# Protein Engineering of Hydrogen Peroxide-Dependent Cytochrome P450s

過酸化水素駆動型シトクロムP450の蛋白質工学

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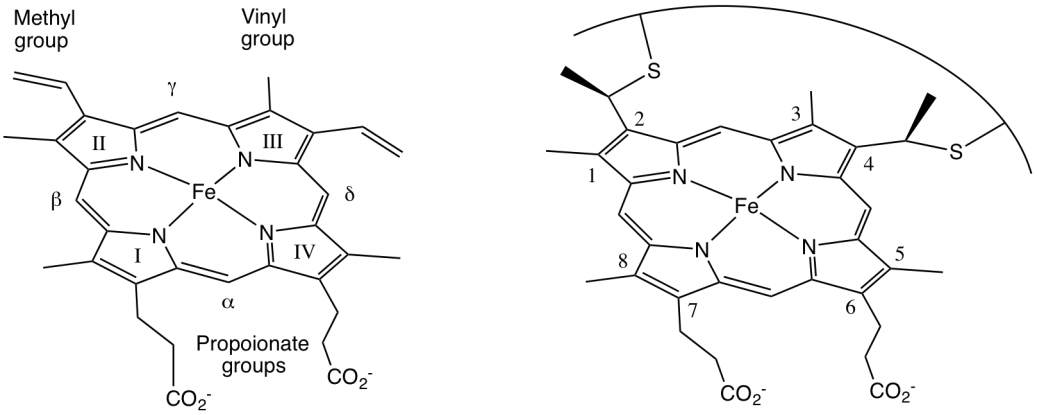
# CHAPTER 1. GENERAL INTRODUCTION

## 1.1 Hemoprotein

Hemoproteins are metalloproteins containing iron-protoporphyrin IX (heme) as the prosthetic group. Hemoproteins are one of the most important metalloproteins in nature. The common types of heme found in the active site of hemoproteins are heme *b*[1] and heme *c*[2] (Figure 1). Heme *b* is iron–protoporphyrin IX noncovalently bound to protein, while heme *c* is characterized by its two thioether covalent bonds between




heme *b* heme *c*

**Figure 1.** Comparison of chemical structures of *b*-type (left) and *c*-type (right) heme. Iron-protoporphyrin IX (heme) is composed of four pyrrole rings (I–IV) linked by bridges of methyl (α, β, γ, δ) forming a tetrapyrrole ring. Pyrroles I and IV convey two propionate groups which utilize their carboxy terminal with the protein surrounding in electrostatic interactions. The center of the porphyrin is a ferrous ($Fe^{2+}$) or ferric ($Fe^{3+}$) iron which is coordinated by the four nitrogens of pyrrole. The Fisher numbering system for substituents of heme is shown in heme c. The curve in heme c indicates as a peptide section. In the majority of cases, this peptide section is separated by two residues, *i.e.* position 4 is attached by cysteine, and the histidine axial ligand follows the cysteine. The symmetry of the heme resolves because of the two vinyl groups and four methyl groups which link the edge protruding of heme from the plane of four pyrrole rings.

**Table 1.** Biological functions of hemoproteins

| Hemoproteins | Functions |
|---|---|
| Myoglobin, hemoglobin | $O_2$ transport/storage |
| Cytochrome *c* oxidase | Electron carrier |
| Cytochrome P450 | Monooxygenation |
| Peroxidase | $H_2O_2$ activation |
| Catalase | $H_2O_2$ dismutation |
| NO synthase (NOSs) | NO synthesis |
| P450nor | NO reduction |
| Heme oxygenase | Heme metabolism |
| FixL, CooA | Gas Sensing |

two cysteine side chains and the vinyl groups at positions 2 and 4 of heme *c*.

Hemoproteins perform diverse functions in physiological systems, including oxygen storage/transport, electron transfer/carrier, gas sensing, and catalysis (Table 1.) [3],[4] although the heme serves as a common active center of hemoproteins. Myoglobin[5] is an oxygen storage protein and hemoglobin[6] is an oxygen transport protein (Figure 2A and 2B). The proximal histidine residue of myoglobin and hemoglobin is very important for oxygen binding. Cytochrome *c*[7] and cytochrome $b_5$[8] (Figure 2C and 2D) serve as an electron transfer protein. In contrast to myoglobin and hemoglobin, there are no enough space for gaseous molecular binding in their proximal side of the heme, that is consistent with their function (electron transfer). Several hemoproteins with function of gas-sensing have been reported, such as FixL[9] and CooA[10] (Figure 2E and 2F). The FixL senses the concentration of molecular oxygen to control the kinase activity.

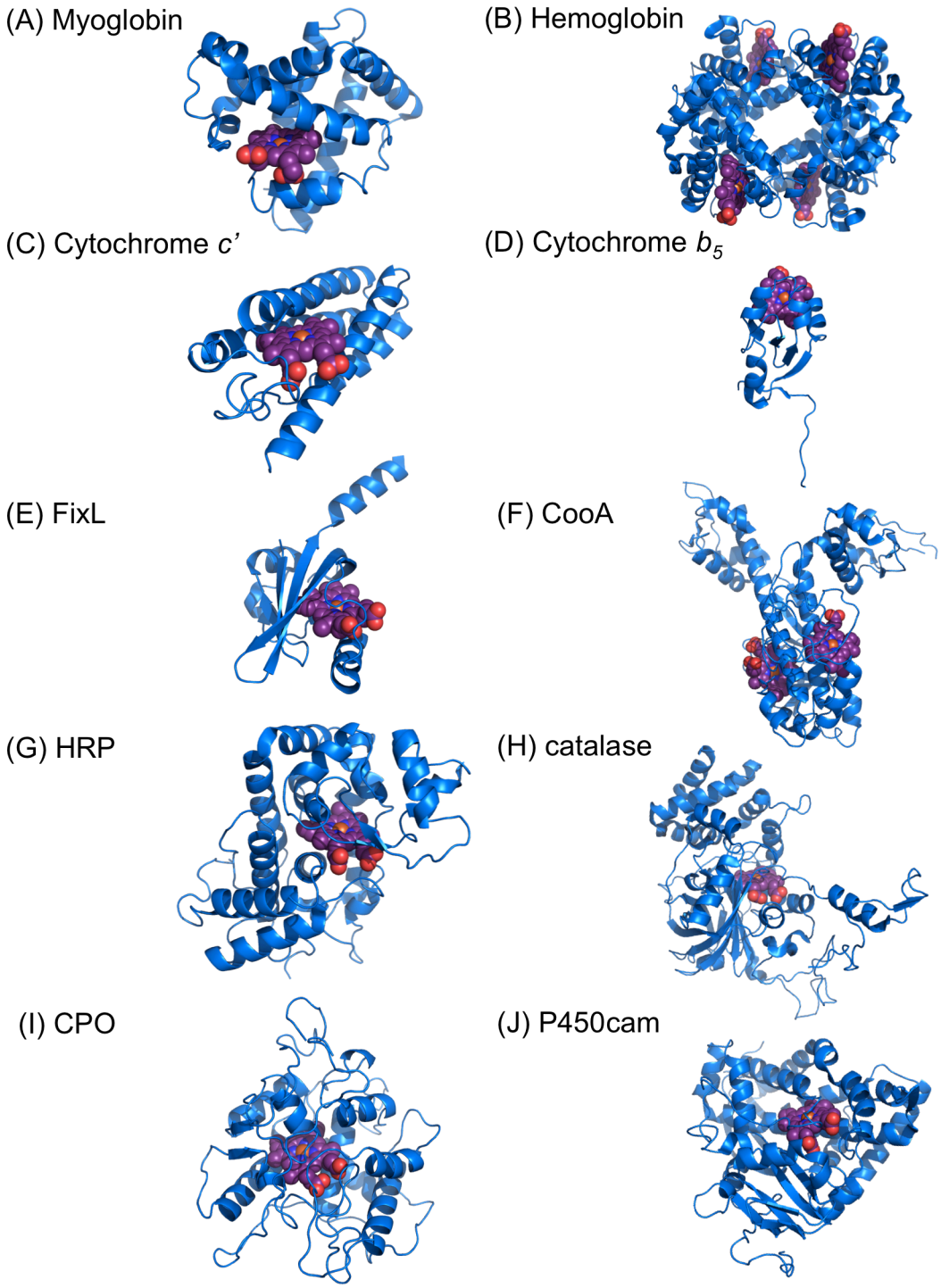



**Figure 2.** Crystal structures of hemoproteins. Hemes are shown as purple space-filling models. (A) myoglobin, PDB ID: 1MBN, (B) human hemoglobin, PDB ID: 2HHB, (C) phototrophic bacteria cytochrome *c'*, PDB ID: 1RCP, (D) human cytochrome $b_5$, PDB ID: 3NER, (E) heme domain of root nodule bacteria FixL, PDB ID: 1DP6, (F) bacteria CooA, PDB ID: 1FT9, (G) horseradish peroxidase (HRP), PDB ID: 1H58, (H) bacteria catalase from *Enterococcus faecalis*, PDB ID: 1SI8, (I) chloride peroxidase (CPO), PDB ID: 1CPO, and (J) P450cam, PDB ID: 3L61.

