

Title	Studies of roles of the amino acid residues in the vicinity of the active site in cytochrome P450cam
Author(s)	Sakurai, Keisuke
Citation	大阪大学, 2009, 博士論文
Version Type	VoR
URL	https://hdl.handle.net/11094/793
rights	
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

The University of Osaka

DEP 13192

# Studies of roles of the amino acid residues in the vicinity of the active site in cytochrome P450cam

A Doctoral Thesis by Keisuke Sakurai

Submitted to the Graduate School of Science, Osaka University Japan

February, 2009

# Studies on roles of the amino acid residues in the vicinity of the active site

# in cytochrome P450cam

チトクロム P450cam の活性部位近傍のアミノ酸残基の役割の研究

# A Doctoral Thesis

by

Keisuke Sakurai

# Submitted to the Graduate School of Science,

Osaka University

Japan

February, 2009

## Acknowledgements

This work has been carried out under the direction of Professor Tomitake Tshukihara of Institute for Protein Research, Osaka University, Professor Takashi Hayashi of Graduate School of Engineering, Osaka University, and Professor Hideo Shimada of Picobiology Institute, Graduate School of Life Science, University of Hyogo. Without each incessant guidance from another field, I could not have completed this work. I appreciate Professor Atsushi Nakagawa and Dr. Tsunehiro Takano of Institute for Protein Research, Osaka University and Professor Kazumasa Muramoto of University of Hyogo for teaching me about the basic X-ray crystallography. I really thank Dr. Kunitoshi Shimokata of Keio University and Dr. Sachie Kayatama of Picobiology Institute, University of Hyogo for giving me preparation method of protein. I appreciate Dr. Takako Hishiki of Keio University for giving me crystallization method of protein. Especially, I deeply thank Dr. Eiki Yamashita, and Professor Mamoru Suzuki of Institute for Protein Research, Osaka University for the instruction and help about data collection at SPring-8 and Photon Factory, respectively. I really appreciate all members of Laboratory of Protein Crystallography and Laboratory of Supramolecular Crystallography in Institute for Protein Research for their useful help and discussion.

Finally, I deeply thank my family for appreciating and supporting me.

Keisuke Sakurai

February, 2009

## **Table of Contents**

Chapter 1: General Introduction	1
1-1. Cytochrome P450	1
1-2. Cytochrome P450cam	6
1-3. Purpose of this work	11
Chapter 2: Experimental Procedures	18
2-1. Overview	18
2-2. Preparations of proteins	18
2-3. Crystallization and crystal manipulating	21
2-4. X-ray experiments and structure determination	22

# Chapter 3: Substrate Binding Induced Protein Structural Changes of Cytochrome P450cam Related to Redox Potential Elevation

	29
3-1. Introduction	29
3-2. Materials and methods	30
3-3. Results and discussion	32

# Chapter 4: Role of Asp297 of Cytochrome P450cam in Exclusion of Waters From the Active Site Occurring Upon the Substrate Binding

42

4-1. Introduction	42
4-2. Materials and methods	43
4-3. Results and discussion	46
Chapter 5: Conclusion	96
References	98
List of Publications	102

## Abbreviations

CCP4	Collaborative Computational Project 4, 1994
DTE	dithioerythritol
E. coli	Escherichia coli
FAD	flavine adenine dinucleotide
HS	high-spin species
IPTG	isopropyl-β-D(-)-thiogalactopyranoside
K <sub>d</sub>	dissociation constant
KEK	High Energy Accelerator Research Organization
KPi	potassium phosphate buffer
LB	lysogeny broth
MAD	multiple wavelength anomalous dispersion
MIR	multiple isomorphism replacement
MPD	2-Methyl-2,4-pentandiol
MR	molecular replacement method
NADH	nicotinamide adenine dinucleotide
NHE	normal hydrogen electrode
P450	cytochrome P450
P450cam	cytochrome P450cam
PDB	protein database
PEG	polyethylene glycol
P. putida	Pseudomonas putida
r.m.s.	root mean square
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ТВ	terrific broth
Tris	tris (hydroxymethyl) aminoethane

## **Chapter 1**

#### **General introduction**

#### 1-1. Cytochrome P450

Cytochrome P450 (P450) is a very large and diverse superfamily of hemoproteins found in all domains of life (Danielson, 2002). P450s use a plethora of both exogenous and endogenous compounds as substrates of the enzymatic reactions. They usually form a part of multicomponent electron transfer chains, called P450-containing systems. The most common reaction catalyzed by cytochrome P450 is a monooxygenase reaction, e.g. insertion of one atom of molecular oxygen into an organic substrate (RH) while the other oxygen atom is reduced to water:

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O$$
(1)

P450s have been identified in all lineages of life, including mammals, birds, fish, insects, worms, sea squirts, sea urchins, plants, fungi, slime molds, bacteria and archaea. More than 7,700 distinct P450 sequences were known by September 2007, and the structures of some P450s were determined by X-ray crystallography (Figure 1-1). Human has 57 P450 genes (Human Cytochrome P450s). A lot of P450 genes are found in plants. For instance, Rice (*Oryza sativa*) has over 400 genes, of which functions are hardly understood.

All P450 consist of about 500 amino acid residues and have heme b in the active site. A conserved cysteine residue and a water molecule are ligands of the iron atom of heme in the resting state of the enzyme. The enzyme releases the water

molecule when it accepts the substrate to raise the redox potential of the heme iron, which permits the reduction of heme by the electron transfer system.

The name cytochrome P450 is derived from the fact that these are colored ('chrome') cellular ('cyto') proteins with a "pigment at 450 nm", so named for the characteristic Soret peak near 450 nm when the enzyme having the reduced heme iron forms the complex with carbon monoxide.



Figure 1-1. A part of crystal structures of P450 superfamily. Each model was drawn so that the propionate side chain comes under heme.

P450 enzymes play crucial roles in the oxidation of endogenous and exogenous compounds in biological systems (Ortiz de Montellano, 2005). P450s are involved in numerous biological processes including the biosynthesis of lipids, steroids, antibiotics, and the degradation of xenobiotics (Table 1-1). P450s are major enzymes involved in drug metabolism, accounting for about 75 % of the total metabolism (Guengerich, 2008). Hence, the P450 enzymes have been extensively studied in the field of medicine and pharmacy. Furthermore, P450 family are involved in the biosynthesis of a variety of hormones in plants and the biosynthesis of hormones regulating the metamorphosis and the expression of tolerance toward agricultural chemicals in insects. Therefore, they are also studied in the field of agriculture. In addition, because P450 can catalyze regio- and stereo- selective hydroxylation of substrates under mild conditions such as normal temperature and pressure (Dawson *et al*, 1996), it has been tried to apply its catalysis (Arnold *et al*, 1996, Jones *et al*, 2002, Sligar *et al*, 1993, and Rao *et al*, 2003).

P450 species are ligated by thiolate of cysteine and its carbonmonooxide (CO) complex shows the characteristic absorption around 450nm. On the other hand, P420 species is the denatured state of P450 with no catalytic activity and shows absorption around 420 nm when it binds CO and has no catalytic activity. CO-ligated hemoglobin and myoglobin, where fifth ligand is imidazole of histidine, also show absorption maxima at 420 nm. Hence, it has been proposed that the fifth ligand thiolate is replaced with imidazole of histidine or that the thiolate ligand is protonated in P420 species (Figure 1-2).



Table 1-1. Typical reactions catalyzed by cytochrome P450 (Harada, 2008)



Figure 1-2. The heme ligation structures of cytochrome P450, P420.

#### 1-2. Cytochrome P450cam

Cytochrome P450cam (P450cam) from *Pseudomonas putida* (Figure 1-3) catalyzes the regio- and stereospecific hydroxylation of camphor to 5-exo-hydroxycamphor as shown in Scheme 1. Its primary structure consists of 415 amino acids including unprocessed precursor, and its molecular weight is 46669 Da. P450cam is used as a model for many cytochrome P450s and is the first member of the P450 superfamily whose three-dimensional structure has been determined. Both the binary enzyme-substrate (Poulos *et al*, 1987) and enzyme-product complexes have been so characterized (Poulos *et al*, 1995).



Figure 1-3. The picture of *Pseudomonas putida*. *P. putida* which is a gram-negative rod-shaped non-spore forming saprotrophic soil bacterium, metabolizes camphor to sugar under the oligotrophic environment. Catalysis of P450cam is the first step of the cascade reaction to consume camphor as energy.

The P450cam molecule has a triangular shape with a side of 60 Å and a thickness of 30 Å including the active site heme plane nearly parallel to the plane of the triangle. The heme is deeply embedded in the hydrophobic interior with no significant exposure of the protein outer surface. The heme iron is ligated by the thiolate derived from Cys357 as a fifth ligand. The three NH of main chains of the loop form three hydrogen boundings with the sulfur atom of the ligand cysteine and

stabilize the Fe-S bond with the distances of 3.2-3.6 Å. The identity of the proximal heme iron ligand as a cysteine has been established based on earlier spectroscopic works by Dawson *et al* (1996). Furthermore, it was confirmed that the specific cysteine was bound to heme iron by the crystal structure analysis reported by Poulos *et al.* (1987). The sixth ligand binding side is located opposite side of the fifth ligand relative to the heme plane, where various exogenous ligands (water, gaseous molecules ( $O_2$ , CO, NO, etc.), substrates and inhibitors.) can be found. The long I-helix exists above the heme binding side and the V-shaped F and G helices are above the I-helix (Figure 1-4).



Figure 1-4. The structure of cytochrome P450cam (PDB ID: 2CPP). Each alphabet is the helix identifier. Helix F, G and I represent cyan, beige and lime, respectively.

The resting state of the enzyme possesses the hexacoordinated low-spin ferric heme with the water as the sixth ligand opposite to the proximal cysteine. Substrate binding to the binding site of heme excludes the water molecule coordinated to heme iron and leads to the generation of the pentacoordinated high-spin ferric heme state with a vacant coordination site which is available for dioxygen binding over the heme iron (Figure 1-5 II). The conversion of the ferric heme from low- to high-spin results in a significant increase of the redox potential of the heme iron from -330 to -170 mV (vs. NHE) (Gunsals et al, 1976). This positive shift of the redox potential facilitates the electron transfer from the reduced putidaredoxin  $(Pdx^{red})(E = -196 \text{ mV vs. NHE})$ (Gunsals et al, 1976)), the redox partner of P450cam, to the ferric heme of P450cam. Subsequently, the first electron transfer occurs from Pdx to P450cam and the pentacooedinated high-spin ferrous P450cam is generated (Figure 1-5 III). The dioxygen molecule binds to the ferrous heme of P450cam to generate the oxygenated intermediate (Figure 1-5 IV). The second electron from the reduced Pdx reduces the oxygenated enzyme to the ferric peroxy species (Figure 1-5 V). The protonation of the distal oxygen in the peroxo-iron complex produces a hydroperoxo species, and the subsequent protonation leads to the heterolytic O-O cleavage releasing a water molecule to form the oxyferryl species (Figure 1-5 VI). Oxygen atom transfer from the iron-oxo complex to the substrate (Figure 1-5 VII), presumably by the oxygen rebound mechanism (Groves et al, 1976), yields the oxidized product and regenerates the resting state of the enzyme (Figure 1-5 I). The electrons necessary to the reaction catalyzed by P450cam originated from NADH, whose electrons are mediated by putidaredoxin and putidaredoxin reductase (Schlichting et al, 2004). Electrons from NADH are delivered to the putidaledoxin reductase, which contains flavine adenine

dinucleotide (FAD) (Mr = 45.6 kDa). The putidaredoxin (Mr = 11.6 kDa) serves as a one-electron shuttle between putidaredoxin reductase and P450cam (Scheme 1).



**Figure 1-5.** The proposed catalytic reaction mechanism of P450cam. In the substrate-free state (the resting state), the substrate binding site is occupied by water molecules and heme is ferric low-spin species (I). When camphor, the substrate, binds to P450cam, heme is ferric high-spin species (II). Next, heme changes ferric to ferrous with an electron from putidaredoxin (PdX) (III). Afterwards, heme binds to oxygen (IV) and oxygenated species (IV) are changed activated oxygen species (VI) called "compound I" by accessing one electron and two protons. Camphor is hydroxylated by the activated oxygen with nucleophilic attack (VII). 5-exo-hydroxycamphor, the product, is expeled from the substrate binding site rapidly.



Scheme 1. Hydroxylation of *d*-camphor catalyzed by cytochrome P450cam.

#### 1-3. Purpose of this work

Although about 20 years have passed since the first structure of ferric cytochrome P450cam was determined (Poulos *et al*, 1987), the enzyme reaction of P450cam is not completely understood yet. P450cam has unique structure, of which the substrate binding site is located at the center of the protein interior, and is not accessed from the outer bulk solvent. Few structures of the proteins whose substrate binding sites were buried in the protein interior like P450cam have been determined. Therefore, the substrate binding mechanism of P450cam has been extensively investigated as a key step of the reaction mechanisms. However, the mechanism of the water expulsion filled in the substrate binding site in the resting state was hardly studied. Here, the author will discuss the reaction mechanism in the vicinity of the reaction site when the substrate is bound to P450cam.

# 1-3-1. Substrate binding induced protein structural changes of cytochrome P450cam

It had been reported that threonine101 (Thr101) of P450cam located in the vicinity of the substrate binding site and was hydrogen bonded to heme-6-propionate (Schlichting *et al*, 1997). However, in another paper, Thr101 bound to Tyr96, not to 6-propionate (Poulos *et al*, 1987). In the previous study, it has been understood that Thr101 had a multiple conformation bound to Tyr96 and the 6-propionate (Harada *et al*, 2008). In this study, the conformation of Thr101 in wild type p450cam was determined and the functional meaning of the rotamer of Thr101 was discussed.

#### 1-3-2. Elucidation of water expelling mechanism in cytochrome P450cam

Water molecules often occupy the substrate-binding site in the interior of enzymes when the substrate is absent. The water cluster might be essential not only for the stabilization of the resting state structure but also for the binding substrate, because the exclusion of the water molecules from the substrate binding site would be thermodynamically important trigger for the substrate binding event. However, there has been no study on the mechanism of the water exclusion from the substrate binding site, although in the case of cytochrome P450, a specific water exclusion pathway was proposed from the theoretical consideration of the structures (Oprea *et al*, 1997).

In 1987, the three-dimensional structure of P450cam-substrate complex was determined by X-ray crystallography (Poulos *et al*, 1987). The substrate *d*-camphor deeply buries in the protein interior and the outer molecular surface is not connected to the inner active site surface (Figure 1-6), indicating that there is no apparent pathway for *d*-camphor from the molecular surface to the binding site. It is suggested that P450cam must undergo structural fluctuations to allow the substrate access and the product exit. The structural analyses of P450cam complexed with artificial substrate analogues (Figure 1-7) have elucidated the pathway extending from the molecular surface to the substrate additional substrate access and the product surface to the substrate binding site (Dunn *et al*, 2001, and Hays *et al*, 2004). The elucidated *d*-camphor pathway is consisted of many hydrophobic residues such as Tyr29, Phe87, Phe98, Phe193, Val247, Ile395, and Val396, etc. (Figure 1-8).



