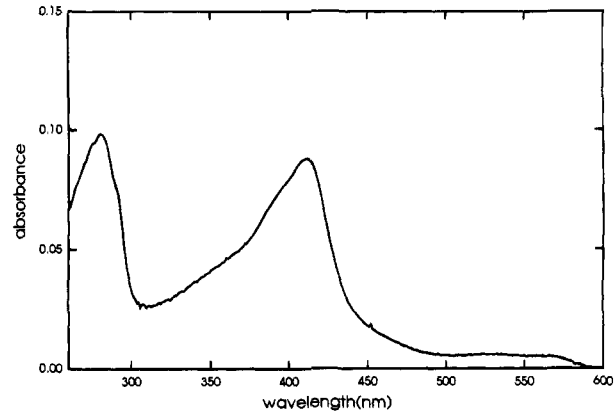

Figure 2. The absorption spectrum taken with 1.1 μM $P450_{cam}$ (redissolving the substrate-free crystals in potassium phosphate buffer) in 50 mM potassium phosphate, 250 mM KCl, and 2 mM 5-*exo*-hydroxycamphor, pH 7.0. The Soret absorption appears at 412 nm with a shoulder on the side of the shorter wavelength, which results from the absorption peak overlaps of the majority low-spin species (417 nm) and the minority high-spin species (391 nm). The high K^+ concentration favors the conversion to the high-spin state, similar to the observation found in the camphor−$P450_{cam}$ complex.[15] The high-spin species was minor in the product−$P450_{cam}$ complex in contrast to the substrate−$P450_{cam}$ complex wherein the high-spin form is predominant at similar ionic strengths.

(9) Brunger, A. T. *X-PLOR, Version 3.1, A System for X-ray Crystallography and NMR*; Yale University Press: New Haven, CT, 1992.
(10) Cambillau, C.; Horjales, E. *J. Mol. Graph.* **1987**, *5*, 174.
(11) Hendrikson, W. A. *Methods Enzymol.* **1985**, *115*, 252.
(12) Pederson, T. C.; Austin, R. H.; Gunsalus, I. C. In *Microsomes and Drug Oxidations*; Ullrich, V., Roots, I., Hildebrandt, A. H., Estabrook, R. W., Cooney, A. H., Eds.; Pergamon Press: New York, 1977; p 275.
(13) Lipscomb, J. D. *Biochemistry* **1980**, *19*, 3590.
(14) Atkins, W. M.; Sligar, S. G. *J. Biol. Chem.* **1988**, *263*, 18842.
(15) Peterson, J. A. *Arch. Biochem. Biophys.* **1971**, *144*, 678.