The CooA from *Rhodospirillum rubrum* senses the level of carbon monoxide and induces the certain genes expression in response to increasing carbon monoxide concentration. There are numbers of hemoproteins with catalytic function. Horseradish peroxidase[11] (HRP, Figure 2G) catalyzes one-electron oxidation of substrates utilizing hydrogen peroxide as an oxidant. Chloroperoxidase[12] (CPO, Figure 2I) also uses hydrogen peroxide, but CPO catalyzes dechlorination reaction and monooxygenation reactions. Cytochrome P450s[13] (Figure 2J) are monooxygenases that catalyze monooxygenation of less reactive organic substrates by activating molecular oxygen. Catalase[14] (Figure 2H) does not catalyze oxidation but disproportionation of hydrogen peroxide into water and oxygen.

The diverse functions of hemoproteins could be attributed to the environment of the active site composed of the heme and proteins including the coordination structure of the heme. In Figure 3, the distal site structures of horseradish peroxidase and catalase are shown for comparison. Histidine residue in the distal side of the heme of HRP and catalase are well known for its function as a general acid-base catalyst for the generation active species using hydrogen peroxide. The axial (proximal) ligand of HRP is histidine but that of catalase is tyrosine (tyrosinate), suggesting different catalytic function could be associated with the axial (proximal) ligand in this case. The coordination structures of CPO and cytochrome P450s (Figure 3D) are similar, i.e., a cysteine residue serves as the axial heme ligand. This suggests that the cysteine ligation to the heme is important for monooxygenation reactions. The arrangement of amino acid residues in the active site of hemoproteins, both in the distal side and in the proximal side, is determine the function of hemoproteins. Many efforts have been devoted to clarifying the structure-function relationship of hemoproteins. A well-established strategy to study the structure-function relationship is investigation of the critical amino acids for catalytic activity by site-directed mutagenesis.

Mutagenesis studies have provided fundamental understandings on the active site of hemoproteins. Furthermore, it also gives us a strategy to improve functions of hemoproteins.

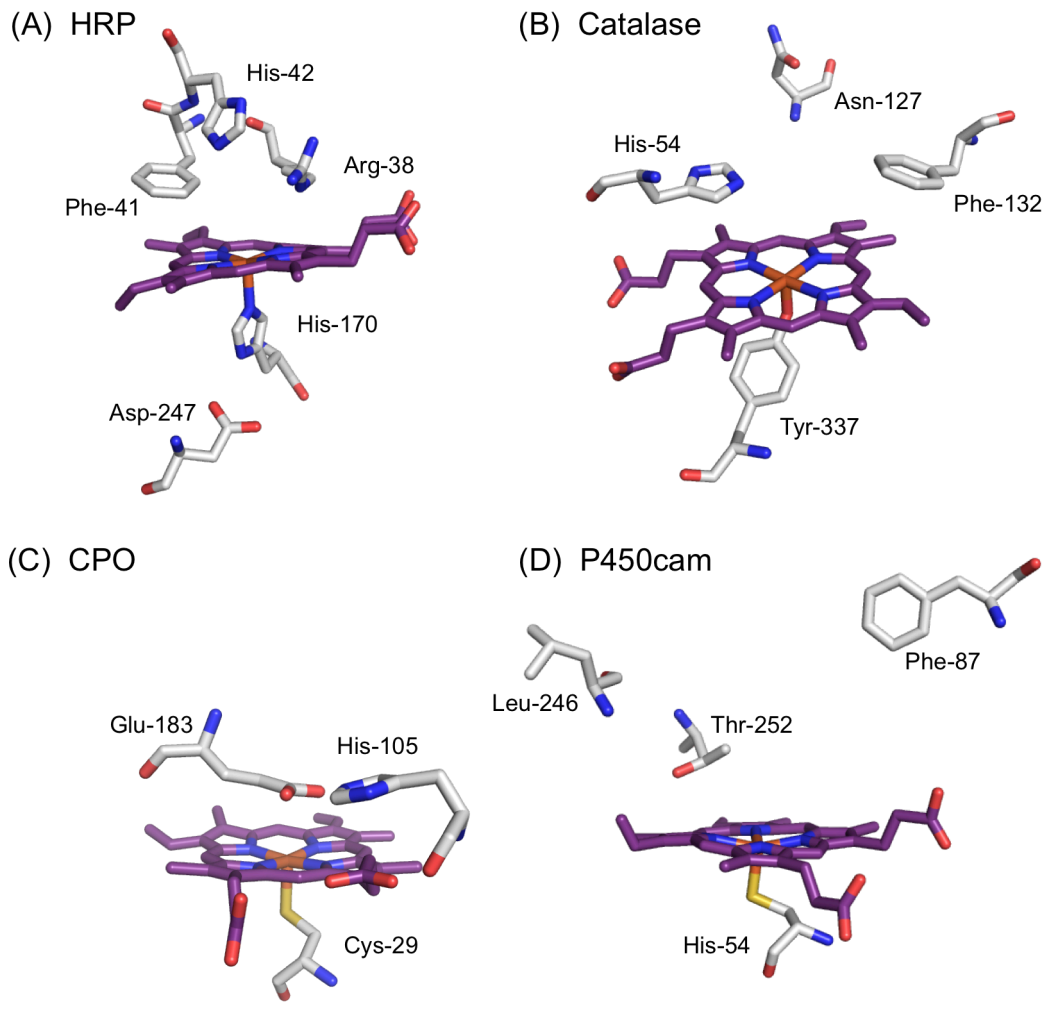



**Figure 3.** Active site structures of (A) HRP, (B) catalase, (C) peroxidase, and (D) P450cam.

### 1.1.1 Cytochrome P450

Cytochrome P450s (CYPs or P450s) constitute a superfamily of enzymes with the ability to metabolize (oxidize) various exogenous and endogenous substrates such as eicosanoids, steroids, pharmaceuticals, pollutants, pesticides, and carcinogens.[13] More than 21,000 distinct P450 proteins are known nowadays.[15] In the human genome, 57 P450s are encoded.[16] P450s involve in the formation of steroid hormones[17] and in the metabolism for external and internal substances.[18],[19] P450s is concerned with the metabolism of the drug around 75% on the market currently.[20] The research on P450s has thus attracted much attention of researchers in the fields of biology, chemistry, and pharmaceutical sciences.

P450 was first found in microsomal fractions of pig[21] and rat liver[22] in 1958. P450s was first characterized in 1962 by Omura and Sato.[23] After two years, the name of P450s was created by the same group based on that the CO bound-form of P450s gave particular absorption at 450 nm.[24] P450 was classified at the same year as a hemoprotein. The standard protocols for purification of P450s from rats were established in 1975-1977 by Lu, Levin, and Guengerich.[25],[26]and[27] Guengerich also carried out the first identification and purification of the human P450s.[28] NADPH-cytochrome P450s reductase that required for reductive activation of oxygen was analyzed by Coon and Lu when they found the role of fatty acid ω-hydroxylation of P450s in 1968.[29]

The first P450 crystal ($P450_{cam}$) was crystallized by Poulos *et al.* in 1985,[30] and the structure was solved in 1987.[31] After 10 years later, the structures of $P450_{BM3}$[32] and P450eryF[33] were solved. Following these results, the number of crystal structures of P450s are deposited to protein data bank (PDB). The whole structure of P450s is a triangular shape with a length of 60 Å and a thickness of 30 Å.[34] The crystal structure of $P450_{cam}$ is shown in Figure 4. The whole structure of P450 can be