**Figure 1-6.** Molecular surface analysis of camphor-bound P450cam. The active-site molecular surface and the outer molecular surface of P450cam are not connected with each other. Camphor is shown in the active site above the heme group. The molecular surface was computed with the PyMOL (version 0.99r6, using a standard probe radius of 1.5Å).



Figure 1-7. Elucidation of d-camphor pathway. Upper model is P450cam with delta-bis(2,2'-bipyridine)-(5-methyl-2-2'-bipyridine)-c2-adamantane ruthenium (II) (PDBID: 1K2O). This model was solved by Dunn et al. The adamantane derivative, which is the artificial with conformation. Lower model is P450cam substrate-analog has multi adamantane-1-carboxylic-acid-5-dimethylamino-naphthalene-1-sulfonylamino-butyl-amide (PDBID: 1RF9). This model was solved by Hays et al. The substrate analogs and heme are shown in stick models (with black carbons, red oxygens, blue nitrogens, orange iron, and cyan ruthenium), and proteins are shown in rainbow cartoon models.



Figure 1-8. Camphor-free ferric P450cam and *d*-camphor pathway. It is assumed that the black hydrophobic residues leaded *d*-camphor to binding site and camphor pathway is indicated by red arrow.

In the resting state of the enzyme, the *d*-camphor binding site is occupied by a water cluster (6 molecules), one of them is the heme-iron bound water. The *d*-camphor binding to the active site eliminates the water cluster from the site, changing the spin state of heme iron from low to high, which allows rapid reduction of the enzyme by the electron transfer system. Thus, the water elimination is essential for the efficient catalytic reaction. The waters might be expelled through the pathway for *d*-camphor access channel formed transiently when *d*-camphor binds to the enzyme. A theoretical study reported by Opera *et al.* postulated that the water

molecules in the substrate binding site could be expelled not through the *d*-camphor binding pathway but through a space transiently formed by metastable rotamer of Arg299, which forms salt-bridge with the heme-7-propionate in the X-ray structure (Opera et al, 1997). However, any experimental evidences of the rotamer of Arg299 have never been provided. Recently, the author's group found that removal of the heme-7-propionate in cytochrome P450cam results in the formation of water array connecting the bulk water and the active site, accompanying the conformational changes of nearby amino acid residues (Asp297 and Gln322) (Hayashi et al, 2009), These two residues form a hydrogen bonding network with the heme-7-propionate and Arg299 (the hydrogen bonding tetrad network) in the wild type protein The tetrad blocks water inlet from the bulk the water exodus from the active site. This removal of the heme peripheral side chain decreases over 1000-fold the d-camphor binding affinity of the enzyme, presumably due to the stabilization of the water cluster at the substrate-binding site by the water array extending from bulk water (Hayashi et al, 2009). These finding led us to hypothesize that the hydrogen boding tetrad network regulates the water exodus from the active site and the water inlet from the bulk and that Asp297 is essential to the transient formation of the water gate.

Here, to study the mechanism of the water exclusion occurring upon *d*-camphor binding to the enzyme, P450cams was mutated at Asp297 to asparagine, alanine, and leucine. The properties and X-ray structure of the mutant proteins strongly support our working hypothesis that Asp297 and nearby residues transiently forms the pathway for the waters expelled from the active site upon the *d*-camphor binding.



Figure 1-9. Plausible water pathway. The water cluster in the substrate binding side could be expelled through a space transiently formed by a cleavage of the 7-propionate-Arg299 or -Asp297salt-bridge.

## Chapter 2

#### **Experimental Procedures**

#### 2-1. Overview

In this work, preparation, X-ray diffraction data harvesting, and structure analyses on many conditions were performed.

#### 2-2. Preparations of proteins

#### 2-2-1. Preparations of the wild-type P450cam

The wild-type cytochrome P450cam was expressed in *Escherichia coli*, strain JMI09, and purified by a previously described procedure with minor modifications (Imai *et al*, 1989). The *E. coli* carrying a gene for the wild-type cytochrome P450cam was grown for 10 hours at 37 °C in 7.5 mL of LB media containing ampicillin (50  $\mu$ g / mL) with vigorous shaking (200 rpm). The culture was then added to 150 ml of LB media containing ampicillin (50  $\mu$ g / mL) with vigorous shaking (200 rpm). The culture was then added to 150 ml of LB media containing ampicillin (50  $\mu$ g / ml) in 500 ml flask with baffle and was incubated at 37 °C for 13 hours with vigorous shaking (200 rpm). Then, 30 mL of the culture was added to 1.5 L of TB media containing ampicillin (50  $\mu$ g / mL) and isopropyl- $\beta$ -D(-)-thiogalactopyranoside (IPTG) (0.24 g / L) in 5 L flask with baffle and was incubated at 37 °C for 10 hours with vigorous shaking (220 rpm). The bacteria were harvested by centrifugation (22,000 × g for 20 min at 4 °C). The total wet weight of bacteria obtained from 6 L of culture media was ca. 90 g (wet weight).

It was stored at -80 °C. The purification of the wild-type P450cam was done at 4 °C unless otherwise indicated. It was lysed by treatments with lysozyme (50 mg of lysozyme per 50 g wet weight of bacteria) (At the same time, RNase A (10 mg/ml  $\times$ 100  $\mu$ L) and DNase I (5 U /  $\mu$ L × 160  $\mu$ L) were added) and with ultrasonication in 40 mM potassium phosphate buffer (pH 7.4) containing 1 mM d-campbor (buffer A) (200 mL of buffer per 50 g wet weight of bacteria). The bacterial lysate was loaded on an anion-exchange column DE52 ( $\varphi$  2.5 cm  $\times$  47 cm, volume = 230 ml) equilibrated with buffer A, and the column was developed with a linear 0-0.4 M KCl gradient (1.5 L total volume) in the same buffer (flow rate was about 1.5 mL/min.). The eluate containing the cytochrome P450cam was precipitated using ammonium sulfate from 40% (supernatant) to 60% (precipitate), and dialyzed against buffer A. The cytochrome P450cam purified by DE52 anion-exchange column chromatography and ammonium sulfate fractionation was further purified by gel filtration on a Sephacryl S-200 column (  $\varphi$  2.6 cm × 120 cm, volume = 640 mL) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM d-camphor and 50 mM KCl. The fractions containing the cytochrome P450cam with  $A_{391}/A_{280}$  ratio of more than 0.8 were collected and concentrated. The enzyme was further purified by an affinity column Blue Sepharose 6 Fast Flow ( $\varphi$  1.5 cm  $\times$  34 cm, volume = 60 mL) equilibrated with 20 mM potassium phosphate buffer (pH 7.4) containing 0.2 mM d-camphor. The column was washed with an column volume of 20 mM potassium phosphate buffer (pH 7.4) containing 0.2 mM al-camphor and was developed with a linear 0-0.2 M KCl gradient (500 mL total volume) in the same buffer (flow rate was about 0.7 mL / min.). The fractions containing the cytochrome P450 with  $A_{391}/A_{280}$ ratio of mere than 1.5 were collected and concentrated. The buffer was exchanged to

50 mM potassium phosphate buffer (pH 7.4) containing 1 mM *d*-camphor and 50 mM KCl, and concentrated into a 1 mM solution. Further, the concentrated sample was frozen with liquid nitrogen and stored at -80 °C. The enzyme samples with an absorption ratio of  $A_{391}/A_{280} > 1.5$  were used for this study. Concentration of the wild-type protein was spectrophotometrically detemined using the extinction coefficient of 102 mM<sup>-1</sup>cm<sup>-1</sup> at 391 nm (Gunsauls *et al*, 1978).

#### 2-2-2. Preparations of the camphor-free wild-type P450cam

The camphor-free sample was prepared by passing the camphor-bound P450cam through a Sephadex G-25 column equilibrated with 50 mM Tris-HCl (pH 7.4). The fractions containing the camphor-free P450cam with  $A_{535} > A_{569}$  were collected.

#### 2-2-3. Camphor affinity determination

#### Dissociation constant:

3 ml of camphor-free P450cam solution (ca. 1.7  $\mu$ M in 20 mM KPi (pH 7.4) containing 100 mM KCl) in 10 mm quarz cell was titrated with aliquots of 3 mM *d*-camphor in 20 mM KPi (pH 7.4) containing 100 mM KCl at 20 °C. Binding was followed by monitoring the decrease in absorbance at 417 nm, the Soret peak of the camphor-free protein. Kd value of P450cam for *d*-camphor was determined using a plot for an equation  $\Delta A = \Delta$ [substrate] / ([substrate] + K<sub>d</sub>), where  $\Delta A$  and  $\Delta A_{\infty}$  are absorption changes upon addition of substrate at 0 < [substrate] <  $\infty$  and [substrate] =  $\infty$  (extrapolated), respectively, and [substrate] is free substrate concentration.

#### Population of high spin species:

The *d*-camphor-free P450cam has a low spin heme with the absorption maxima at wavelength of 570, 540, and 417 nm. Upon *d*-camphor binding, P450cam change the spin state from low to high with the absorption maxima at 646, 512, and 391 nm. 3 ml of P450cam solution (ca. 1.7  $\mu$ M in 20 mM KPi (pH 7.4) containing 100 mM KCl) in 10 mm quarz cell was titrated with aliquots of 3 mM *d*-camphor in 20 mM KPi (pH 7.4) containing 100 mM KCl at 20 °C. The population of high spin species is determined by measuring the absorbance at 391 nm. The wild type camphor-bound p450cam is calculated as 100%.

#### 2-3. Crystallization and crystal manipulating

#### 2-3-1. Crystallization

Crystals of the protein were grown using the sitting-drop vapor diffusion method. Three to five  $\mu$ l of P450cam solution [30 mg/ml P450cam, 250 mM KCl, 10 mM DTE, 1 mM *d*-camphor] was mixed with an equal volume of the reservoir solution [50 mM tris-HCl (pH 7.4), 250 mM KCl, 10 mM DTE, 1 mM *d*-camphor, and 22 to 30 % (w/v) PEG 4000] and allowed to stand at 268 K (-5 °C) for 2 days.

#### 2-3-2. Camphor soaking

Crystals of proteins grown in the crystallization buffer were transferred to the reservoir solution saturated with *d*-camphor and allowed to stand at 268 K for 1 day. The *d*-camphor saturated solution was prepared by adding excess amount of fine powdered *d*-camphor to the reservoir solution and stirred extensively for one day.

Undissolved *d*-camphor was removed from the solution.

#### 2-3-3. Crystal freezing

The crystals were equilibrated with a solution containing 20 %(v/v) MPD (2-Methyl-2,4-pentandiol) as a cryoprotectant and they were frozen in liquid nitrogen.

#### 2-4. X-ray experiments and structure determination

#### 2-4-1. Overview

In a X-ray diffraction experiment, electrons of the ordered atoms in a crystal diffract the X-rays in defined directions of space. We measure the intensities of each reflection by recording the diffraction pattern on a detector. To reconstruct the electron density (and thus the shape of the atoms and molecules diffracting the X-rays) by Fourier transformation, however, we need two components for each reflection, hkl:

$$\rho(\mathbf{x}\mathbf{y}\mathbf{z}) = V^{1}\Sigma\Sigma\Sigma \left| F(hkl) \right| \exp[-2\pi i(hx + ky + lz) + i\alpha(hkl)]$$
(2)

In this equation, |F(hkl)| is structure factor corresponding to the intensity of diffraction spot. The *h*, *k*, and *l* are miller indices. *V* and  $\alpha(hkl)$  means a volume of unit cell and a phase angle of each reflection, respectively. For determination of crystal structure, |F(hkl)| and  $\alpha(hkl)$  must be determined. I(hkl), which is an intensity of diffraction spot, directly proportional to  $|F(hkl)|^2$  and can be determined from experimental data. In the case of macromolecule crystal,  $\alpha(hkl)$  cannot be

determined from a data set. Therefore, to solve crystal structure of macromolecule, phases have to be determined by another methods. There are two methods to overcome the phase problem. One is experimental method such as multiple isomorphism replacement method (MIR) (Green *et al.* 1954) and multiple wavelength anomalous dispersion method (MAD) (Okaya and Pepinsky 1956). The other one is molecular replacement (MR) method (Rossmann and Blow 1962). Since MR method is efficiently applicable to homologous protein structure, the author used MR method to determine all structures with previously determined P450cam structure. Once initial phases was determined by placing the known P450cam structure in the unit cell of a target crystal, we build an initial model structure by tracing a calculated electron density. Some program packages were used for refinement of the structures. By rebuilding the model structure by manual and refinement program, the model structure approaches to real structure. To monitor a refinement going well or not, *R* factor and free *R* factor are calculated at each steps.

#### 2-4-2. Intensity data havesting

To calculate electron density map, structure factor amplitude should be determined. Described in section 2-4-1, |F(hkl)| can be obtained from intensity data.

A program of truncate (French and Wilson, 1978) is for evaluating structure factor amplitude, |F(hkl)|, from observed intensity data using truncate procedure by making intensity statistics.

After truncate procedure, a unique list of reflections was generated with program Unique. The program is used to tag each reflection with a flag. The resulting

reflection file is used for calculating of free *R* factors ( $R_{\text{free}}$ ) (Brünger 1992). In this study, 5% randomly selected reflections were used for calculating  $R_{\text{free}}$ .

#### 2-4-3. Molecular replacement

The structure determination by the molecular-replacement method, consists of two steps. In the first step, the rotation function is calculated to determine rotation matrix, and in second step, the translation function is calculated to determine translation matrix. Both steps, the Patterson function is used, which is the function directly calculable from observed diffraction. The equation of the Patterson function is shown:

$$P(\boldsymbol{u}) = P(\boldsymbol{u}, \boldsymbol{v}, \boldsymbol{w}) = V^{1} \Sigma_{h} \Sigma_{k} \Sigma_{l} |F(hkl)|^{2} \cos[2\pi (h\boldsymbol{u} + k\boldsymbol{v} + l\boldsymbol{w})]$$
(3)

In this equation, (u, v, w) is Patterson vector corresponding to an inter-atomic vector. The Patterson function in a crystal may be considered to have two components; vectors between scattering canters in the same subunit, and those between different subunits. The intra-molecule vectors are necessarily shorter than the maximum distance of atoms in a subunit in the crystal, so they are mostly magnitude of the subunit dimension or longer. By considering the region closer to origin of Patterson function, it is possible to include a high proportion of intra-molecule vectors.