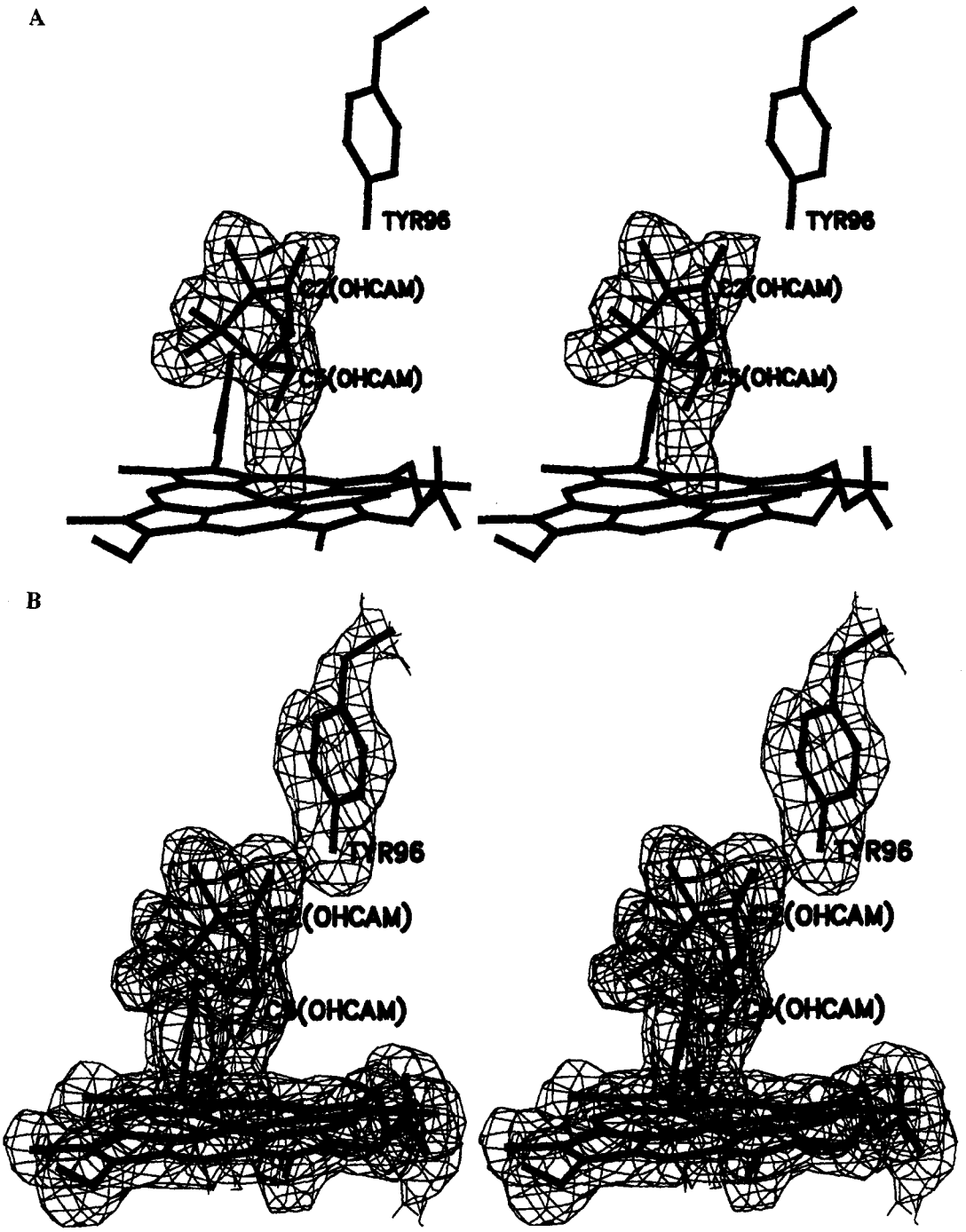

Figure 3. Stereoscopic views of the active site in the enzyme-product complex. (A) The initial $F_o - F_c$ electron density contoured at 3σ where σ is the root-mean-square difference electron density computed over the entire asymmetric unit. The model used for the phase calculation is the refined $P450_{cam}$-camphor complex[4] except the substrate was eliminated from the structure factor calculation. Note the continuous electron density between the product 5-OH group and the iron atom. (B) The final $2F_o - F_c$ electron density map at 2.2 Å showing the fit of the product, heme, and Tyr96. Again the electron density between the product 5-OH and iron atoms is continuous. The side chain OH group of Tyr96 forms an H-bond with the product C2 carbonyl group exactly as it does in the $P450_{cam}$-substrate complex.[4]

Fe−O interaction then would be insufficient to overcome the unfavorable desolvation of the product OH group required to enter the active site. Hence, the explanation may be differences in solvation properties.

A weak interaction between the product OH and iron could provide a possible control mechanism for electron flow. $P450_{cam}$ can accept electrons from its iron−sulfur protein donor only when the enzyme is in the high-spin substrate-bound complex.[16] Substrate binding also leads to an increase in redox potential which makes the reduction of the substrate-bound $P450_{cam}$ thermodynamically favorable.[16] Without substrate bound to the heme iron, electron transfer is thermodynamically unfavorable. Formation of the Fe−O interaction in the product complex results in a low-spin heme, which will inhibit reduction of the iron in $P450_{cam}$ and, possibly, lower the redox potential thereby preventing the possible further nonspecific oxidation of product or wasted electron flow.

Acknowledgment. This work was supported in part by the NIH Grant No. GM 33688 (T.L.P.). We thank Dr. J. R. Cupp-Vickery for kindly providing the purified recombinant $P450_{cam}$ sample for this study.

JA950593R

(16) Sligar, S. G.; Gunsalus, I. C. *Proc. Natl. Acad. Sci U.S.A.* **1976**, *73*, 1078.