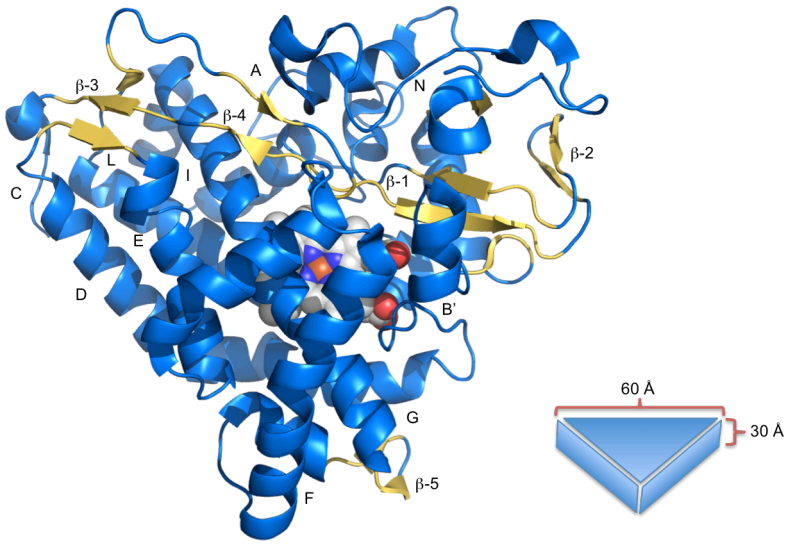
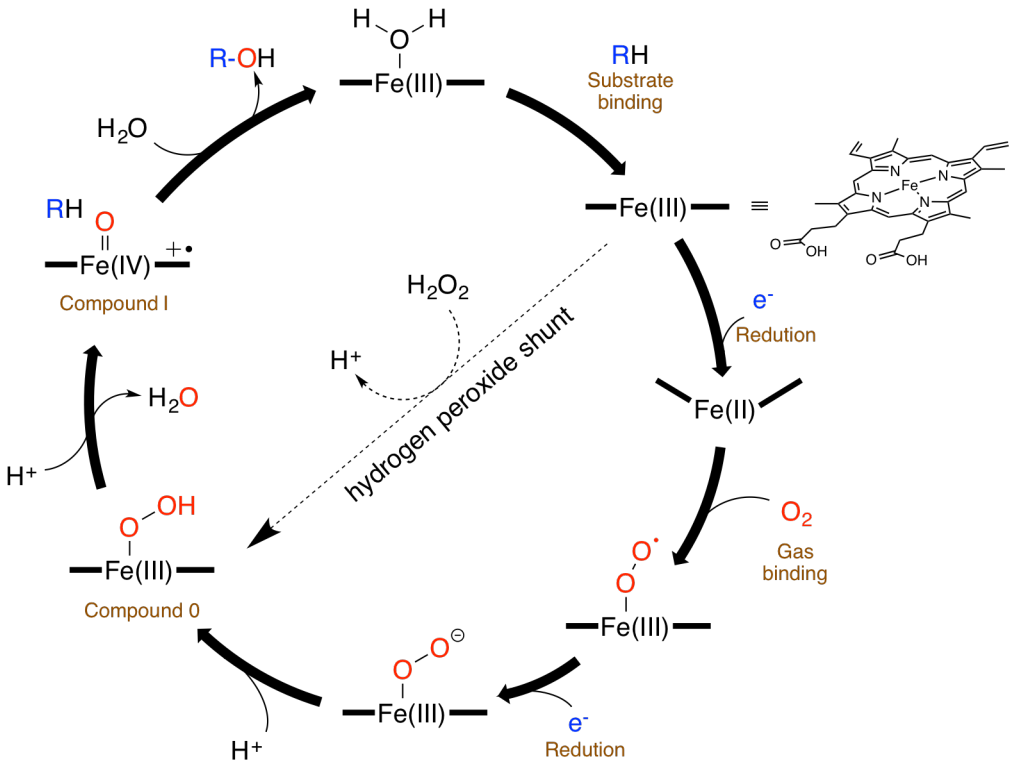



**Figure 4.** Overall crystal structure and schematic representation of P450cam (CYP101A1, PDB ID: 3WRH) which is the first crystal structure of P450 to be solved. The active site heme is shown in space-filled models. The bond distance between Fe (heme) to S (Cys-345) is 2.3 Å. Secondary structure elements are labeled (helices = A to L; β-sheets = 1 ~ 2) and highlighted: β-sheet = yellow, and helices A ~ L = marine.

divided into a smaller β-sheet rich domain consisting of mostly antiparallel β strands and an α-helix rich domain containing the heme.[35] The heme in the active site of P450s is coordinated by the cysteine thiolate. It is also well-known that a very reactive intermediate called compound I (ferryl-oxo porphyrin π–cation radical, $Por^{\cdot+}Fe^{IV}=O$) plays the most important role to carry out the most of reactions catalyzed by P450s.

The general catalytic mechanism of P450s for the activation of molecular oxygen to generate the active species (compound I) is shown in Scheme 1. Most P450s utilize NADPH or NADH as an electron donor for the activation of oxygen.[36],[37] Some of P450s utilize hydrogen peroxide as an oxidant. These P450s are called hydrogen peroxide-dependent P450.[38],[39] We will discuss them separately in the next chapter

(1.1.2.) The catalytic cycle starts from the resting state, a ferric ($Fe^{III}$) 6-coordinate low-spin heme with weakly bound water molecule ($H_2O$). Binding of a substrate (RH) removes the water molecule to give a 5-coordinate high-spin heme. The ferric iron is reduced by a redox partner to give a ferrous iron ($Fe^{II}$) followed by binding of dioxygen to form the ferric-superoxo species. A further reduction accompanied by a proton transfer leads to the generation of ferric-hydroperoxo species called compound 0. The heterolysis of the O–O bond after protonation of Compound 0 give Compound I. The compound I hydroxylates substrates through a "radical rebound mechanism."[40],[41]




**Scheme 1.** The catalytic cycle of cytochrome P450s.

Construction of biocatalysts based on P450s such as that can efficiently catalyze monooxygenation of less reactive organic compounds under mild conditions,[13],[42] has attracted much attention. Recently, P450s have received attention not only from academia but also from industry in conjunction with the development of drugs.

## 1.1.2 Hydrogen Peroxide-Dependent P450s

In the catalytic cycle of P450s shown in Scheme 1, the arrow from the resting state of P450s to compound 0 is called "hydrogen peroxide shunt pathway." $H_2O_2$-dependent P450s can interact directly with $H_2O_2$ and produce compound I, ferryl-oxo porphyrin π–cation radical. $P450_{BS\beta}$ (CYP152A1) from *Bacillus subtilis* [43],[44] and $P450_{SP\alpha}$ (CYP152B1) from *Sphingomonas paucimobilis*,[45],[46] which be classified to the CYP152 family of enzymes, efficiently utilize $H_2O_2$ instead of electrons and molecular oxygen from NAD(P)H.[47] The substrate specificities of $P450_{BS\beta}$ and $P450_{SP\alpha}$ are very high; in fact, these enzymes catalyze the oxidation of long-chain fatty acids exclusively. The analysis of the first crystal structure of $H_2O_2$-dependent P450, $P450_{BS\beta}$, showed that the enzyme lacks general acid–base residues in the heme distal side (Figure 5), although general acid–base amino acid residues are highly conserved in both peroxygenases and peroxidases.[48],[49]and[50]

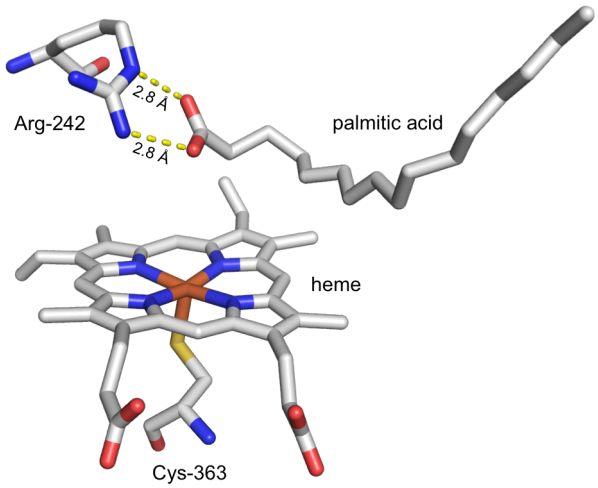
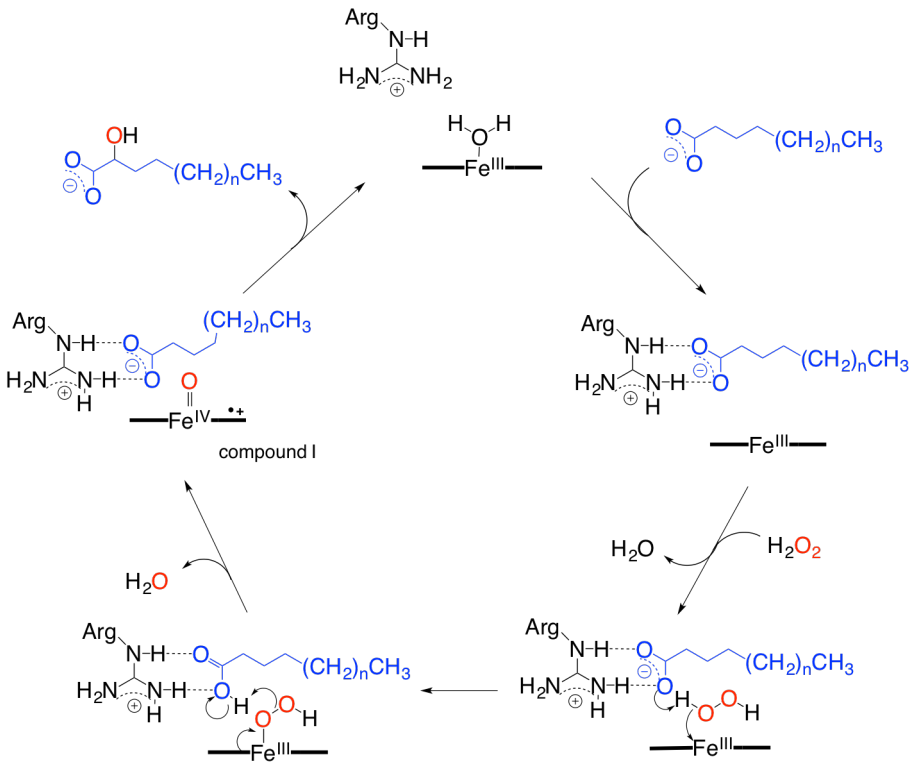



**Figure 5.** The active site structure of palmitic acid-bound form $P450_{BS\beta}$ (PDB ID: 1IZO).

A reaction mechanism was suggested based on the X-ray crystal structure analysis of the fatty acid-bound form of $P450_{BS\beta}$ (Scheme 2);[51],[52]and[53] namely, in place of the general acid–base amino acid residues, the terminal carboxylate group of the bound fatty acids interacting with Arg-242 located in the heme distal side is assumed to serve as the general acid–base catalyst. It was also reported that CYP152L1 ($OleT_{JE}$, PDB ID: 4L40)[54] capture the carboxylate at the "distal arginine." Recently, it was reported that carboxylate-arginine salt bridges of *Aae*APO and *Aau*DyP accelerate the formation of heme active spices.[55],[56] Phylogenetic tree shows the relationship between the bacterial CYP152 family members and other




**Scheme 2.** Proposed mechanisms of catalytic reaction for hydroxylation of long-alkyl-chain fatty acids by $H_2O_2$-dependent P450s.