#### 2-4-4. Rotation function

A search model that is similar to the target molecule in tertiary structure is

selected from known structures. The intra-molecular vectors of the search molel are included in the Patterson function vectors of the target crystal structure. In the first step, we have to know the orientation of model molecule in the target crystal. The matrix relating between the search model and the target molecule can be determined by calculating a rotation function. The rotation function is described as follows:

$$R(\alpha, \beta, \gamma) = \int_{\mathbf{u}} P_{\text{obs}}(\boldsymbol{u}) \times P_{r}(\boldsymbol{u}_{r}) d\boldsymbol{u}$$
(4)

In this equation,  $(\alpha, \beta, \gamma)$  means Euler angles.  $P_{obs}(u)$  and  $P_r(u_r)$  are observed Patterson function and rotated model Patterson function, respectively. The Patterson function of search model molecule can be overlapped well with the Patterson function of target crystal by rotating its Patterson function. The rotation function gives a high value if the model Patterson function would be rotated to the similar orientation of a target molecule in the crystal. Rotation function can determine only the relative orientation between model molecule and target molecule, then next step we should determine the position in cell in the next step.

#### 2-4-5. Translation function

As with the rotation problem, the translation problem is solved by a search using Patterson function. The equation of translation function is shown:

$$T(t) = \int_{V} P_{\text{calc}}(u, t) \times P_{\text{obs}}(u) du$$
(5)

Translation function involves a comparison between the observed Patterson

function  $(P_{obs})$  and calculated Patterson function  $(P_{calc})$  calculated by moving a search model with a orientation determined by the Rotation function in an asymmetric unit. A *t* is the translational vector for the model molecule in a crystallographic asymmetric unit, and *t* includes the information of real position.  $P_{calc}(u,t)$  should be almost same as P(u), when parameter *t* indicates correct position, which means T(t) exhibiting high value.

In process searching the translation position, R factor and correlation coefficient are evaluated. The equations are described below:

R factor

$$R = \sum_{hkl} \left| \left| F_{obs} \right| - k \left| F_{calc} \right| \right| / \sum_{hkl} \left| F_{obs} \right|$$
(6)

Correlation coefficient (CC)

$$CC = \sum_{hkl} (F_{obs} - |F_{obs}|) (F_{calc} - |F_{calc}|) / [\sum_{hkl} (F_{obs} - |F_{obs}|)^2 (F_{calc} - |F_{calc}|)^2]^{1/2}$$
(7)

If a solution approaches to correct value, R factor becomes lower and correlation coefficient becomes higher.

#### 2-4-6. Molecular replacement with a program MOLREP

The calculations for molecular replacement were performed using program *MOLREP*, which a program contained in CCP4 (Collaborative Computational Project 1994) suite. The previously determined ferric P450cam (PDB ID: 2CPP) by Poulos *et al.* (1987) was used as a search model. After calculation, the author got an unique

solution as an initial structure for further refinement.

#### 2-4-7. Structure refinement

After solving the phase problem by the molecular replacement method, refinements of the structures were performed using the program REFMAC5 (Murshudov et al, 1997) in the CCP4 suite and by manual modeling. REFMAC5 refines protein structures with the maximum likelihood method under the geometric restraint condition. The refinement by the program *REFMAC5* is usually followed by the manual revision of the structure. The author used composite omit maps for the manual refinement. When an initial phase was obtained by the molecular replacement method, the reference structure model might affect the calculated phases. It is called the model bias. To reduce the influence of an initial model to the calculated electron density as much as possible, a composite omit map is composed as follows; (1) A small part of the unit cell is assigned and atomic coordinates of the protein in the small part are omitted from the phase calculation. (2) To remove the bias of omitted part from remained model, simulated annealing was performed. (3) Electron density map for the small part is calculated with the phases. (4) These procedures are repeated for any part of unit cell. (5) A composite omit map of the unit cell is generated by merging all the part of electron density. Because electron density calculation of each part is free from the atomic parameters in the concerning part, the merged electron density of the unit cell is free from the model bias. The composite omit map was generated using the program CNS (Brunger et al, 1998). Manual refinement was performed with the program COOT (Emsley et al, 2004) as model visualization and manipulation. Structure refinement was performed by repeating the

computing and manual refinement. Difference Fourier maps were calculated with coefficients of  $(|F_0| - |F_c|)\exp(2\pi i\alpha_c)$  to detect the *d*-camphor molecule at the active site, where  $|F_0|$  and  $|F_c|$  were observed and calculated structure amplitudes, respectively, and  $\alpha_c$  was the calculated phase.

During refinement steps described above, R factor and  $R_{\text{free}}$  factor were monitored. Shown in equation (6) R factor is used to confirm a building model structure being proper or not. But sometimes R factor is low value with a wrong model structure in a refinement step because of over fitting problem. To prevent this problem, the  $R_{\text{free}}$  is calculated. The equation is shown in (6). The important thing is that the data for calculating is 5-10% randomly selected data which are not used in refinement. If correct model were built, both R factor and R free factor would exhibit low value. In this study, we use 5% of data for R free calculation, and isomorphous crystal.

#### 2-4-8. The Ramachandran plot

Soundness of the refined structure is inspected in respect of the stereochemistry of the main chain folding. The Ramachandran plot is applied for inspection of the stereochemistry of main chain folding. In the Ramachandran plot the dihedral angles of  $\Phi$  and  $\Psi$  for each residue are plotted. Assuming poly-alanine chain, short contacts between atoms of adjacent residues prevent  $\Phi$  and  $\Psi$  angles from taking possible angles between  $-\pi$  to  $\pi$ . For the adequately refined structure almost all the  $\Phi$  and  $\Psi$ angles are in the allowed region. The Ramachandran plot was calculated with *PROCHECK* (Laskowski *et al*, 1993) in the CCP4 suite.

### Chapter 3.

# Substrate Binding Induced Protein Structural Changes of Cytochrome P450cam Related to Redox Potential Elevation

#### **3-1. Introduction**

Cytochrome P450cam (P450cam) is a thiolate-heme containing monooxygenase that catalyzes the regino- and stereo-selective hydroxylation of d-camphor to produce 5-exo-hydroxycamphor (Gunsalus, et al, 1974). The reaction is initiated by the binding of d-camphor to the resting state of the enzyme. Substrate binding changes the spin state of the heme iron from low to high, and raises its redox potential (Fe<sup>3+</sup>/Fe<sup>2+</sup>couple) by about  $\approx 100$  mV (Gunsalus, *et al*, 1974, Sligar and Gunsalus 1976), which allows reduction of the enzyme by an electron transfer system NADH-putidaredoxin reductases putidaredoxin. comprising and X-ray crystallographic studies on the substrate-free (Poulos et al, 1986) and -bound (Poulos et al, 1987) forms of the enzyme at 2.2 and 1.6 Å resolutions, respectively, revealed that a cluster of waters (6 molecules) at the active site of the substrate-free form, one of which binds to the heme iron, is expelled from the active site upon d-camphor binding. This occurs without an accompanying conformational change of the protein except for a slight shift (by about 0.3 Å) of a phenylalanine residue near the bound substrate. These crystallographic results explain the change in spin state and entropy driven substrate binding (Griffin and Peterson, 1972). Recent X-ray structures of the substrate-bound form at 1.4-1.6 Å resolution (Schlichting et al, 2000 and Meilleur et al. 2005). demonstrate that Thr101 forms a hydrogen bond with the heme-6-propionate, which is different from the previous conformation (Poulos et al,
1987). The hydrogen bond formation of Thr101 with heme-6-propionate is important because it will raise the redox potential of heme iron. However, the conformational change of Thr101 remains unknown whether it is tightly coupled with the substrate binding or not. To understand the mechanisms of the large redox potential changes, elimination of the water cluster from the active site, and other events that take place upon substrate binding, a higher resolution X-ray structure is necessary for the substrate-free form.

Here, we provide X-ray structures of the substrate-free and -bound forms of the enzyme at 1.30-1.35 Å. Substrate binding induces hydrogen bond formation between Thr101 and the ionized heme-6-propionate side chain. This hydrogen bond may significantly raise the redox potential of the heme.

#### 3-2. Materials and methods

#### **3-2-1.** Preparation of protein

Preparation of wild-type P450cam was described in chapter 2-1. However, enzyme samples with an absorption ratio of  $A_{391}/A_{280} > 1.6$  were used herein to harvest higher resolution data.

## 3-2-2. Crystallization

Crystals of the ferric form of P450cam were grown using the sitting-drop vapor diffusion method. Three to five  $\mu$ l of P450cam solution [30 mg/ml P450cam, 250 mM KCl, 10 mM DTE, 1 mM *d*-camphor] was mixed with an equal volume of the reservoir solution [50 mM tris-HCl (pH 7.4), 250 mM KCl, 10 mM DTE, 1 mM *d*-camphor, and 22 to 30 % (w/v) PEG 4000] and allowed to stand at -5 °C for 2 days.

#### 3-2-3. Camphor soaking

Crystals of ferric P450cam grown in the crystallization buffer were transferred to a reservoir solution saturated with *d*-camphor and allowed to stand at -5 °C for one day. The *d*-camphor saturated solution was prepared by adding excess fine powdered *d*-camphor to the reservoir solution and stirring extensively for one day. Undissolved *d*-camphor was removed from the solution.

## 3-2-4. X-ray experiments and structure determination

After the crystals were equilibrated with a solution containing 20 %(v/v) MPD (2-Methyl-2,4-pentandiol), they were frozen in liquid nitrogen. X-ray diffraction data for crystals that had or had not been soaked in *d*-camphor saturated buffer were collected at beamlines BL41XU and BL44XU at SPring-8, respectively. The diffraction images were indexed, integrated, scaled, and merged using the programs *HKL2000* (Otwinoski and Minor, 1997).

Initial phasing of the crystals was performed by molecular replacement using the structure of ferric P450cam (PDBID: 2CPP) as a reference molecule and the program *MOLREP* (Collaborative Computational Project, 1994). The crystal structures were refined using the program *REFMAC* (Collaborative Computational Project, 1994). Difference Fourier maps were calculated according to the previously described procedures (chapter 2). The refined structures were inspected using the program *PROCHECK* (Laskowski *et al*, 1993).

#### 3-3. Results and discussion

Crystals of ferric P450cam were grown in crystallization buffer containing 1 mM d-camphor, and then the grown crystals were soaked in crystallization buffer saturated with d-camphor ( $\approx 8$  mM). The structures of the soaked and unsoaked ferric P450cam crystals were solved at 1.30 Å (PDBID: 2ZWT) and 1.35 Å (PDBID: 2ZWU) resolution, respectively (Table 3-1). Figures 3-1 and 3-2 show the Ramachandoran plots of the camphor unsoaked and soaked model, respectively. These indicate that there is no conformational change by camphor soaking. The structure of the unsoaked P450cam shows an active site that is partially occupied by d-camphor and a water molecule liganded to the heme iron, and rotamers of Thr101 (Figure 3-3). The water molecule bound to the heme iron is 1.56 Å from the C-5 atom of d-camphor. Hence, the water does not coexist with d-camphor in one protein structure, indicating that the crystals are a mixture of d-camphor-bound and -free forms. It is noted that the electron density arising from the keto group of d-camphor is much higher than that of the bound water, suggesting that the *d*-camphor bound form is the major component, while the water bound form is the minor component. The two rotamers of the Thr101 side chain also showed unequal electron density; the form with the hydroxy group directed toward the peripheral heme-6-propionate showed much higher electron density than the form with the hydroxy group directed toward Tyr96. The structures of the minor and major components are depicted in Fig. 3-4A and B, respectively. In the soaked P450cam, the population of the major component was increased, while the minor decreased.

## Table 3-1.

Data collection, processing, and refinement statistics. Values in parentheses are for the highest resolution shell.

	unsoaked	soaked
Data collection		
X-ray source	SPring-8 BL41XU	SPring-8 BL44XU
Wavelength (Å)	0.8	0.7
Space group	P4 <sub>3</sub> 2 <sub>1</sub> 2	P4 <sub>3</sub> 2 <sub>1</sub> 2
Unit cell parameters (Å)	<i>a=b=</i> 63.38, <i>c</i> =247.30	<i>a</i> = <i>b</i> =63.61, <i>c</i> =250.39
Resolution (Å)	50.00-1.35 (1.40-1.35)	50.00-1.30 (1.35-1.30)
Observed resolutions	779,984 (80,388)	610,735 (61,650)
Unique reflections	113,101 (11,165)	125,182 (12,324)
Completeness (%)	99.5 (100.0)	98.6 (98.9)
Redundancy	6.9 (7.2)	4.9 (5.0)
<i>/&lt;{(I)&gt;</i>	41.4 (4.8)	34.4 (4.9)
$R_{ m merge}$	0.064(0.400)	0.075 (0.407)
Refinement		
R factor (%)	16.3	16.6
$R_{\rm free}$ (%)	19.0	18.4
R.m.s. deviation		
from ideal values		
Bond lengths (Å)	0.010	0.010
Bond angles (° )	1.5	1.5



**Figure 3-1.** The Ramachandran plot of the unsoaked wild type P450cam. Marking with A, a, and  $\sim a$  indicates most appropriate, additional allowed, and generously allowed regions, respectively, of the dihedral angles (Psi and Phi) of amino acid residue forming  $\alpha$ -helix. Similarly, B, b, and  $\sim b$  are for  $\beta$ -sheet and L, l, and  $\sim l$  for left-handed helix. Marking with p, and  $\sim p$  is for proline. Glycine does not have sidechain, and thus its allowed dihedral angles are wider than those of the other amino acid residues, therefore glycine residue is unrelated to these regions (see triangle). These regions are based on an analysis of 118 structures at least 2.0 Å resolution and *R* factor no greater than 20%. In a good quality model, over 90 % of the amino acid residues in a protein are expected to be in the most favored regions.



**Figure 3-2.** The Ramachandran plot of the camphor soaked P450cam. Marking with A, a, and  $\sim a$  indicates most appropriate, additional allowed, and generously allowed regions, respectively, of the dihedral angles (Psi and Phi) of amino acid residue forming  $\alpha$ -helix. Similarly, B, b, and  $\sim b$  are for  $\beta$ -sheet and L, l, and  $\sim l$  for left-handed helix. Marking with p, and  $\sim p$  is for proline. Glycine does not have sidechain, and thus its allowed dihedral angles are wider than those of the other amino acid residues, therefore glycine residue is unrelated to these regions (see triangle). These regions are based on an analysis of 118 structures at least 2.0 Å resolution and *R* factor no greater than 20%. In a good quality model, over 90 % of the amino acid residues in a protein are expected to be in the most favored regions.



**Figure 3-3.** Stereo view of the structure of the active site and its vicinity of ferric cytochrome P450cam determined from the unsoaked crystals. Active site residues (Cys357 and Tyr96), heme (iron represented by a large orange sphere), substrate *d*-camphor, a water molecule (a small red sphere) liganded to heme, and Thr101 are shown in stick models (with green carbons, red oxygens, and a blue nitrogen). *d*-Camphor, the water molecule, and Thr101 are represented by electron density from the composite omit map (*cyan*, contoured at  $1.5\sigma$ ). The Thr101 side chain exhibits two rotamer structures (Note the two red sticks extended in different directions; one toward the hydroxy group of Tyr96, the other toward the peripheral heme-6-propionate. The electron density of the latter is much higher than that of the former.). It is also noted that the electron density of the keto group of *d*-camphor is much stronger than that of the bound water. These figures were drawn using PyMOL (V0.99rc6).



Figure 3-4. The active site structures and their vicinity of the minor (A) and major (B) component of ferric cytochrome P450cam represented by stick models with green carbons and a red oxygen. (A) A water molecule bound to heme iron is shown by a red sphere. Thr101 is hydrogen bonded to Tyr96. (B) Tyr96 and Thr101 are hydrogen bonded to d-camphor and peripheral heme 6-propionate, respectively. Dotted lines indicate hydrogen bonds. Figures were drawn using PyMOL (V0.99rc6).

To determine the occupancy of each Thr101 side chain rotamer, we refined the structures of the soaked and unsoaked crystals under several different occupancy values. Refined temperature factors for the Thr101 side chain (average of those for  $C^{\beta}$ ,  $C^{\gamma}$ , and  $O^{\gamma}$  atoms) were found to highly correlate with occupancy (Fig. 3-5A and B), and we assumed that each rotamer would have the same temperature factor. When the occupancies of the rotamers represented by the major and minor component structures of the unsoaked crystals were 66 and 34%, respectively, they have the same temperature factor of 10.6 Å<sup>2</sup> (Fig. 3-5A). Similarly, the occupancies of the major and minor rotamers of the soaked crystal were determined as 78 and 22%, respectively (Fig. 3-5B).

Refinements for the *d*-camphor molecule and the heme bound water under several sets of occupancies were performed for the unsoaked and soaked crystals to determine their occupancies, assuming that the temperature factors of the *d*-camphor and water are almost equal to the temperature factor of the heme group. The occupancies and temperature factors highly correlate (Figure 3-6A and B). For the unsoaked crystal, when the respective occupancies of the *d*-camphor and the water were 66 and 35%, their temperature factors were almost equal to the averaged temperature factor of the heme group of 9.5 Å<sup>2</sup> (Figure 3-6A). Since the *d*-camphor molecule cannot coexist with the water molecule, 35% occupancy of the water molecule is equivalent to 65% occupancy of the *d*-camphor molecule. Thus, the estimated occupancies of 78 and 24% for the *d*-camphor and water, respectively, resulted in temperature factors nearly equal to that of the heme group (Fig. 4B). Consequently, the estimated occupancy of *d*-camphor in the soaked crystal is 76-78%.



**Figure 3-5.** Plots of occupancy *versus* temperature factor of the two Thr101 side chain conformers in the unsoaked (A) and soaked (B) structures. Plotted temperature factors were the average of three atoms ( $C^{\beta}$ ,  $C^{\gamma}$ , and  $O^{\gamma}$  atoms) of the Thr101 side chain. Diamonds; the rotamer represented by the major component structure, squares; the rotamer represented by the minor component structure. In the unsoaked structure, the occupancies of 64 and 36% for the major and minor rotamers, gave the same temperature factors, while in the soaked structure, the occupancies of 78 and 22% for the major and minor rotamers provided the same temperature factors.



Figure 3-6. Plots of occupancy *versus* temperature factor of the active site *d*-camphor and the water bound to heme iron obtained by refinement of the unsoaked (A) and soaked (B) structures under the given value of occupancy (Occupancy of heme was fixed at 100%). Square, *d*-camphor; diamond, heme; triangle, water. The temperature factors of *d*-camphor and heme were the average of the whole molecules.

These analyses indicate that the occupancy of the major Thr101 rotamer is almost identical to that of the occupancy of *d*-camphor, and that of the minor Thr101 rotamer is almost equal to that of the water molecule. These agreements imply that *d*-camphor binding to the active site changes the conformation of the Thr101 side chain from that of the minor component to that of the major component. Thus, the structure of the major component shown in Figure 3-4B is the structure of *d*-camphor-bound P450cam, while the structure of the minor component shown in Figure 3-4A is the *d*-camphor-free (water-bound) structure. Our substrate-free structure is superposed well with the previously reported structure by Poulos *et al.* (1986). In the free structure, it has been reported that 6 water molecules, including a water molecule bound to heme, are located in the substrate-binding site. Such water molecules are also suggested in this study by observed electron density other than that of the bound *d*-camphor both in the soaked and unsoaked crystals.

The present study indicates that Thr101 hydrogen bonds to Tyr96 in the camphor-free state (Figure 3-4A), and changes conformation upon *d*-camphor binding to form a hydrogen bond with the peripheral 6-propionate of heme (Figure 3-4B). Since Thr101 functions as the hydrogen donor in the hydrogen bond, it raises the redox-potential of the heme iron. This conformational change of Thr101 together with the spin state change contributes to the efficiency of the *d*-camphor hydroxylation catalyzed by this enzyme.

## Chapter 4

# Role of Asp297 of Cytochrome P450cam in Exclusion of Waters From the Active Site Occurring Upon the Substrate Binding

## 4-1. Introduction

Cytochrome P450cam containing heme b as the prosthetic group catalyzes monooxygenation of d-camphor. d-Camphor binding to the active site situated deep inside of the protein eliminates a water cluster (6 molecules) bound to the active site (Poulos *et al*, 1986), changing the spin state of heme iron from low to high. This spin state change allows rapid reduction of the enzyme by the electron transfer system. Thus, the water exclusion is essential to the efficient catalytic reaction. However, no pathway has been detected in the crystal structure of the enzyme. The waters might be expelled through the d-camphor access channel formed transiently when d-camphor binds to the active site (Dunn *et al*, 2001, and Hays *et al*, 2004). However, Oprea *et al.* postulated from a theoretical study that waters at the active site could be expelled through a space transiently formed between a metastable rotamer of Arg299 and the heme-7-propionate (Oprea *et al*, 1997). In the crystal structure, Arg299 form a salt bridge with the heme-7-propionate. Moreover, any experimental evidence for the rotamer of Arg299 has never been provided.

Recently, the author's group has found that removal of the heme-7-propionate in P450cam results in the formation of water array connecting bulk water and the active site, accompanying the conformational changes of nearby amino acid residues (Asp297 and Gln322). These two amino acid residues form hydrogen bonding tetrad network with the heme-7-propionate and Arg299 in the wild type protein. This removal of the heme peripheral side chain decreases over 1000-fold the *d*-camphor binding affinity of the enzyme, presumably due to the stabilization of the water cluster at the substrate-binding site by the water array extending from bulk water. These findings suggest that the tetrad block water inlet from bulk water to maintain high *d*-camphor binding affinity (Hayashi *et al*, 2009). Furthermore, conformational changes of Asp297 and Gln322 from those of the wild type protein open a space for the water expelled from the active site to the exterior of the enzyme. Hence, these findings led us to hypothesize that the tetrad regulates the water exodus from the active site and the water inlet from the bulk and that Asp297 is essential to the transient opening of the water gate.

Here, to study on the mechanism of the water exclusion occurring upon *d*-camphor binding to the enzyme, Asp297 was mutated to asparagine, alanine, and leucine. We determined *d*-camphor binding affinity and X-ray structures of the mutant as well as wild type enzymes. The present studies suggest that the waters are expelled through a specific pathway involving Asp297, which is distinct from the *d*-camphor access channel.

## 4-2. Materials and methods

## 4-2-1. Preparation of wild type P450cam

Wild type P450cam was expressed in *Escherichia coli* stain JM109, and purified to homogeneous state by SDS-PAGE according to the previously described procedures (chapter 2). The enzyme samples were dissolved in 50 mM potassium

phosphate buffer (pH 7.4) containing 50 mM KCl and 1 mM *d*-camphor, frozen in liquid N<sub>2</sub> and stored at -80 °C until use. The enzyme samples with an absorption ratio of  $A_{391}/A_{280} > 1.5$  were used for the present study. Concentrations of the wild type and mutant proteins were spectrophotometrically determined using the extinction coefficient of 102 mM<sup>-1</sup>cm<sup>-1</sup> at 391 nm (Gunsals *et al*, 1978).

## 4-2-3. Preparation of the mutant P450cam

Site-directed mutagenesis of the P450cam gene was carried out with the conventional methods. Asp297 of P450cam was mutated to Ala, Asn, and Leu. Expression of the mutant proteins in *E. coli* cells and their purification from the cells were performed with the procedures employed for those of the wild type protein (chapter 2).

## 4-2-4. d-Camphor affinity determination

3ml of camphor-free P450cam solution (ca. 1.7µM in 50 mM KPi (pH 7.4) containing 100 mM KCl) in a quartz cell of 10-mm light pathlength was titrated with aliquots of 3mM *d*-camphor in 50 mM KPi (pH 7.4) containing 100 mM KCl at 20 °C according to the previously described procedures (chapter 2). Binding was followed by monitoring the decrease in absorbance at 417 nm, the Soret peak of the camphor-free protein.  $K_d$  value of P450cam for *d*-camphor was determined using a plot for an equation  $\Delta A = \Delta A_{\infty}$  [substrate] / ([substrate] +  $K_d$ ).

## 4-2-5. Crystallization of the mutated protein

Crystals of the mutant P450cam were grown using the sitting-drop vapor

diffusion method. 3-5  $\mu$ l of P450cam solution [30 mg/ml P450cam, 250 mM KCl, 10 mM DTE, 1 mM *d*-camphor] was mixed with an equal volume of the reservoir solution [50 mM tris-HCl (pH 7.4), 250 mM KCl, 10 mM DTE, 1 mM *d*-camphor, and 22 to 30 % (w/v) PEG 4000] and allowed to stand at 268 K (- 5 °C) for 2 days or more.

## 4-2-6. Camphor soaking

The crystals of mutant P450cam was soaked in the *d*-camphor saturated crystallization buffer as described previously (Chapter 2).

## 4-2-7. X-ray experiments and structure determination

After the crystals of the mutant proteins were equilibrated with a solution containing 15 % (v/v) and 20 % (v/v) MPD (2-Methyl-2,4-pentandiol), respectively, they were frozen in liquid nitrogen. The X-ray diffraction data of crystals were collected at BL41XU in SPring-8 (Hyogo, Japan) and NW-12 in PF-AR (KEK, Tsukuba, Japan). The diffraction images were indexed, integrated, scaled and merged using the program *HKL2000*.

The initial phases of the crystals were obtained by the molecular replacement with the structure of ferric P450cam (PDB ID: 2CPP) as a reference molecule by using the program *MOLREP* of CCP4. The crystal structures were refined by the program *REFMAC*. Difference Fourier maps were calculated according to the previously described procedures (chapter 2). The refined structures were inspected by a program *PROCHECK* (CCP4).

# 4-2-8. Determination of the occupancy of *d*-camphor and others in the crystal structures of P450cam

Crystal structure of wild type P450cam was a mixture of camphor-free and -bound forms as described in the previous chapter and others (Sakurai *et al*, 2009). Occupancies of *d*-camphor and one of two rotamers of Thr101 in the protein structure is found to be the same between the two, indicating that one of the two rotamers of Thr101 belong to the camphor-bound form and the other to the camphor-free form. Crystal structures of the mutant proteins were also the mixture of camphor-free and -bound forms. Electron density arising from two rotamers of Thr101 could be spatially separable in contrast to those from the bound *d*-camphor and the water cluster, which occupy the almost same location. The occupancies of two Thr101 rotamers and others were determined employing the methods described already (Chapter 3 and Sakurai *et al*, 2009). The determined value for the Thr101 rotamer was used as that of *d*-camphor in the present study.

## 4-3. Results and Discussion

## 4-3-1. d-Camphor affinity

The *d*-camphor-free P450cam has a low spin heme with the absorption maxima at wavelength of 570, 540, and 417 nm. Upon *d*-camphor binding, P450cam change the spin state from low to high with the absorption maxima at 646, 512, and 391 nm. Dissociation constant of *d*-camphor for the mutant P450cam was determined by spectrophotometric titration of the *d*-camphor-free protein by increasing amount of *d*-camphor except for the D297L-mutant enzyme, which did not allow the above

determination due to its very low affinity toward *d*-camphor. The dissociation constant of the D297L mutant was estimated from the contents of high and low spin species comprising the absorption spectra at an appropriate concentration of *d*-camphor, which were determined by deconvolution of the spectra using the absorption spectra of the camphor-free mutant enzyme and the camphor-bound wild type enzyme. Dissociation constants of *d*-camphor for the wild type, and D297N and D297A mutants of P450cam (Table 4-1) were 1.70, 12.5, 9.2  $\mu$ M, respectively. The value of the wild type enzyme is very similar to the reported values. The dissociation constant of the D297N mutant was estimated to be lower than 10<sup>4</sup>  $\mu$ M. The population of high-spin species of the wild type (*d*-camphor-bound form), and D297N, D297A, and D297L mutants of P450cam under 1 mM *d*-camphor in 50 mM potassium phosphate, pH 7.4, containing 100 mM KCl were 100, 88, 78, and 4±1 %, respectively (Table 4-1).

Table 4-1.	Camphor	affinity and	l high spin r	ate for the w	vild type and	mutated P450cams <sup>a</sup>

	Wild Type	D297N	D297A	D297L
$Kd(\mu M)^{b}$	$1.70 \pm 0.04$	$12.5 \pm 0.4$	$9.2 \pm 0.2$	>10 <sup>4</sup>
HS (%) <sup>c</sup>	100	88	78	$4 \pm 1$

<sup>a</sup>pH 7.4 50 mM KPi buffer containing 100 mM KCl at 20 °C.

<sup>b</sup>Dissociation constant of *d*-camphor.

<sup>c</sup>Population of high-spin species.

## 4-3-2. Crystal structure

## 4-3-2-1. Crystallographic data of P450cam mutants

Crystals of the wild type and mutant P450cams were grown in the crystallization buffer containing 1 mM *d*-camphor (Figs. 4-1, 4-2, 4-3, and 4-4). The hexagonal bipyramid like crystals of tetragonal ( $P4_32_12$ ) space group were used for the X-ray crystallographic analyses. In addition, the hexagonal bipyramid like crystals were further soaked for two days in the crystallization buffer saturated with *d*-camphor (> 8 mM). The X-ray structures of the wild type and mutant proteins were determined at resolutions higher than 1.6Å. The crystallographic data are listed in Table 4-2 and 4-3.