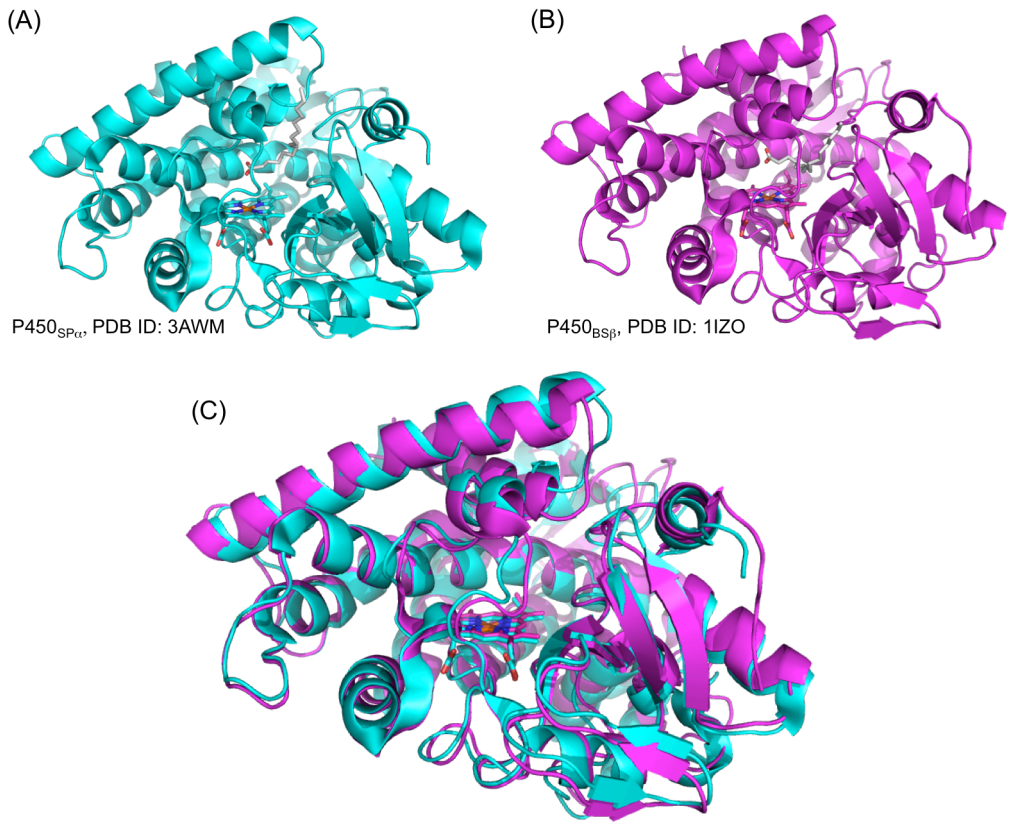



**Figure 6.** The substrate-bound forms of $P450_{SP\alpha}$ and $P450_{BS\beta}$. (A) $P450_{SP\alpha}$ (in cyan; PDB ID: 3AWM), (B) $P450_{BS\beta}$ (in magenta; PDB ID: 1IZO). (C) Superposed structures of $P450_{SP\alpha}$ and $P450_{BS\beta}$. The heme groups and fatty acids are represented in stick models.

bacterial CYP superfamily (Figure 7).[54] CYP152A1 ($P450_{BS\beta}$) is closely related to CYP152B1 ($P450_{SP\alpha}$) within 44% amino acid identity. In addition, the crystal structures of $P450_{Sp\alpha}$ and $P450_{BS\beta}$ are very similar (Figure 6). In fact, CYP152Al, CYP152A2 ($P450_{CLA}$),[57] CYP152B1, CYP152L1[58] and CYP152P1 (CYP-MP)[59] specifically catalyze fatty acid oxidation although each enzyme produces different products: α-, β-, γ-, δ- and ε-hydroxy fatty acids, terminal olefins. Many researchers

discussed the stereo- and regioselectivity of CYP152s using the mutation study,[60] MQ/MM study and MD study.[61],[62] We also reported that the G290F mutant of $P450_{BS\beta}$ exclusively catalyze α-hydroxylation of fatty acids.[63]

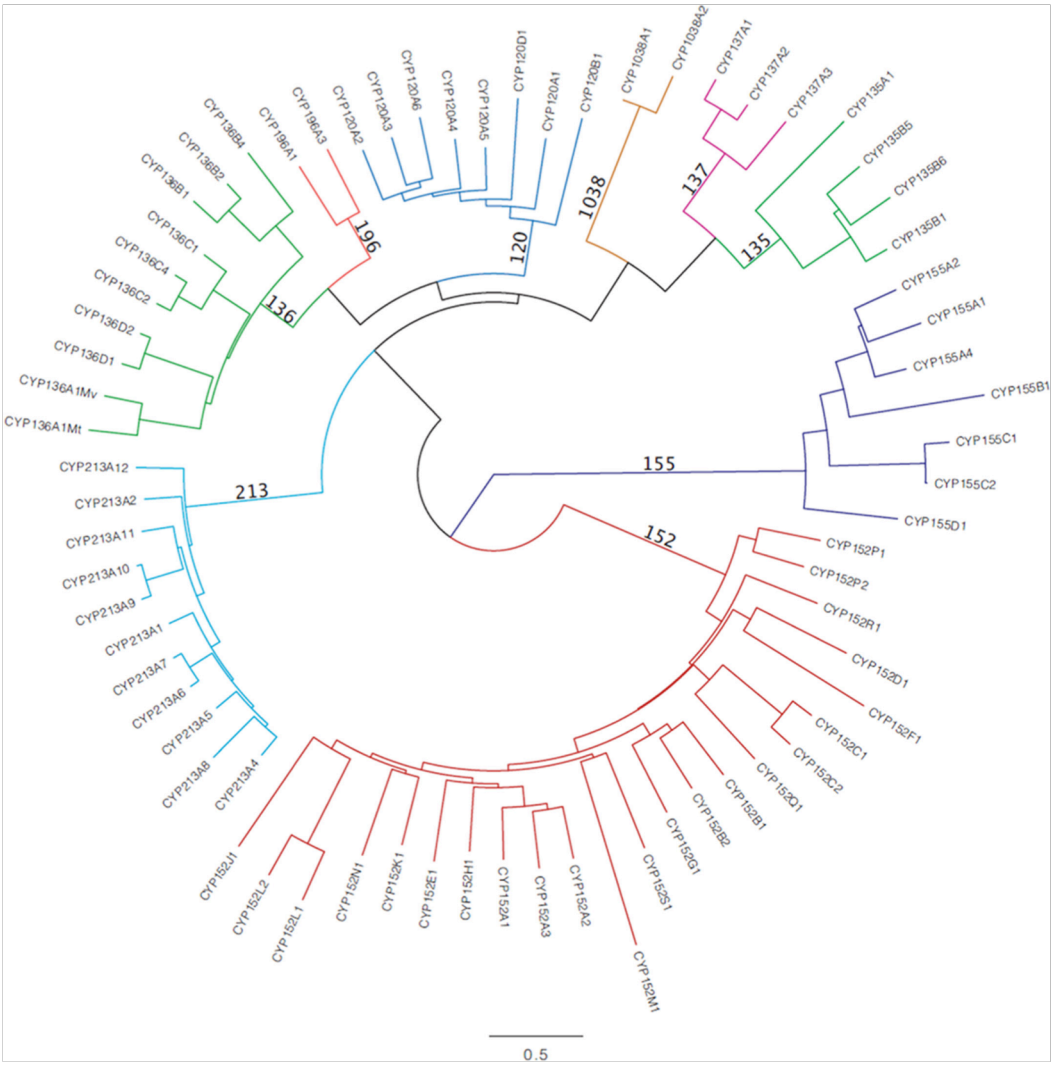



**Figure 7.** Phylogenetic tree of the bacterial CYP152s and other bacterial P450s.

## 1.2 Engineering of Hemoproteins

Protein engineering is a promising strategy to improve native functions of hemoproteins or to develop artificial hemoproteins with different functions. In the case of hemoproteins, not only replacement of amino acids, but also replacement of the heme with synthetic metal complexes can be applied (Figure 8).[64] By site-directed and random mutagenesis, it is possible to replace amino acids in the hemoproteins. Myoglobin (Mb) has been used as a model protein to study the relationship between structure and function of hemoproteins. A variety of Mb mutants