Figure 4-1. Crystals of the wild type P450cam. The bar represents 1 mm. Reddish-brown color of the crystals is caused by the prosthetic group, heme b.



Figure 4-2. Crystals of the D297N mutant P450cam. The bar represents 1 mm. Bipyramid-like crystals were used for data collection. Stick-like crystals were not used for data collection.



**Figure 4-3.** Crystals of the D297A mutant P450cam. The bar represents 100  $\mu$ m. The bipyramid-like crystal located at the center of the picture has tetragonal ( $P4_32_12$ ) spacegroup and was found to be the best sample for diffraction data measurement. The square plate-like crystals were found to have triclinic (P1) spacegroup and to be multi-layered crystal complex. Thus, they were not suitable for the data collection.



Figure 4-4. Crystals of the D297L mutant P450cam. The bar represents 1 mm. Bipyramid-like crystals were used for data collection, while stick-like crystals were not. This mutant enzyme samples formed precipitations during the crystallization as seen at the bottom of the vessel.

 Table 4-2.
 Data collection, processing, and refinement statistics for the P450cam crystals without soaking with *d*-camphor-saturated buffer.

P450cam	wild type	D297L	D297A	D297N
Data collection				
X-ray source	SPring-8	SPring-8	SPring-8	Photon Factory
	BL44XU	BL44XU	BL44XU	PF-AR NW12
Wavelength (Å)	0.7	0.7	0.7	1.0
Space group	P4 <sub>3</sub> 2 <sub>1</sub> 2	P4 <sub>3</sub> 2 <sub>1</sub> 2	$P4_{3}2_{1}2$	P4 <sub>3</sub> 2 <sub>1</sub> 2
Unit cell parameters (Å)	<i>a=b=</i> 63.522	<i>a=b=</i> 63.952	<i>a=b=</i> 63.687	<i>a=b</i> =63.670
	<i>c</i> =249.670	<i>c</i> =248.673	<i>c</i> =247.284	<i>c</i> =250.014
Resolution (Å)	31.77-1.60	27.96-1.60	39.07-1.60	33.46-1.50
	(1.66-1.60)	(1.66-1.60)	(1.66-1.60)	(1.55-1.50)
Observed reflections	486,890	409,542	486,824	1,113,352
	(47,782)	(40,572)	(49,618)	(66,535)
Unique reflections	68,576	69,210	68,601	83,561
	(6,730)	(6,762)	(6,705)	(8,215)
Completeness (%)	99.5 (100.0)	99.7 (99.9)	99.9 (100.0)	99.9 (100.0)
Redundancy	7.1 (7.1)	5.9 (6.0)	7.1 (7.4)	13.3 (8.1)
< <i>I&gt;/&lt;</i> σ( <i>I</i> )>	n/a (n/a)	47.3 (6.1)	54.9 (8.0)	63.7 (6.4)
R <sub>merge</sub>	0.064 (0.35)	0.048 (0.409)	0.056 (0.387)	0.058 (0.350)
Refinement				
R factor (%)	0.196	0.163	0.160	0.169
$R_{\rm free}$ (%)	0.222	0.196	0.191	0.196
R.m.s. deviation			<b>、</b>	
from ideal values				
Bond lengths (Å)	0.011	0.013	0.011	0.011
Bond angles (°)	1.502	1.546	1.461	1.502

Values in parentheses are for the highest resolution shell.

Values in parentheses are fo	r the highest resol	ution shell.	
P450cam	D297L	D297A	D297N
Data collection			
X-ray source	SPring-8	SPring-8	SPring-8
	BL44XU	BL44XU	BL44XU
Wavelength (Å)	0.7	0.7	0.7
Space group	P4 <sub>3</sub> 2 <sub>1</sub> 2	P4 <sub>3</sub> 2 <sub>1</sub> 2	P4 <sub>3</sub> 2 <sub>1</sub> 2
Unit cell parameters (Å)	<i>a=b=</i> 63.758	<i>a=b=</i> 63.749	<i>a</i> = <i>b</i> =63.604
	<i>c</i> =248.353	<i>c</i> =250.943	<i>c</i> =250.105
Resolution (Å)	33.39-1.60	33.54-1.55	33.45-1.50
	(1.66-1.60)	(1.61-1.55)	(1.55-1.50)
Observed reflections	310,261	535,213	558,641
	(31,199)	(41,624)	(33,676)
Unique reflections	66,343	75,680	80,010
	(6,638)	(7,433)	(5,908)
Completeness (%)	96.5 (98.9)	99.1 (99.9)	95.7 (72.1)
Redundancy	4.7 (4.7)	7.1 (5.6)	7.0 (5.7)
< <i>I&gt;</i> /<σ( <i>I</i> )>	39.0 (5.0)	38.7 (3.8)	55.6 (8.8)
R <sub>merge</sub>	0.057 (0.398)	0.058 (0.366)	0.042 (0.185)
Refinement			
R factor (%)	0.160	0.160	0.158
$R_{\mathrm{free}}$ (%)	0.203	0.185	0.178
R.m.s. deviation			
from ideal values			
Bond lengths (Å)	0.013	0.011	0.010
Bond angles (°)	1.525	1.488	1.467

Table 4-3. Data collection, processing, and refinement statistics for the P450cam crystals soaked in *d*-camphor saturated buffer for one day. 37.1 ... . . . . ..

## 4-3-2-2. The Ramachandran plots of P450cam mutants

X-ray structures of the wild type and mutant P450cams were analyzed by Ramachandran plots to examine whether they have allowed conformations. No residue is located in disallowed region (Figures 4-5~11). Dihedral angles (phi and psi) of the amino acid residue at the mutation site (297th residue from the N-terminus) are listed in Table 4-4. The value for the Asn297 of the D297N mutant protein is almost equal to that of Asp297 of the wild type protein, while those of the others (Ala297 and Leu297 of the D297A and D297L mutant enzymes) are significantly deviated from the wild type value. However, the other residues of each mutant protein have almost the same dihedral angles with those of the corresponding residues of the wild type protein, indicating that the mutation causes a minimal change in the protein structure. These results are consistent with normal UV-visible spectra of the ferric mutant enzymes at the camphor-free and -bound states.

Table	4-4.	Dihedral	angles	of	the	amino	acid	residue	at	297th	position	of	the	wild	type	and
mutan	t P45	0cams														
							(0)			. (0)						

protein	phi (°)	psi (°)	
Wild type	-119.26	-159.89	
D297N	-119.97	-159.62	
D297A	-100.94	-170.37	
D297L	-108.57	174.71	



**Figure 4-5.** The Ramachandran plot of the ferric wild type P450cam. Red circle indicates Asp297. Marking with A, a, and ~a indicates most appropriate, additional allowed, and generously allowed regions, respectively, of the dihedral angles (Psi and Phi) of amino acid residue forming  $\alpha$ -helix. Similarly, B, b, and ~b are for  $\beta$ -sheet and L, l, and ~l for left-handed helix. Marking with p, and ~p is for proline. Glycine does not have sidechain, and thus its allowed dihedral angles are wider than those of the other amino acid residues, therefore glycine residue is unrelated to these regions (see triangle). These regions are based on an analysis of 118 structures at least 2.0 Å resolution and *R* factor no greater than 20%. In a good quality model, over 90 % of the amino acid residues in a protein are expected to be in the most favored regions.



reamour of chartesidaes (excit of y and (10)	5
Number of glycine residues (shown as triangles)	25
Number of proline residues	30
Total number of residues	100
	400

**Figure 4-6.** The Ramachandran plot of the ferric D297N mutant P450cam without soaking. Red circle indicates Asn297. Marking with A, a, and ~a indicates most appropriate, additional allowed, and generously allowed regions, respectively, of the dihedral angles (Psi and Phi) of amino acid residue forming  $\alpha$ -helix. Similarly, B, b, and ~b are for  $\beta$ -sheet and L, l, and ~l for left-handed helix. Marking with p, and ~p is for proline. Glycine does not have sidechain, and thus its allowed dihedral angles are wider than those of the other amino acid residues, therefore glycine residue is unrelated to these regions (see triangle). These regions are based on an analysis of 118 structures at least 2.0 Å resolution and *R* factor no greater than 20%. In a good quality model, over 90 % of the amino acid residues in a protein are expected to be in the most favored regions.



Residues in most favoured regions [A,B,L]	315	90.5%
Residues in additional allowed regions [a,b,l,p]	33	9.5%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	348	100.0%
Number of end-residues (excl. Gly and Pro)	812	
Number of glycine residues (shown as triangles)	25	
Number of proline residues	30	
Total number of residues	1215	

**Figure 4-7.** The Ramachandran plot of the ferric D297N mutant P450cam with soaking. Red circle indicates Asn297. Marking with A, a, and  $\sim$ a indicates most appropriate, additional allowed, and generously allowed regions, respectively, of the dihedral angles (Psi and Phi) of amino acid residue forming  $\alpha$ -helix. Similarly, B, b, and  $\sim$ b are for  $\beta$ -sheet and L, l, and  $\sim$ l for left-handed helix. Marking with p, and  $\sim$ p is for proline. Glycine does not have sidechain, and thus its allowed dihedral angles are wider than those of the other amino acid residues, therefore glycine residue is unrelated to these regions (see triangle). These regions are based on an analysis of 118 structures at least 2.0 Å resolution and *R* factor no greater than 20%. In a good quality model, over 90 % of the amino acid residues in a protein are expected to be in the most favored regions.



**Figure 4-8.** The Ramachandran plot of the ferric D297A mutant P450cam without soaking. Red circle indicates Ala297. Marking with A, a, and ~a indicates most appropriate, additional allowed, and generously allowed regions, respectively, of the dihedral angles (Psi and Phi) of amino acid residue forming  $\alpha$ -helix. Similarly, B, b, and ~b are for  $\beta$ -sheet and L, l, and ~l for left-handed helix. Marking with p, and ~p is for proline. Glycine does not have sidechain, and thus its allowed dihedral angles are wider than those of the other amino acid residues, therefore glycine residue is unrelated to these regions (see triangle). These regions are based on an analysis of 118 structures at least 2.0 Å resolution and *R* factor no greater than 20%. In a good quality model, over 90 % of the amino acid residues in a protein are expected to be in the most favored regions.



Total number of residues

Figure 4-9. The Ramachandran plot of the ferric D297A mutant P450cam with soaking. Red circle indicates Ala297. Marking with A, a, and ~a indicates most appropriate, additional allowed, and generously allowed regions, respectively, of the dihedral angles (Psi and Phi) of amino acid residue forming  $\alpha$ -helix. Similarly, B, b, and  $\sim$ b are for  $\beta$ -sheet and L, l, and  $\sim$ l for left-handed helix. Marking with p, and ~p is for proline. Glycine does not have sidechain, and thus its allowed dihedral angles are wider than those of the other amino acid residues, therefore glycine residue is unrelated to these regions (see triangle). These regions are based on an analysis of 118 structures at least 2.0 Å resolution and R factor no greater than 20%. In a good quality model, over 90 % of the amino acid residues in a protein are expected to be in the most favored regions.



Residues in most favoured regions [A,B,L]	313	89.9%
Residues in additional allowed regions [a,b,l,p]	35	10.1%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	348	100.0%
Number of end-residues (excl. Gly and Pro)	5	
Number of glycine residues (shown as triangles)	25	
Number of proline residues	30	
Total number of residues		
TOTAL HUMUCE OF TESTIQUES	408	

**Figure 4-10.** The Ramachandran plot of the ferric D297L mutant P450cam without soaking. Green circle indicates Leu297. Marking with A, a, and ~a indicates most appropriate, additional allowed, and generously allowed regions, respectively, of the dihedral angles (Psi and Phi) of amino acid residue forming  $\alpha$ -helix. Similarly, B, b, and ~b are for  $\beta$ -sheet and L, l, and ~l for left-handed helix. Marking with p, and ~p is for proline. Glycine does not have sidechain, and thus its allowed dihedral angles are wider than those of the other amino acid residues, therefore glycine residue is unrelated to these regions (see triangle). These regions are based on an analysis of 118 structures at least 2.0 Å resolution and *R* factor no greater than 20%. In a good quality model, over 90 % of the amino acid residues in a protein are expected to be in the most favored regions.



**Figure 4-11.** The Ramachandran plot of the ferric D297L mutant P450cam with soaking. Green circle indicates Leu297. Marking with A, a, and ~a indicates most appropriate, additional allowed, and generously allowed regions, respectively, of the dihedral angles (Psi and Phi) of amino acid residue forming  $\alpha$ -helix. Similarly, B, b, and ~b are for  $\beta$ -sheet and L, l, and ~l for left-handed helix. Marking with p, and ~p is for proline. Glycine does not have sidechain, and thus its allowed dihedral angles are wider than those of the other amino acid residues, therefore glycine residue is unrelated to these regions (see triangle). These regions are based on an analysis of 118 structures at least 2.0 Å resolution and *R* factor no greater than 20%. In a good quality model, over 90 % of the amino acid residues in a protein are expected to be in the most favored regions.

## **4-3-2-2. Crystal structure of the D297N mutant P450cam** Structure of Thr101:

The crystal structure of the ferric D297N mutant P450cam superimposes well to that of the ferric wild type protein. The active site structure exhibits bound *d*-camphor and a molecule of water ligated to the heme iron as seen in that of the wild type protein (Chapter 3). As discussed already, *d*-camphor and the water molecule does not coexist in a single protein structure, indicating that the structure is the mixture of the camphor-free (water-bound) and -bound forms. The protein structures of the two forms are identical to each other except for Thr101 as described previously in the case of the wild type protein (Chapter 3). Thr101 hydrogen bonded to Tyr96 in the camphor-free form breaks the hydrogen bond upon *d*-camphor binding and changes its conformation and forms a hydrogen bond with the heme-7-propionate. These different conformations of Thr101 between the two forms coexist. The present study of the mutant protein supports *d*-camphor binding induced conformational change of Thr101, which the author have found recently (Chapter 3 and Sakurai *et al.* 2009).

## Hydrogen bonded tetrad network:

The amide group of Asn297 side chain hydrogen bonds to the heme-7-propionate (Figures 4-12, 4-13). We have been proposed that the hydrogen bonded tetrad network comprising of Asp297, Gln322, heme-7-propionate, and Arg299 in the wild type enzyme (Figure 4-12) has an essential role in maintaining a high *d*-camphor binding affinity by block the access of the bulk water to the active

site (Hayashi *et al*, 2009). This tetrad is also formed in the mutant enzyme having Asn297 in place of Asp297 (Figure 4-13). Furthermore, superimposition of the wild type structure on the mutant structure revealed that the hydrogen bonded tetrad network structure is almost identical between the two (Figures 4-12, 4-13), being consistent with the high *d*-camphor binding affinity.



Figure 4-12. The stereopair representations of the structure of the active site and its vicinity of the ferric wild type P450cam. Gln322, Ala296, Asp297, Gly298, Arg299, and Cys357 (heme ligand), and heme b (iron represented by a large orange sphere) are shown with stick models (Green, red and blue sticks represent carbon, oxygen, and nitrogen atoms, respectively). Dashed lines indicate hydrogen bonds. The upper figure is the view from the distal side of the heme and the lower figure is from the proximal side.



Figure 4-13. The stereopair representations of the structure of the active site and its vicinity of the ferric D297N mutant P450cam. Gln322, Ala296, Asn297, Gly298, Arg299, and Cys357 (the internal ligand), and heme b (iron represented by a large orange sphere) are shown in stick models (Green, red and blue sticks represent carbon, oxygen, and nitrogen atoms, respectively). Dashed lines indicate hydrogen bonds. Yellow sticks are superimposed ferric wild type P450cam (Figure 4-12). The upper figure is a view from the distal side of the heme and the lower figure from the proximal side.

#### 4-3-2-3. Crystal structure of the D297A mutant P450cam

*Water cluster at the active site of the camphor-free form:* 

The crystal structure of the ferric D297A mutant P450cam superimposes well to that of the ferric wild type P450cam except for the close proximity to the mutation site (Figure 4-14). The crystal structure is the mixture of the camphor-free (water bound) and -bound forms as seen in the wild type and D297N-mutant crystal structures (Figure 4-15). This mutant contains 20 and 80 % camphor-bound and -free forms (see below). A high content of the camphor-free forms reveals 7 water molecules including the molecule bound to the heme iron (Figure 4-15) at the active site, supporting for the first time existence of a water cluster (six molecules of water including a water bound to the heme iron) in the camphor-free wild type enzyme (Poulos *et al*, 1986).

## Structural changes by mutation to Alanine:

Soaking the crystals in *d*-camphor saturated buffer increased the population of the *d*-camphor-bound form to 33 %, while it decreased the *d*-camphor-free form. The protein structures of the camphor-free and -bound forms are almost identical between the two except for Thr101 as in the wild type and D297N-mutant proteins. Thr101 undergo conformational change as found in the wild type and Asp297Asn mutant proteins. Mutation of Asp297 to less bulky Ala leads to insertion of a water molecule into the site corresponding to that for the carbonyl group of the Asp297 side chain (Figure 4-14). This water molecule hydrogen bonds to the heme-7-propionate (Figure 4-16). Moreover, the mutation leads to rotamer structures of Phe87, Tyr96, and Ile395 located in the active site and its vicinity (Figure 4-15, 4-16) of the proteins. In
contrast to Thr101 that shows "apparent" rotamers due to two forms of the enzyme (Chapter 3), the rotamers of these residues are found to be real rotamers detected in both the camphor-free and -bound forms coexisted in the single crystal structure (see below). The rotamer structures of these three residues suggest that they are capable of associated conformational changes. In other word, undergoing Asp297 conformational changes of these three residues, which have been proposed to occur to make space for *d*-camphor during its penetration to the active site from the enzyme's surface, could inversely induce that of Asp297. Near Phe87, there detected electron densities arising from disordered water (O-O distance of 2.33Å indicates multi-location of a single water molecule). These waters surrounded by Phe87, Met184, Thr185, Val247, and d-camphor are not detected in the wild type and other mutant enzymes, supporting rotamer structures of Phe87, presumably one of which forms a space sufficient for the water existence.

#### Hydrogen bonded tetrad network:

The mutation to Ala disrupts hydrogen bonds connecting Asp297 side chain carboxyl with Glu322 and heme-7-propionate, although the heme-7-propionate forms hydrogen bond with an unique water identified at the site corresponding to that for the carboxyl group of Asp297 (Figure 4-16). Therefore the hydrogen bonded tetrad network is partially collapsed. However, the mutation does not form a space for the bulk water accessible to the active site in contrast to P450cam reconstituted with des-7-propionate-hme b where the water array is formed extending from bulk to the active site Tyr96 (Hayashi *et al*, 2009). This structural evidence is consistent with the high *d*-camphor binding affinity of the Asp297Ala mutant P450cam.

## d-Camphor binding:

In a solution, the D297A mutant protein exhibits the camphor-bound high spin spectrum in the presence of 1 mM *d*-camphor in 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl. Crystallization buffer also contains 1 mM *d*-camphor and potassium ion, which stabilizes bound *d*-camphor. These conditions are expected to be sufficient to form camphor-bound high spin form. However, camphor-bound form is 20 %, which is much less than that expected from the solution property of the mutant protein. Visible spectral measurements of the crystals show much less intense absorption peak at 646 nm (A peak arising from the camphor-bound form) than that of the wild type protein, supporting the low content of the camphor-bound form. Discrepancy of *d*-camphor binding between solution and crystalline states remains to be studied.



Figure 4-14. Effect of mutation of Asp297 to Ala on the hydrogen bonded tetrad network. Gln322, Ala296, Ala297, Gly298, Arg299, and Cys357, and heme b (iron represented by a large orange sphere) are shown in stick models (Green, red, blue, and yellow sticks represent carbon, oxygen, nitrogen, and sulfur atoms, respectively). Dashed lines indicate hydrogen bonds. Yellow sticks are superimposed ferric wild type P450cam (Figure 4-12). The upper figure is a view from distal side of the heme and the lower figure is from the proximal side.



**Figure 4-15.** The structure of the active site and its vicinity of the D297A mutant P450cam. Gln322, Ala297, Gly298, Thr101, Ile395, Phe87, and Tyr96, and heme *b* (iron represented by a large orange sphere) are shown in stick models (Green, red and blue stick represent carbon, oxygen, nitrogen atoms, respectively). Amino acid residues except for Gly298 and a water molecule are shown with electron density from the composite omit map (*cyan*, contoured at  $1.5\sigma$ ). Thr101, Ile395, Phe87, and Tyr96 exhibit rotamer structures. Thr101 rotamers are "apparent" rotamers caused by coexistence of the camphor-free and -bound forms of P450cam, where Thr101 has different conformation between the two forms, while rotamers of the other residues are real rotamers seen each of the camphor-free and -bound forms.



**Figure 4-16.** Water cluster at the active site of the ferric D297A mutant P450cam. Ala297, Gly298, Gly299, Thr101, Ile395, Phe87, Cys357, and Tyr96, *d*-camphor, and heme *b* (iron represented by a large orange sphere) are shown in stick models (Green, red, blue, and yellow represent carbon, oxygen, nitrogen, and sulfur atoms, respectively). *d*-Camphor and water molecules are represented with electron density from the composite omit map (*cyan*, contoured at  $1.5\sigma$ ). The electron density of *d*-camphor is lower than that of the water cluster (7 molecules of water including a water bound to heme iron), indicating that the water bound form (camphor-free form) is dominant over the camphor-bound form. Black arrows indicate the disordered waters, which are surrounded by Phe87, Met187, Thr181, Val247, and *d*-camphor (Met188, Thr181, and Val247 are omitted to show the waters).

## 4-3-2-4. Crystal structure of the D297L mutant P450cam

### Structure of Thr101:

The crystal structure of the ferric D297L mutant P450cam superimposes well to that of the ferric wild type P450cam except for the mutation site and its vicinity where the main chain is evidently deviated from that of the wild type protein (Figure 4-17). Similar to the wild type and the other 297-mutant proteins, the crystal structure of the D297L mutant protein is the mixture of the camphor-free and -bound forms. In the camphor-unsoaked crystals, the free and bound forms have 34 and 66 % occupancies, respectively (see below). By soaking the crystals into the d-camphor saturated buffer, two forms change the occupancy to 50 % (see below). Two forms show an identical protein structure except for Thr101, Leu297 and heme-7-propionate. In the camphor-free form, Thr101 forms a hydrogen bond with Tyr96 as found in the wild type and the other 297-mutant proteins. In the camphor-bound form, Thr101 changes its conformation accompanying breakage of the hydrogen bond with Tyr96 and forms a hydrogen bond with water (water A, see Figures 4-18, 4-20, 4-21) identified between heme peripheral two propionate groups (Figure 4-20, 4-21). This water A uniquely found in the camphor-bound form hydrogen bonds to each of the two heme-propionates. Distance between Thr101 and heme-6-propionate in the camphor-bound form is 3.8 Å, which is longer than that detected in the wild type and the other 297 mutant proteins. This elongation is the result of the slight deviation of the main chain by the mutation. So Thr101 does not form a hydrogen bond with the heme-6-propionate (see Figures 4-18, 4-20).

# Structure of Leu297:

The electron density of the Leu297 side chain (contoured at  $1.0\sigma$ ) in the camphor-unsoaked crystal does not fit to the density of single structure of Leu side chain (Figure 4-18A). Two rotamer structures of Leu297 around the  $\beta$ - $\gamma$  axis of the side chain best fit the electron density as seen in the figure. However, they are not sufficient to fully explain the observed electron density. Furthermore, electron density contoured at a value larger than  $1.0\sigma$  suggests that the Leu297 side chain is fairly disordered. Soaking the crystals in *d*-camphor saturated buffer enhances the electron density (contoured at  $1.0\sigma$ ) of Leu297. Moreover, the density extends toward the heme-7-propionate (Figure 4-18B). Again two rotamers of Leu297 around the  $\beta$ - $\gamma$  best fitted to the electron density are not sufficient to fully explain the conformation of the  $\alpha$ - $\beta$  axis is slightly different from that of the camphor-unsoaked structure (Figure 4-18B), supporting that electron density derived from Leu297 could not be explained by two rotamers around the  $\beta$ - $\gamma$  of the side chain.

When *d*-camphor binds to the active site, the  $C^{\delta}$  atom of one of the two Leu297 rotamer (see asterisk in Figures 4-18 A and B and Figure 4-19) is 2.56Å from *d*-camphor, which is shorter than allowed van der Walls distance of interatomic contact (3.0 and 3.2 Å for contact types C-C and C-CH<sub>3</sub>, respectively), indicating that one of the two Leu297 rotamers depicted in Figures 4-18 A and B does not coexist with *d*-camphor. As the results, electron density of disordered Leu297 extends toward the heme-7-propionate (Figure 4-18 B).

## Structures of the heme peripheral 7-propionate:

The heme-7-propionate exhibits two rotamer structures (rotamers 1 and 2)

(Figures 4-19, -20, and -21) of the carboxyl groups; the axis connecting the terminal oxygen atoms of rotamer 1 is rotated clockwise by 7.25 degree from the heme normal, while that of rotamer 2 by 69 degree as seen clearly in Figures 4-20 and -21. Electron density of rotamer 2 increases by camphor soaking, while that of rotamer 1 decreases, suggesting that rotamers 1 and 2 originate from the *d*-camphor-free and -bound forms, respectively. Occupancies of rotamers 1 and 2 in the camphor unsoaked crystals were determined to be 63 and 37 %, respectively (Figure 4-26A), which are similar to those of the camphor-free and -bound form (67 and 34 %, respectively, Figure 4-24A), supporting that each rotamer of the heme-7-propionate derives from the two protein forms. However, in the camphor-soaked crystals, occupancies of rotamers 1 and 2 change to 39 and 61 %, respectively (Fgure4-26B), which are significantly different from those for the camphor-free and -bound forms (50 %, Fgure4-24B). This disagreement suggests that other factors, presumably disordered structure of Leu, modify the population of rotamers 1 and 2.

The  $C^{\delta}$  atom of one of the Leu297 rotamer presented in Figures 4-18 A and B (see double asterisks) is 2.9 and 2.7 Å, respectively, from a oxygen atom of the heme-propionate rotamer 1, which are shorter than allowed van der Walls distance of interatomic contact (3.2 Å for a contact type CH<sub>3</sub>-O). Furthermore, the electron density of Leu297 (Figure 4-18B) extended toward the rotamer 1 of the heme-7-propionate upon camphor soaking indicates the presence of disordered Leu297 side chain in a region closer to the propionate rotamer 1 than the location of the above Leu297 rotamer. This disordered Leu297 also sterically hinders the rotamer 1 and thus promotes rotation of the carboxyl group to the rotamer 2. Hence, conversion of rotamer 1 to 2 is apparently driven sterically by the Leu297 side chain,

which is highly disordered in irrespective of the camphor-free and -bound forms. It is highly possible that disordered structure of Leu297 side chain modify the population of the rotamers 1 and 2 from that determined by the presence and absence of d-camphor at the active site.

#### Hydrogen bonded network involving two rotamers of the heme-7-propionate:

The propionate group of rotamer 1 forms hydrogen bonds with waters B, D, and E (waters D and E are detected in the wild type protein) and also forms a hydrogen bond with Arg299, which in the wild type protein is connected to the heme-7-propionate via two hydrogen bonds (Figure 4-20A, 4-21A). Water B is unique to this mutant and stabilized by a hydrogen bond with water C, which in turn hydrogen bonds to Tyr96 (Figure 4-20A, 4-21A). Water D connects to Gln322 and Tyr75 by a hydrogen bond and  $\pi$ - $\sigma$  bond, respectively, in irrespective of camphor-free and -bound forms. Water E connects via hydrogen bond to the hydroxyl group of Ser83, and the main chain carbonyl of Thr101, and also a side chain of His355 via a  $\pi$ - $\sigma$  bond. This water E is present both in the camphor-free and -bound forms. In the d-camphor-bound form (Figure 4-20B, 4-21B), the propionate group of rotamer 2 forms hydrogen bonds with waters A, D, and E and also forms a hydrogen bond (salt bridge) with Arg299. As described already, unique water A is located between two propionate groups of the heme and hydrogen bond to each of the two heme-propionates and to the hydroxyl group of Thr101. Two rotamers of the heme-7-propionate are oriented differently with respective to the heme normal. Each rotamer has four hydrogen bonds; three hydrogen bonds with waters, which are stabilized by main chain carbonyl and the amino acid side chain via hydrogen bond,

and one hydrogen bond (salt bridge) with the guanidium group of Arg299. Hence, two rotamers of the heme-7-propionate could be stabilized to an extent similar to each other.

#### Waters:

Water A is unique to the camphor-bound form of the Leu297 mutant protein. Rotamer 2 of the heme-7-propionate is essential to the presence of water A. The space between two heme propionates is accessible from the active site, although it is isolated from the bulk water. Therefore, water A might be forms the water cluster at the active site of the camphor-free form. On the other hand, waters B and C are evidently identified in the camphor-free form. Hydrogen bond between water B and rotamer 1 of the heme-7-propionate is important for the stability of waters B and C.

#### d-Camphor binding:

In solution, this mutant protein forms about 4 % the camphor-bound high spin species in 50 mM potassium phosphate, pH 7.4 containing 1 mM *d*-camphor and 100 mM KCl. In the crystals (unsoaked crystals), content of the camphor-bound form is 34 % under the similar conditions with those in solution, which is much higher than that expected from the properties of the protein in solution. Visible spectral measurements of the crystals of the mutant proteins are consistent with the results of X-ray crystallographic study. This large discrepancy of content of the *d*-camphor-bound form is favored for the crystallization. However, further studies are necessary to elucidate the large discrepancy.