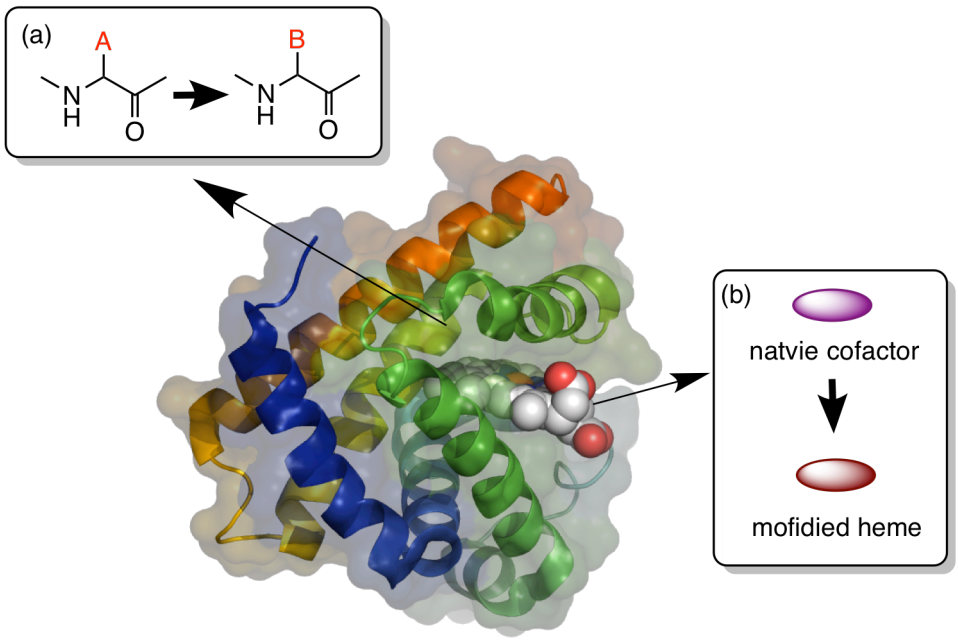



**Figure 8.** Two types of hemoprotein modifications. Model of hemoprotein: myoglobin, PDB ID: 1MBN. (a) Replacement of an amino acid residue with the other B amino acid residue and (b) insertion of an artificial prosthetic group into apo-protein.

have been constructed to develop artificial enzymes and to construct model heme proteins by replacing amino acids located around the heme. For example, H64D mutant of Mb was prepared to simulate the active site of chloroperoxidase (CPO) which has the carboxylate group in the heme distal side (Figure 9D). H64L/F43H was also constructed as a model of the active site of horseradish peroxidase (HRP) which has histidine residue at the distal side of the heme (Figure 9F). Although Mb does not show any catalytic activity, H64D and H64L/F43H mutants of Mb catalyze oxidations of styrene and sulfoxide using hydrogen peroxide as an oxidant, indicating that the amino acid around the heme is very important to determine the function of hemoproteins. H93C and H93Y Mb mutants were also prepared as the models for CPO and catalase (Figure 9H).[65]

The amino acid residues around the heme are also related to the substrate specificity of P450s. The substrate specificities of bacterial P450s are generally very high and thus the catalytic activity toward non-native substrates is very low. According to the P450s catalytic reaction mechanism (Scheme 1), proper binding of the substrate to the active site of P450s is indispensable for beginning the reaction. For this reason, molecules with different structures from those of the native substrates such as gaseous alkanes could not initiate the first step of the catalytic cycle, resulting in a very low catalytic activity. To provide space to accommodate nonnative substrates, a variety of P450 mutants, such as P450cam[66],[67] and P450BM3[68], have been prepared. These mutants have been found to catalyze hydroxylation of inert alkanes such as propane and ethane.[69],[70],[71]and[72] $P450_{PMO}$ (mutant of P450BM3) showed 33,400 total turnovers on propane hydroxylation with a high coupling efficiency of 93%.[70] These studies showed that the mutagenesis of P450s to make a binding site for non-native substrates is a promising method.

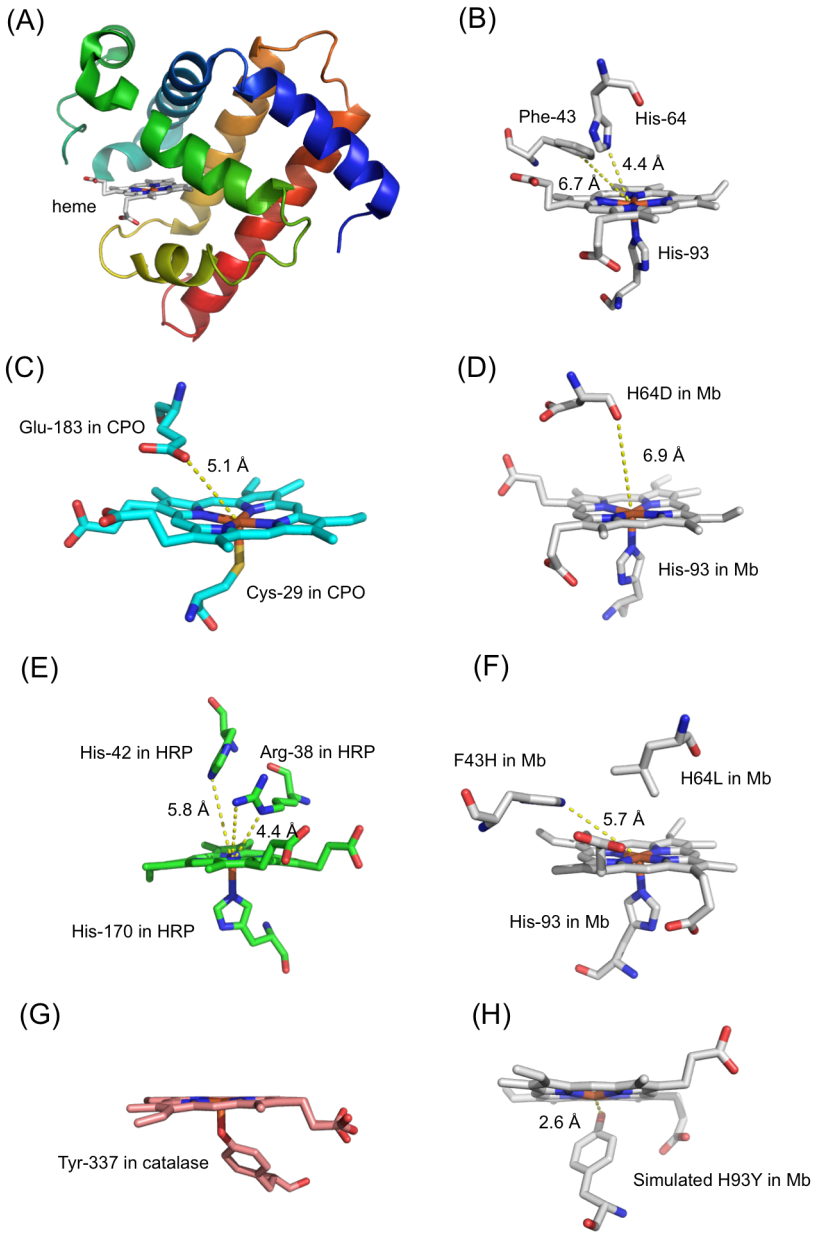



**Figure 9.** (A) Overall structure of wild-type myoglobin and (B-H) the active site structures of selected hemoproteins. (B) The heme pocket of WT Mb. (C) CPO (PDB ID: 1CPO), (D) H64D Mb (PDB ID: 2E2Y), (E) HRP (PDB ID: 1H58), (F) F43H/H64L Mb (PDB ID: 1OFK), (G) catalase (PDB ID: 1SI8), and (H) H93Y Mb created by PyMOL. Key amino acid residues in the distal side and proximal side of the heme are shown as stick models.

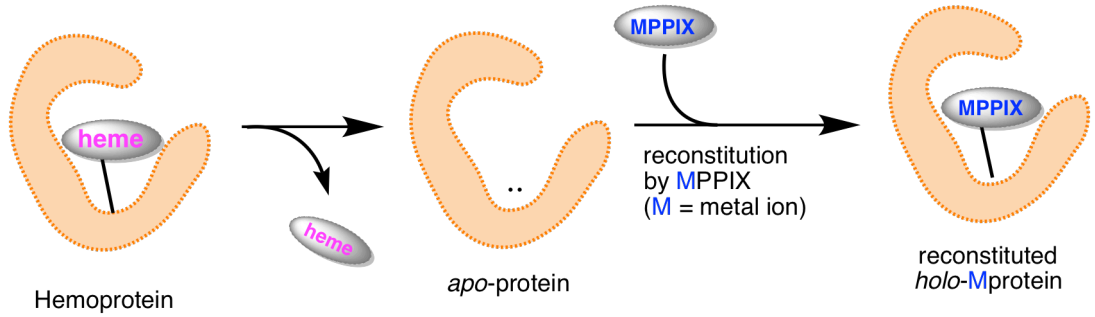



**Figure 10.** Schematic illustration of apo-hemoprotein reconstitution. Preparation of artificial metalloenzymes is reconstituted an apo-hemoprotein with an artificial analogue of the native heme.