Figure 4-17. Stereopairs of the structures of the ferric D297L mutant P450cam focusing on the mutation site viewed from the distal (upper) and proximal (lower) side of the heme. Gln322, Ala296, Leu297, Gly298, Arg299, and Cys357, and heme b (iron represented by a large orange sphere) are shown in stick models (Green, red, and blue sticks represent carbon, oxygen, nitrogen, and sulfur atoms, respectively). Dashed lines indicates hydrogen bond. Yellow sticks are superimposed structure of the ferric wild type P450cam (Figure 4-12).



**Figure 4-18.** The structure of the active site and its vicinity of *d*-camphor unsoaked (A) and soaked (B) D297L mutant P450cam. Gln322, Leu297, Gly299, Thr101, Cys 357, and Tyr96, and heme *b* (iron represented by a large orange sphere) are shown in stick models (Green, red, blue, and yellow sticks represent carbon, oxygen, nitrogen, and sulfur atoms, respectively). The red sphere indicates water with arrowed alphabet. Leu297, Thr101, and the heme are represented with electron density from the composite omit map (*cyan*, contoured at  $1.0\sigma$ ). The water D located in the proximal side of the heme is not drawn. Asterisks indicate Leu297 rotamer identifiers.



Figure 4-19. The rotamer structure of Leu297 and heme-7-propionate in camphor soaked model. Leu297 and heme (iron represented by a large orange sphere) are shown in stick models (with green carbons, red oxygens, and blue nitrogens). Leu297, *d*-camphor, and heme are represented by electron density from the composite omit map (*cyan*, contoured at  $1.5\sigma$ ). Black dash represents the shortest distance between Leu297 and *d*-camphor, 2.56Å. Numbers represent the oxygens of 7-propionate rotamers.



Figure 4-20. Hydrogen bonding network at the active site of camphor-free (A) and -bound (B) forms of the D297L mutant P450cam viewed from the distal side of the heme. Gln322, Leu297, Gly299, Thr101, Tyr75, His355, and Tyr96, and heme b (iron represented by a large orange sphere) are shown in stick models (Green, red and blue blue stick represent carbon, oxygen, and nitrogen atoms, respectively). Red spheres with arrowed alphabets indicate the water molecules. Dashed lines represent hydrogen bond.



Figure 4-21. Hydrogen bonding network at the active site of camphor-free (A) and -bound (B) forms of the D297L mutant P450cam viewed from the proximal side of the heme. Gln322, Leu297, Gly299, Thr101, Tyr75, His355, and Tyr96, and heme b (iron represented by a large orange sphere) are shown in stick models (Green, red and blue blue stick represent carbon, oxygen, and nitrogen atoms, respectively). Red spheres with arrowed alphabets indicate the water molecules. Dashed lines represent hydrogen bonds.

#### 4-3-3. Occupancy of camphor and some residues

#### Thr101 in the 297-mutant P450cam:

Two rotamers of Thr101 identified in the crystal structure of the wild type P450cam is derived from the two forms of the protein, d-camphor-free and -bound forms that coexist in the crystals. The occupancy of each rotamer of Thr101 in the crystal is exclusively determined by bound *d*-camphor as described in chapter 3 and others (Sakurai et al, 2009). The crystals of the mutant proteins also show two rotamers of Thr101 due to a mixture of camphor-free and -bound forms. The occupancies of the rotamers of Thr101 of the mutant proteins were determined by the plot presented in Figures 4-22, -23, and -24. The rotamer (1) is the structure of Thr101 bound to Tyr96 and the rotamer (2) is that of Thr101 hydrogen bonded to heme-6-propionate (in the wild type, and the D297N, and D297A mutant proteins) or to the water molecule A (in the D297L mutant protein). To determine the occupancy of each Thr101 side chain rotamer, we refined the structures of the camphor soaked and unsoaked crystals under several different occupancy values. Refined temperature factors for the Thr101 side chain (average of those for  $C^{\beta}$ ,  $C^{\gamma}$ , and  $O^{\gamma}$  atoms) were found to highly correlate with the occupancy. We could reasonably assume that each rotamer would have the same temperature factor. In the D297N mutant protein, when the occupancies of the rotamers represented by the rotamer (1) and rotamer (2) component structures of the unsoaked crystals were 35 and 65%, respectively, they have the same temperature factor of 10.1 Å<sup>2</sup> (Figure 4-22A). Similarly, the occupancies of the rotamer (1) and rotamer (2) of the camphor soaked crystal were determined as 33 and 67%, respectively (Figure 4-22B). In the D297A mutant, when the occupancies of the rotamers represented by the rotamer (1) and rotamer (2)

component structures of the unsoaked crystals were 80 and 20%, respectively, they have the same temperature factor of 17.0 Å<sup>2</sup> (Figure 4-23A). The occupancies of the rotamer (1) and rotamer (2) of the soaked crystal were determined as 67 and 33%, respectively (Figure 4-23B). In the D297L mutant protein, the occupancies of the rotamer (1), and rotamer (2) rotamers, and the water molecule A for the unsoaked crystal were determined as 66, 34%, and 25%, respectively, and of soaked crystal they were determined as 50%, 50%, and 47%, respectively (Figure 4-24). From the occupancy values for the Thr101 rotamers, those of the bound *d*-camphor were estimated to be 65, 67, 20, 33, 34 and 50%, respectively, for unsoaked D297N, soaked D297A, unsoaked D297A, unsoaked D297L and soaked D297L mutant.



**Figure 4-22.** Plots of occupancy *versus* temperature factor of the two Thr 101 side chain conformers of D297N mutant in the unsoaked (A) and soaked (B) structures. Plotted temperature factors were the average of three atoms ( $C^{\beta}$ ,  $C^{\gamma}$ , and  $O^{\gamma}$  atoms) of the Thr101 side chain. Diamonds; rotamer (1) represented by the structure of Thr101 bound to heme-6-propionate, squares; rotamer (2) represented by the structure of Thr101 bound to Tyr96. In the unsoaked structure, occupancy of 65 and 35% for the (1) and (2) rotamers, gave the same temperature factors, while in the soaked structure, occupancy of 67 and 33% for the (1) and (2) rotamers provided the same temperature factors.



**Figure 4-23.** Plots of occupancy *versus* temperature factor of the two Thr101 side chain conformers of D297A mutant in the unsoaked (A) and soaked (B) structures. Plotted temperature factors were the average of three atoms ( $C^{\beta}$ ,  $C^{\gamma}$ , and  $O^{\gamma}$  atoms) of the Thr101 side chain. Diamonds; rotamer (1) represented by the structure of Thr101 bound to heme-6-propionate, squares; rotamer (2) represented by the structure of Thr101 bound to Tyr96. In the unsoaked structure, occupancy of 20 and 80% for the (1) and (2) rotamers, gave the same temperature factors, while in the soaked structure, occupancy of 33 and 67% for the (1) and (2) rotamers provided the same temperature factors.



**Figure 4-24.** Plots of occupancy *versus* temperature factor of the two Thr101 side chain conformers of D297L mutant and water molecule between heme-propionates in the unsoaked (A) and soaked (B) structures. Plotted temperature factors were the average of three atoms ( $C^{\beta}$ ,  $C^{\gamma}$ , and  $O^{\gamma}$  atoms) of the Thr101 side chain. Diamonds; rotamer (1) represented by the structure of Thr101 bound to the water molecule A, squares; rotamer (2) represented by the structure of Thr101 bound to Tyr96, and triangles; the water molecule A.

## Phe87 in the D297A mutant protein:

The occupancy for the rotamer of Phe87 in the D297A mutant protein was determined with the same procedures described in the previous section by the correlation between temperature factors and various occupancies for the Phe87 side chain (average of those for  $C^{\beta}$ ,  $C^{\gamma}$ ,  $C^{\delta_1}$ ,  $C^{\delta_2}$ ,  $C^{\epsilon_1}$ ,  $C^{\epsilon_2}$ , and  $C^{\zeta}$  atoms). The occupancies of the rotamers represented by the major and minor component structures of the Phe87 of the unsoaked crystals were 55 and 45%, respectively (Figure 4-25A). Similarly, the occupancies of the major and minor rotamers of the soaked crystal were determined as 66 and 34%, respectively (Figure 4-25B). The occupancy of Phe87 is different from those of Thr101, but the occupancy of Phe87 increased with camphor soaking.



**Figure 4-25.** Plots of occupancy *versus* temperature factor of the two Phe87 side chain conformers of D297L mutant and water molecule between heme-propionates in the unsoaked (A) and soaked (B) structures. Diamonds; rotamer represented by the major component structure, squares; rotamer represented by the minor component structure. In the unsoaked structure, occupancy of 55 and 45% for the major and minor rotamers, gave the same temperature factors, while in the soaked structure, occupancy of 66 and 34% for the major and minor rotamers provided the same temperature factors.

## Heme-7-propionate side chain in the D297L mutant protein:

The occupancy for the rotamers of the heme-7-propionate in the D297L mutant protein was determined as above by the correlation between temperature factors and various occupancies for the carbonyl group. The occupancies of the rotamers represented by the major and minor component structures of the 7-propionate of the unsoaked crystals were 63 and 37%, respectively (Figure 4-26A). Similarly, the occupancies of the major and minor rotamers of the soaked crystal were determined as 39 and 61%, respectively (Figure 4-26B).



**Figure 4-26.** Plots of occupancy *versus* temperature factor of the heme-7-propionate side chain rotamers of D297L mutant in the unsoaked (A) and soaked (B) structures. Plotted temperature factors were the average of carbonyl group of the propionate side chain. Squares; rotamer (1) represented by the structure of the propionate side chain near those of wild type, diamonds; rotamer (2) represented by the structure of the propionate side chain bound to the water molecule A.

## Difference of camphor affinity between the solution and the crystal:

The author have described in chapter 3 that there is correlation between the rotamer structure of Thr101 and the occupancy of the substrate, d-camphor. Here the occupancy of Thr101 rotamer in the mutant proteins was used as that for the substrate d-camphor. The occupancies for d-camphor in the mutant proteins are listed in Table 4-5.

Table 4-5. The occupancy of the *d*-camphor of P450cam mutants

Camphor	wild type	D297N	D297A	D297L
Unsoaked (%)	66	65	20	34
Soaked (%)	78	67	33	59

There is clear discrepancy in the content of the camphor-bound form between proteins in solution (Table 4-1) and in crystals. Contents of the camphor-bound form are found to be consistent with absorption spectra of the crystals, supporting that camphor-free and -bound forms generate low and high spin species, respectively. The discrepancy between two states is currently unexplainable. Further studies are necessary to solve this problem.

## 4-3-4. Water cluster at the active site of P450cam

In the substrate d-camphor-free state, the active site of the wild type protein was reported to contain a cluster of 6 water molecules including a water bound to the heme iron, generating ferric low spin species, which is less reactive to the electron transfer system. In the substrate-bound state, any water is not detected at the active site, yielding a high spin species that is readily reduced by the electron transfer system. Hence, exclusion of the water cluster from the active site is essential to the initiation of the *d*-camphor hydroxylation.

Present studies on the 297-mutant proteins also revealed a water molecule bound to the heme iron in addition to the electron density presumably arising from multiple molecules of water. In particular, in the Ala297 mutant protein, 7 water molecules are identified including a heme bound water at the active site, supporting the water cluster detected in the wild type protein. The substrate-bound forms of the mutant proteins do not contain any water molecule at the active sites, being consistent with the *d*-campbor binding induced water exclusion found in the wild type protein. How these water molecules are expelled from the active site? The waters would be transferred to the outside of the enzyme through a pathway (or pathways) and/or would be retained at some sites in the protein interior. The crystal structures of the wild type and mutant proteins do not demonstrate a specific pathway for the water extending from the active site to the enzyme's surface. It has been proposed that d-camphor binds to the active site situated deep inside of the protein through a specific pathway transiently formed during the passage (Dunn et al, 2001, and Hays et al, 2004). Thus it might be possible that the water cluster is expelled through the d-camphor access channel.

#### 4-3-5. Pathway for the water cluster excluded from the active site

Mutation of Asp297 to Asn and Ala causes a slight change in the *d*-camphor binding affinity (by about 10 folds), while the Leu mutation lower the affinity by over 10,000 folds. The affinity change toward *d*-camphor by the mutations could be caused by structural changes in the *d*-camphor access channel and/or of the binding

site or the stabilization of the bound water at the active site, which directly competes with *d*-camphor. The X-ray structures of the mutant proteins indicate no evident structural changes of the *d*-camphor access channel (Dunn *et al*, 2001, and Hays *et al*, 2004) to inhibit the access. Asn and Ala at the mutation site do not hinder (at least sterically) the bound *d*-camphor. Thus, X-ray structures of the Asn and Ala mutant proteins are consistent with a slight change in the *d*-camphor binding affinity.

Leu297 slightly changes upon *d*-camphor binding the region where its highly disordered side chain occupies in the active site. However, hydrophobic side chain of Leu forms van der Walls contact with bound *d*-camphor, which favors *d*-camphor binding to the mutant protein. Slight change in the region where the Leu side chain occupies induces a structural changes of P450cam represented by the clockwise rotation by about 60 degree of the carboxyl group of the heme-6-propionate ("rotamer 1") around the  $\beta$ - $\gamma$  axis to the another structure, "rotamer 2". As discussed already, the rotated heme-6-propionate, rotamer 2 is stabilized by multiple hydrogen bonds to a similar extent as the rotamer 1 is stabilized by the hydrogen bonds (Figures 4-22, -23), indicating that the rotation causes less change in the enthalpy. Moreover, rotation of the carboxyl group is sterically free except for disruption of a hydrogen bond with a water (water B in Figure 4-22), which is energetically compensated by the formation of a hydrogen bond with a water (Water A in Figure 4-22). Hence, the rotation of the heme-6-propionate carboxyl group gives less effect on the d-camphor binding affinity. Accordingly, a large change in the d-camphor binding affinity is not caused directly by the sterical interference from the bulky Leu side chain. The large change in the *d*-camphor binding affinity by the mutation to Leu strongly suggest that the water cluster bound at the active site is greatly stabilized

presumably due to blockage of the water exclusion from the active site. X-ray structure of the Leu297 mutant protein demonstrates that the Leu side chain blocks the water exclusion through the pathway we have proposed previously as a working hypothesis (Hayashi *et al*, 2009). The present mutation results suggest that the water cluster at the active site of P450cam is expelled through a specific pathway involving Asp297 and not through the *d*-camphor access channel.