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Replacement of the heme of hemoproteins with synthetic metal complexes is a powerful strategy to improve the catalytic activity hemoproteins and to alter properties of hemoproteins (Figure 10).[73] A variety of artificial hemoproteins with heme derivatives shown in Figure 11 as well as various synthetic metal complexes have been reported. Harris *et al.* constructed horseradish peroxidase (HRP) with δ-*meso*-Methylheme or δ-*meso*-ethylheme to investigate the effect of the substitution of the heme on their catalytic activity.[74] Hayashi and Sato *et al*. reported that $k_{cat}/K_m$ remarkably increased when myoglobin was reconstituted with a modified heme having eight carboxyl groups.[75],[76] The reconstituted myoglobin with the modified heme in which the propionate chain on position 6 was instead of arginine-alanine dipeptide or histidine residue. This modifications allowed the substrates to bind more strongly to the protein.[77] Hamachi *et al.* reported a reconstituted myoglobin with the heme containing benzeneboronic acid groups to enable binding of monosaccharides.[78],[79] It is interesting that the monosaccharide binding also enhances the oxygen binding to the reconstituted myoglobin. Hayashi group immobilized apoCYT onto a modified gold electrode surface using a modified dimer

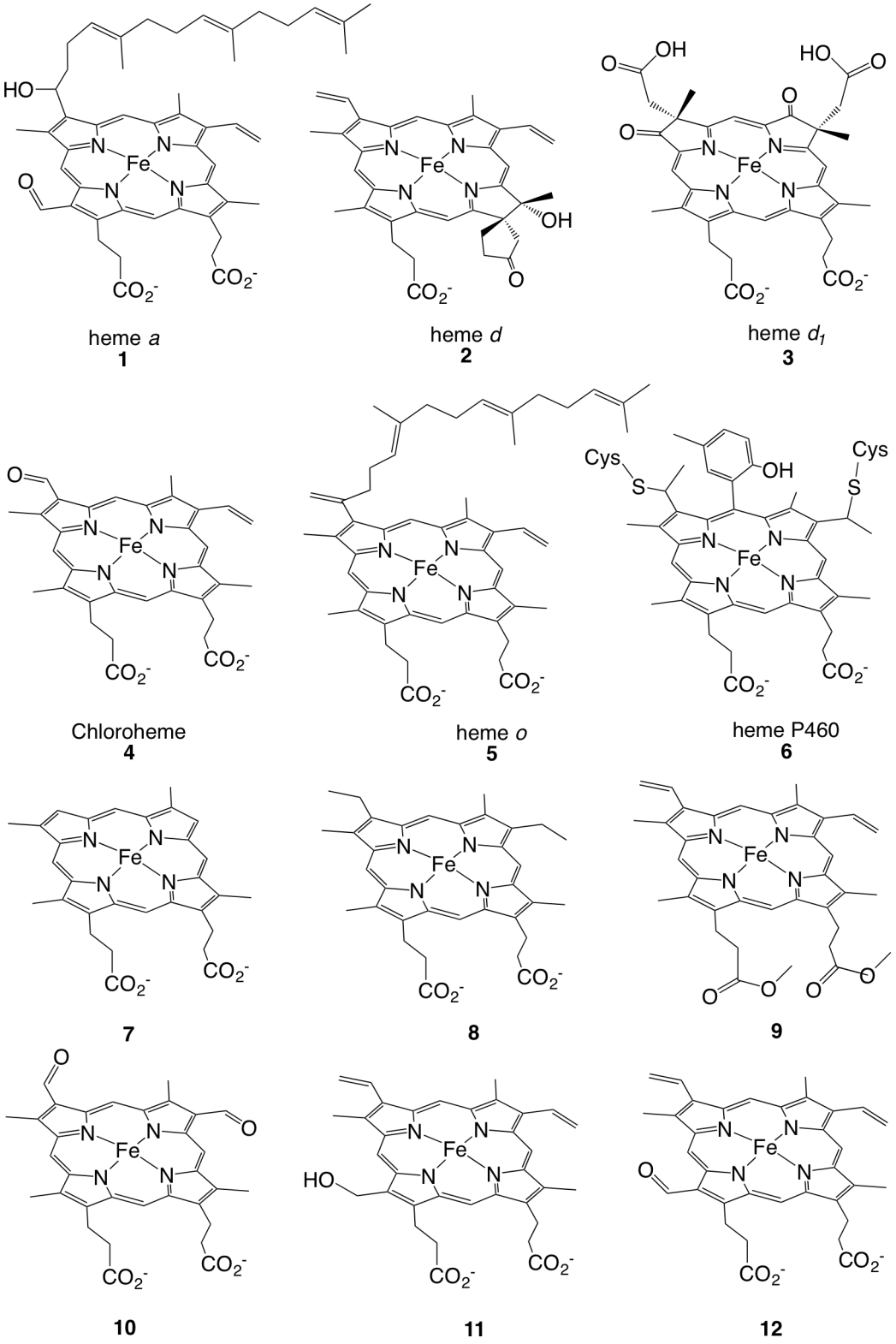


**Figure 11.** Structures of naturally occurring (**1**–**6**, and Fig. 1) and other synthetic heme cofactors (**7**–**12**) widely used in apo-protein reconstitutions.

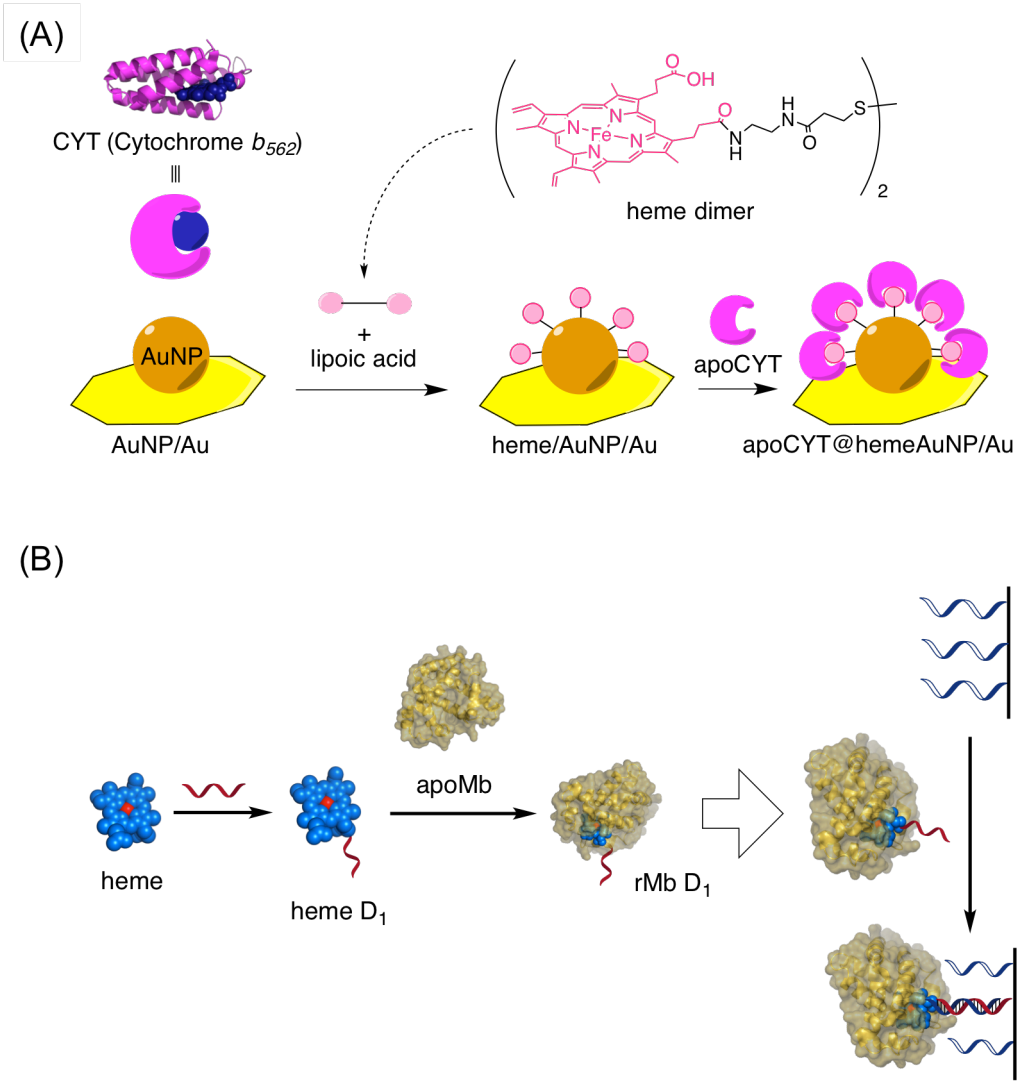


**Figure 12.** (A) Immobilization of apo-CYT through the heme–heme pocket interaction on AuNP-modified gold electrodes. Cytochrome $b_{562}$ is shown in magenta and heme moieties are shown in blue. (B) The use of DNA to immobilize the myoglobin onto surface of substrate.

heme (Figure 12A).[80] Niemeyer group employed a reconstituted apo myoglobin with DNA oligonucleotide-modified heme as a tool for immobilizing the artificial enzymes at the solid surfaces (Figure 12B).[81] Ryabov group reported an electrochemically and catalytically active reconstituted HRP with ferrocene-modified hemin.[82] The replacement of iron of the heme of myoglobin[83],[84], hemoglobin, [85],[86]and[87] and heme enzymes was also reported. The iron of the heme was replaced in order to enhance catalytic activity of HRP,[88] P450cam,[89] and P450BM3[90]. A reconstituted $Co^{II}$PPIX myoglobin is designed as the photoactive center that could be used for $H_2$ evolution and hydrogenation.[91],[92]and[93] A reconstituted myoglobin with zinc-porphyrin was prepared as a model of electron transfer protein.[94],[95]and[96] Other reconstituted hemoproteins in which the propionate group of the heme was modified with relatively large molecules such as lipid,[91] biohybrid surfactants,[97] DNA-modified enzymes,[98] and hemoproteins[99] had been reported.