## 4-3-6. Mechanism of opening the pathway for the water cluster

In the wild type P450cam, the hydrogen bonding tetrad network comprised of Asp297, Gln322, Arg299, and the heme-7-propionate block the access of bulk water to the active site, which maintains high *d*-camphor binding affinity (Hayashi *et al*, 2009). *d*-Camphor binding transiently induces conformational changes of Asp297 and Gln322, leading to breakage of the hydrogen bond between them. This conformational change collapses the hydrogen bond tetrad network and generates a space through which the active site water cluster is expelled to the exterior of the protein (Hayashi *et al*, 2009). However, no mechanism of driving the conformational changes of Asp297 and Gln322 has yet been given.

Mutation of Asp297 to less bulky Ala causes rotamer structures of Phe87, Ile395, and Tyr96. The former two residues form the *d*-camphor access channel, while the latter is the *d*-camphor binding site in the wild type protein. One rotamer of side chain of Phe87 or Ile395 has the structure identical to that of the corresponding residue in the wild type protein. The other rotamer change the structure from the above rotamer in a way to widen the space for *d*-camphor passage (Figure 4-27). X-ray structure of P450cam reconstituted with des-7-propionate heme *b* also

demonstrates that conformational change of Asp297, which forms a hydrogen bond with the heme-7-propionate and Gln322 in the wild type protein, disrupts the hydrogen bond with Gln322 and accompanies conformational changes of Phe87, Ile395, and Tyr96. The conformational changes of the former two residues widen the space for *d*-camphor passage. The present and previous X-ray structural evidence suggest that the conformational changes of Phe87 and Ile395 forced by *d*-camphor penetration (Dunn *et al*, 2001, and Hays *et al*, 2004) generate that of the Asp297 in a way to disrupt a hydrogen bond with Glu322, leading to the opening of the water channel. Phe87 and Ile395 are located close to Tyr96. Accordingly, conformational changes of Phe87 and Ile395 by *d*-camphor passage are almost immediately followed by *d*-camphor binding to the site. Therefore, the passage and binding of *d*-camphor to the enzyme together drives the opening of the pathway for the water and concomitant exclusion of the water cluster from the active site to the outside of the enzyme.







Figure 4-27. The proposed water expelling mechanism. Active site residues (Gln322, Asp297, and Arg299), and heme (iron represented by a large orange sphere) are shown in stick models (with green carbons, red oxygens, and blue nitrogens). The water molecule and cluster are represented by pink ball. Black dashs indicate hydrogen bonds. In camphor binding, tetrad hydrogen bonds with Gln322. Asp297, and heme-7-propionate side chain are cut by the water cluster and Asp297 side chain flipped into the active site (1). The water gate was opened by flipping of Asp297 side chain, and the water cluster flow out through the gate with hydrogen bond (2). When the water cluster finish expelling, Asp297 reflipped to bond to heme-7-propionate side chain, and water expelling channel was closed. Hydrophobic camphor bound to the waterless reactive site (3).

# Chapter 5

## Conclusion

Cytochrome P450cam (P450cam) from Psudomonas putida is а heme-containing monooxygenase that catalyzes the regio- and stereo-specific hydroxylation of d-camphor by using molecular oxygen and accepting two electrons from NADH. The three-dimension structure of P450cam is shown that the active site of P450cam locates in the center of the protein, and the active site has no pathway to outer bulk solvent. Furthermore, in substrate-free state, the active site is occupied with a water cluster. These facts suggest that P450cam has the special mechanisms of expulsion of the water cluster, binding of the substrate, and expulsion of the product. The mechanism of conformational change of the amino acid residue of the active site and the existence of the specific water expelling pathway are solved by the results of this work.

In the previous work, Thr101 of P450cam has been known to have two conformations. However, although Thr101 located in the vicinity of the active site, the function of the rotation of Thr101 did not have be interesting. This study indicates that Thr101 hydrogen bonds to Tyr96 in the camphor-free state, and changes conformation upon *d*-camphor binding to form a hydrogen bond with the peripheral 6-propionate of heme. Since Thr101 functions as the hydrogen donor in the hydrogen bond, it raises the redox-potential of the heme iron. This conformational change of Thr101 together with the spin state change contributes to the efficiency of the *d*-camphor hydroxylation catalyzed by this enzyme.

The water cluster in the active site in camphor-free state is also one of the facts in which many scientists not interesting. They believed that P450cam may expel the water cluster along anywhere in the substrate binding, because no reports about water expelling pathway of P450cam had been established. In this study, Asp297 was mutated some other amino acids and their crystal structure was determined. The result strongly suggests that 450cam has the specific channel of water expelling with Asp297 as the exit site.

#### References

Bell, S. G., Chen, X., Sowden, R. J., Xu, F., Williams, J. N., Wong, L. -L., and Rao, Z., (2003) Molecular recognition in (+)- $\alpha$ -pinene oxidation by cytochrome P450<sub>cam</sub>, *J. Am. Chem. Soc.*, **125**: 705-714.

Brünger, A. T., (1992) Free R value: a novel statistical quantity for assessing the accuracy of crystal structures., *Nature*, **355**: 472-475.

Brünger, A.T., Adams, P.D., Clore, G.M., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.-S., Kuszewski, J., Nilges, N., Pannu, N.S., Read, R.J., Rice, L.M., Simonson, T., and Warren, G.L., (1998) Crystallography & NMR System (CNS), A new software suite for macromolecular structure determination, *Acta Cryst*. **D54**: 905-921.

Collaborative Computational Project, Number 4. (1994) "The CCP4 Suite: Programs for Protein Crystallography". *Acta Cryst.* **D50**: 760-763.

Danielson, P., (2002) the cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans, *Curr. Drug. Metab.*, **6:** 561-97.

Dunn, A.R., Dmochowski, I.J., Bilwes, A.M., Gray, H.B., Crane, B.R. (2001) Probing the open state of cytochrome P450cam with ruthenium-linker substrates. *Proc. Natl. Acad. Sci. USA* 98: 12420-12425.

Emsley, P., and Cowtan, K., (2004) Coot: model-building tools for molecular graphics, *Acta Cryst*. **D60**: 2126-2132.

French, K. J., Rock, D. A., Rock, D. A., Manchester, J. I., Goldstein, B. M., and Jones J. P., (2002) Active site mutations of cytochrome P450cam alter the binding, coupling, and oxidation of foreign substrates, *Arch. Biochem. Biophys.*, **398**: 188-197.

French, S., and Wilson, K. S., (1978) On the treatment of negative intensity observations, *Acta Crist.* A34: 517-525.

Green, D. W., Ingram, V. M., and Pertzu, M. F. (1954), The structure of hemoglobin IV. Sign determination by the isomorphous replacement method. *Proc. R. Soc. London Ser. A* **225**: 287-307.

Griffin, B.W., and Peterson, J.A. (1972) Camphor binding by Pseudomonas putida cytochrome P-450. Kinetics and thermodynamics of the reaction., *Biochemistry* **11**(25): 4740-4746.

Groves, J. T., and McClusky, G. A. (1976) Aliphatic hydroxylation via oxygen rebound. Oxygen transfer catalyzed by ion, *J. Am. Chem. Soc.* **98**: 859-861.

Guengerich, F. P., (2008) Cytochrome P450cam and chemical toxicology, *Chemical Research in Toxicology*, **21**: 70-83.

Gunsalus, I. C., Meeks, J. R., Lipscomb, J. D., Debrunner, P., and Munck, E. (1974). In: Hayaishi, O. (ed). *Molecular Mechanism of Oxygen Activation*, Academic Press, New York.

Gunsalus, I. C., and Wagner, G. C. (1978) bacterial P-450<sub>cam</sub> methylene monooxugenase components: Cytochrome m, putidaredoxin, and putidaredoxin reductase, *Methods Enzymol.* 52: 166-188.

Harada, K., Elucidation of Functional roles of Heme-propionate Side Chains in Hemoproteins, Doctoral Thesis, Osaka Univ., Jan., 2008.

Harada, K., Sakurai, K., Ikemura, K., Ogura, T., Hirota. S., Shimada, H., and Hayashi, T. (2008) Evaluation of the Functional role of the Heme-6-propionate Side Chain in Cytochrome P450cam, *J. Am. Chem. Soc.*, **130**: 432-433.

Hayashi, T., Harada, K., Sakurai, K., Shimada, H., and Hirota, S., (2009) Role of the Heme-7-Propionate Side Chain in Cytochrome P450cam as a Gate for Regulating the Access of Water Molecules to the Substrate-Binding Site, *J. Am. Chem. Soc.*, in Press.

Hays, A-M.A., Dunn, A.R., Chiu, R., Gray, H.B., Stout, C.D., Goodin, D.B. (2004) Conformational States of Cytochrome P450cam Revealed by Trapping of synthetic Molecular Wires *J.Mol.Biol.* 344: 455-469

"Human Cytochrome P450s" http://drnelson.utmem.edu/human.P450.table.html

Imai, M., Shimada, H., Watanabe, Y., Matsushima-Hibiya, Y., Makino, R., Koga, H., Horiuchi, T., and Ishimura, Y. (1989) Uncoupling of the cytochrome P-450cam monooxygenase reaction by a single mutation, threonine-252 to alanine or valine: A possible role of the hydroxy amino acid in oxygen activation, *Proc. Natl. Acad. Sci. USA* **86**: 7823-7827.

Joo, H., Lin, Z., and Arnold, F. H., (1999) Laboratory evolution of peroxidase-mediated cytochrome P450 hydroxylation, *Nature*, **399:** 670-673.

Laskowski, R.A., MacArthur, M.W., Moss, D.S., and Thornton, J.M., (1993) *PROCHECK*: a program to check the stereochemical quality of protein structures, *J. App. Cryst.* **26**: 283.

Li, H., Narasimhulu, S., Havran, L.M., Winkler, J.D., and Poulos, T.L. (1995) J. Am. Chem. Soc., 117: 6297.

Loida, P. J., and Sligar, S. G., (1993) Engineering cytochrome P-450<sub>cam</sub> to increase the stereospecificity and coupling of aliphatic hydroxylation, *Protein. Eng.*, **6**: 207-212.

Lüdemann, S. K., Lounnas, V., and Wade, R. C., (2000) How do substrates enter and products exit the buried active site of cytochrome P450cam? 1. Random expulsion molecular dynamics investigation of ligand access channels and mechanisms, *J. Mol. Biol.*, **303**: 797-811.

Meilleur, F., Dauvergne, M. T., Schlichting, I., and Myles, D. A. (2005) Production and X-ray crystallographic analysis of fully deuterated cytochrome P450cam, *Acta Cryst.* **D 61:** 539-544.

Murshudov, G.N., Wilson, K.S., and Dodson, E.J., (1997) Refinement of Macromolecular Structures by the Maximum-Likelihood method, *Acta Cryst.* **D53**: 240-255.

Nicholls, A. Sharp, K.A. and Honig, B. (1991) Protein Folding and Association: Insights From the Interfacial and Thermodynamic Properties of Hydrocarbons. Proteins, *Stuc., Func. and Genet.* **11**: 281-296.

Okaya, Y., and Pepinsky, R. (1956) new formulation and solution pf the phase problem in X-ray analysis of noncentric crystals containing anomaraous scatter., *Phs. Rev.*, **103**: 1645-1647.

Ortiz de Montellano, P.R. (Ed.) (2005) Cytochrome P450: Structure, Mechanism, and Biochemistry, 3rd Ed., Kluwer Academic/Plenum Publishers, New York

Oprea, T. I., hummer, G., and Garcia A. E. (1997) Identification of a functional water channel in cytochrome P450 enzymes, *Proc. Natl. Acad. Sci. USA* 94: 2133-2138.

Otwinowski, Z., and Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode, *Methods in Enzymol.*, **276**: 307-326.

Poulos, T. L., Finzel, B. C., and Howard, A. J. (1986) Crystal structure of substrate-free *Psudomonas putida* cytochrome P-450, *Biochemistry* **25**(18): 5314-5322.

Poulos, T.L., Finzel, B.C., and Howard, A.J. (1987) High-resolution crystal structure of cytochrome p450cam, J. Mol. Biol., 195: 687-700.

Pylypenko, O., and Schlichting, I. (2004) Structural aspects of ligand binding to and electron transfer in bacterial and fungal P450cams, *Annu. Rev. Biochem.* 73: 991-1018.

"The PyMOL Molecular Graphics System" http://pymol.sourceforge.net

Rossmann, M. G., and Blow, D. M., (1962) The detection of sub-units within the crystallographic asymmetric unit., *Acta Cryst.* **15**: 24-31.

Schlichting, I., Berendzen, J., Chu, K., Stock, A. M., Maves, S. A., Benson, D. E., Sweet, R. M., Ringe, D., Petsko, G. A., and Sligar, S. G. (2000) The catalytic pathway of cytochrome P450cam at atomic resolution, *Science* **287**(5458): 1615-1622.

Schlichting, I., Jung, C., Schulze, H. (1997) Crystal structure of cytochrome P-450cam complexed with the (1S)-camphor enantiomer. *FEBS Letters* **415**(3): 253-257.

Sligar, S. G., and Gunsalus. I. C. (1976) A thermodynamic model of regulation: Modulation of redox equilibria in camphor monooxygenase, Proc. *Natl. Acad. Sci. USA*, **73**: 1078-1082.

Sono, M., Roach, M. P., Coutler, E. D., and Dawson, J. H., (1996) Heme-containing oxygenases, *Chem. Rev*, 96: 2841-2887.

Vagin, A. A., and Teplyakov, A. (1997) MOLREP: an automated program for molecular replacement., *J. Appl. Cryst.* **30**: 1022-1025.
## **List of Publications**

- Sakurai, K., Shimada, H., Hayashi, T., and Tsukihara, T., Substrate binding induces structural changes in cytochrome P450cam, Acta Cryst. Sec. F., 2009, in press.
- Hayashi, T., Harada, K., <u>Sakurai, K.</u>, Shimada, H., and Hirota, S., A Role of the Heme-7-Propionate Side Chain in Cytochrome P450cam as a Gate for Regulating the Access of Water Molecules to the Substrate-Binding Site, J. Am. Chem. Soc., 2009, in Press.

## **Not Directly Related**

- Aoyama, H., Yamashita, E., <u>Sakurai, K.</u>, and Tsukihara, T., Strategy to Obtain high Resolution Structure of Membrane Proteins by X-ray Crystallography, In Cheng R. H. & Hammer L. (Eds.), Conformational Proteomics of Macromolecular Architecture, World Scientific Publishing, 2004, pp.111-132.
- Harada, K., <u>Sakurai, K</u>., Ikemura, K., Ogura, T., Hirota. S., Shimada, H.,
  and Hayashi, T., Evaluation of the Functional role of the
  Heme-6-propionate Side Chain in Cytochrome P450cam, J. Am.
  Chem. Soc., 2008, 130(2); 432-433

• • • • •