The heme replacement has been regarded as a powerful strategy to construct artificial hemoproteins.[100],[101] The most of reconstituted hemoproteins were prepared through preparation of apo-hemoprotein. Hemes can be extracted from hemoproteins using acid organic solvents such as acetone and 2-butanone under the harsh conditions.[102] Unfortunately, these methods using organic solvent are not suitable if the hemoproteins and their apo-forms are not strong enough against organic solvents and/or under acidic conditions. Recently, it was reported that reconstituted hemoprotein can be prepared in bacterial cells. Brustad group has demonstrated that the reconstituted P450BM3 can be prepared by exploiting the ability of a natural heme transport protein, ChuA. ChuA imports heme derivatives promiscuously. Therefore, the heme analog can be incorporated into P450BM3 by using ChuA system (Figure 13A) without preparation of apo-hemoprotein.[103] Our research group also reported a single-step method for preparing reconstituted proteins.[90] In this

method, apo-hemoprotein was expressed by *E. coli.* cell under the iron deficient conditions followed by the reconstitution of apo-hemoprotein at the disruption stage of *E. coli.* By using this method, we can prepare reconstituted hemoproteins without preparation and purification of apo-hemoprotein (Figure 13B). However, the reconstitution of hemoproteins using bacterial cells was limited to metal complexes

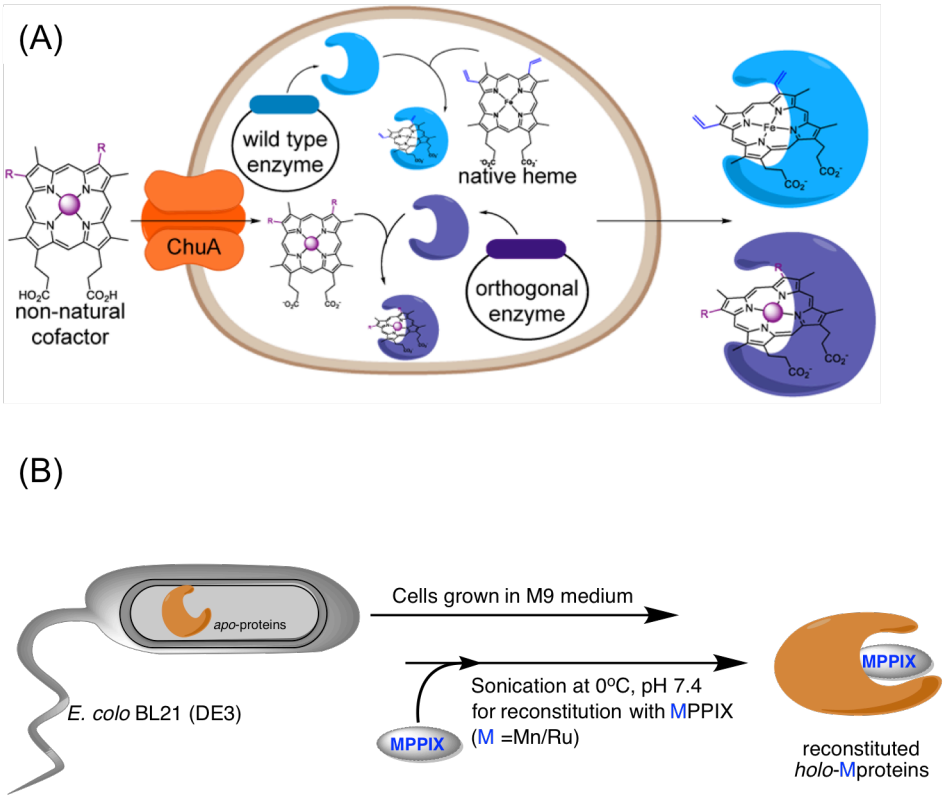


**Figure 13.** Schematic representations for (A) heme transport channel, ChuA (orange), enables cellular import of non-natural heme derivatives (purple) that selectively bind to orthogonal protein scaffolds (purple) without cross-talk with endogenous heme and hemoproteins (black and blue, respectively), and (B) a single-step reconstitution method of hemoproteins.

having a protoporphyrin IX framework. To expand the range of applicable metal complexes, it is important to develop other strategy for removing the heme of hemoproteins without the need for organic solvents and acidic conditions. In Chapter 3, the new strategy to prepared apo-hemoproteins without use of organic solvent is reported.

## 1.3 Outline of Thesis

We are interested in modifying P450s to increase their catalytic activity for non-native substrate oxidation. We have developed a self-sufficient variant of $H_2O_2$-dependent $P450_{SP\alpha}$, A245E mutant of $P450_{SP\alpha}$, which showed a high catalytic ability at styrene catalysis. This result indicating that the self-sufficient $H_2O_2$-dependent P450 can be constructed by site-directed mutagenesis. Although we have succeeded in developing the self-sufficient $H_2O_2$-dependent P450 showing high catalytic activity for styrene epoxidation, its catalytic activates for the hydroxylation of 1-methoxynaphthalene and indole were not as high as that for epoxidation of styrene. Double-sites mutants of $P450_{SP\alpha}$ (L78F/A245E and A245E/F288G) based on the self-sufficient variant have been designed to improve the binding of larger substrates by constricting an active site suitable for binding of 1-methoxynaphthalene and indole. Mutagenesis approach toward $P450_{SP\alpha}$ is described in chapter 2.

$P450_{BS\beta}$ can catalyze oxidation of non-native substrates other than the native substrate, fatty acids.[104] We expected that the catalytic activity of $P450_{BS\beta}$ for non-native substrates can be further improved by replacement of the heme of $P450_{BS\beta}$. However, it has been believed impossible to prepare apo-form of $P450_{BS\beta}$ using acid-butanone method due to irreversible aggregation of $P450_{BS\beta}$. To overcome this limitation, we have developed a new method for preparation of apo-hemoprotein under mild condition. The preparation of apo-$P450_{BS\beta}$ utilizing apo-myoglobin followed by reconstitution with heme analogues is described in chapter 3.

In chapter 4, the summary and prospective of this thesis are described.

## 1.4 References


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# CHAPTER 2.

# Non-native Substrates Oxidation Catalyzed by A Substrate-Binding-State Mimic of $H_2O_2$-Dependent Cytochrome P450

## 2.1 Introduction

Cytochrome P450s (CYPs or P450s)[1] constitute a superfamily of heme-thiolate enzymes that catalyze oxidations of inert organic substrates.[2],[3] Given that bacterial P450s can be obtained in soluble forms by using typical *Escherichia coli* gene expression systems.[4],[5] Since the efficient oxidation of inert substrates C–H bonds is catalyzed by P450s under mild conditions, they attract great attention as the candidates for biocatalysts that can be applied to synthetic chemistry.[6] A wide variety of engineered P450s for the oxidation of inert alkanes and aromatic compounds have thus been constructed by mutagenesis[7],[8] and modification[9],[10]. Most P450s, however, consume a stoichiometric amount of expensive cofactor (NAD(P)H) for the reductive activation of molecular oxygen to generate the active species (Compound I, oxo-ferryl porphyrin with π-cation radical, see Scheme 1).[11],[12]and[13] $H_2O_2$ is known as another oxidant to generate the active species (see

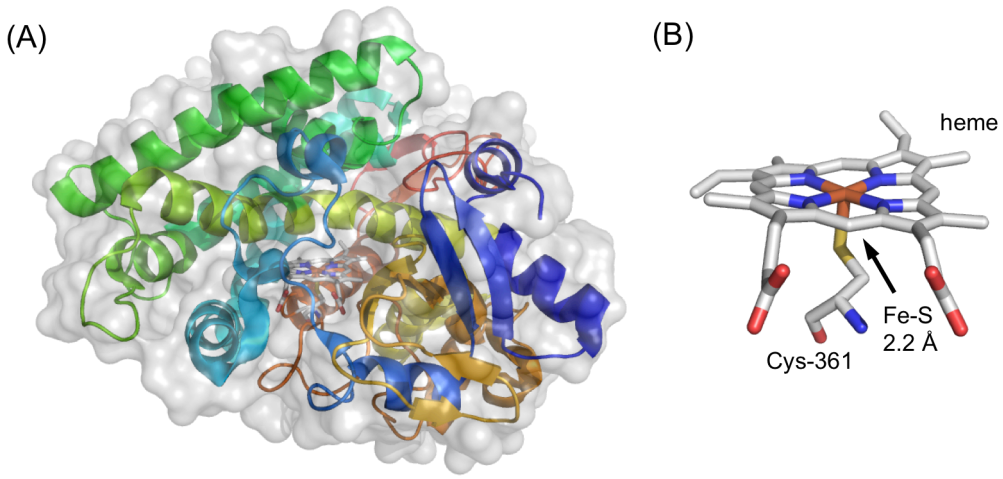
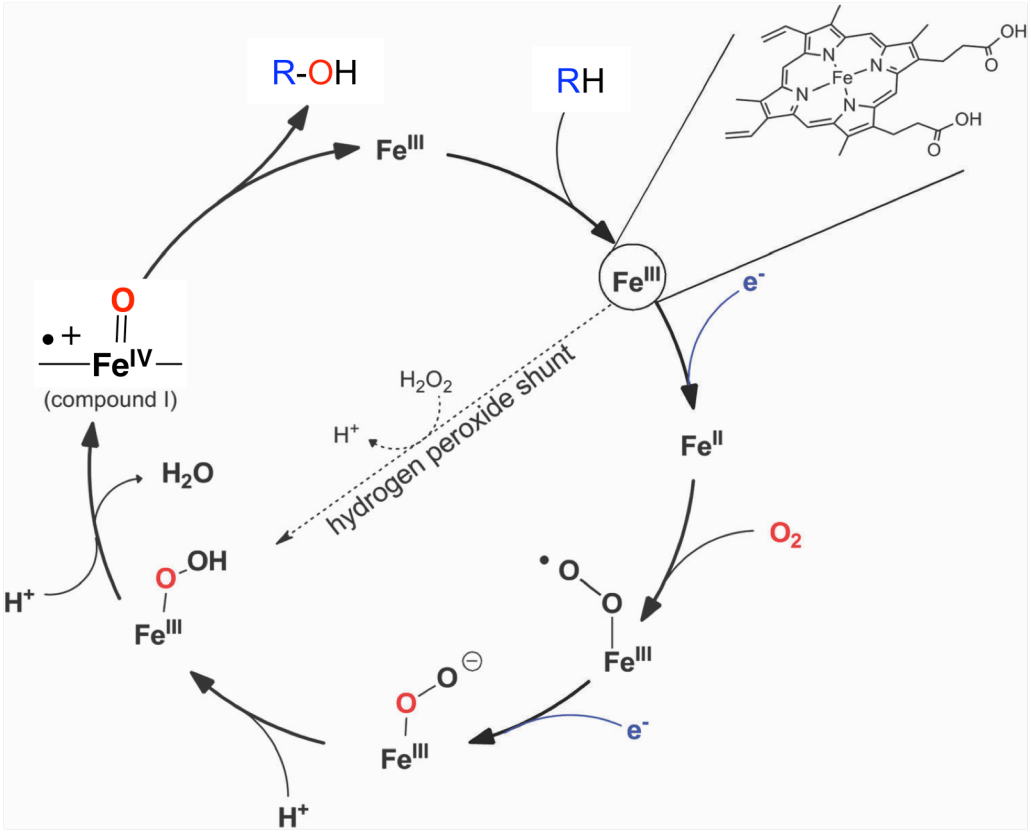



**Figure 1.** Crystal structure of Cytochrome $P450_{SP\alpha}$ (CYP152B1, PDB ID: 3AWM). (A) Whole structure and (B) the active site. The heme and the axial cysteine residue shown as stick models.

Scheme 1, $H_2O_2$-shunt pathway). The $H_2O_2$-shunt reaction is an attractive candidate for monooxygenation reactions catalyzed by P450s,[14] because i) electron transfer partners, such as P450 reductase, are not required for the generation of an active species, and ii) the low cost of $H_2O_2$ allows the reaction to be performed on an industrial scale. However, in general, the $H_2O_2$-shunt reaction is inefficient. The catalytic activities of P450s for the $H_2O_2$-shunt reaction are lower than those when molecular oxygen is used. Thus, site-directed or random mutagenesis has been employed for the evolution of P450s to improve the efficiency via the "peroxide shunt pathway."[15] For example, Arnold group developed a peroxide-drive $P450_{BM3}$ by random mutagenesis.[16]




**Scheme 1.** The catalytic cycle of cytochrome P450s

In contrast to most P450s which use molecular oxygen, $H_2O_2$-dependent P450s, such as $P450_{BS\beta}$ (CYP152A1) from *Bacillus subtilis*,[17],[18]and[19] $P450_{CLA}$ (CYP152A2) from *Clostridium acetobutylicum*,[20] $P450_{SP\alpha}$ (CYP152B1) from *Sphingomonas paucimobilis*,[21],[22]and[23] and OleTJE (CYP152L1) from *Jeotgalicoccus sp.*[24],[25]and[26] $H_2O_2$-dependent P450s efficiently utilize $H_2O_2$ for the hydroxylation of less reactive alkanes. $H_2O_2$-dependent P450s have thus been considered as excellent candidates for practical biocatalysts. $P450_{SP\alpha}$ was the first member to be classified in the family of $H_2O_2$-dependent P450s. Unfortunately, these $H_2O_2$-dependent P450s exclusively catalyze the hydroxylation of long-alkyl-chain fatty acids, and their substrate specificity is very high. The crystal structures of $P450_{SP\alpha}$ (Figure 1 and Figure 2A, PDB ID: 3AWM)[27] and $P450_{BS\beta}$ (Figure 2B, PDB ID: 1IZO)[18] in the palmitic acid-bound forms reveal that $P450_{SP\alpha}$ and $P450_{BS\beta}$ lack any general acid–base residue around the distal side of the heme, whereas the carboxylate group of palmitic acid interacts with the arginine located at the distal side of the heme. Thus, salt bridge formation between the arginine residue and the

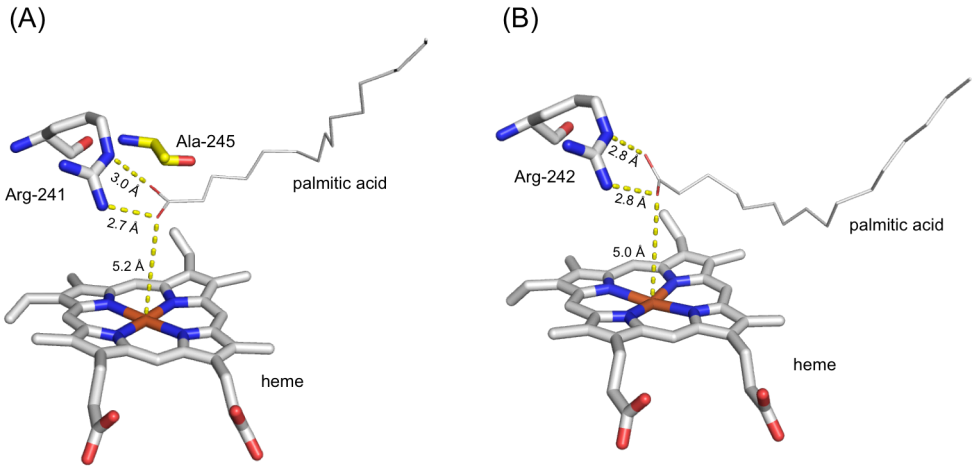
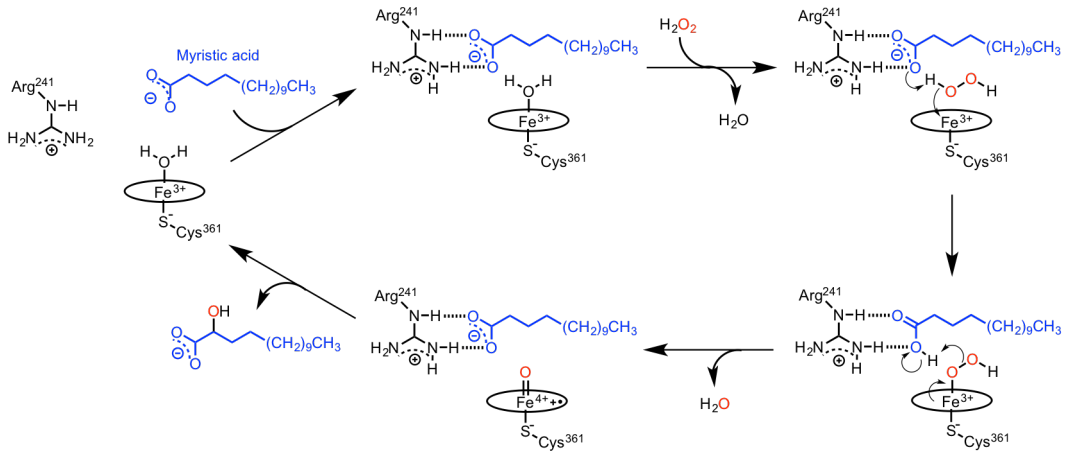



**Figure 2.**The active site structure of (A) $P450_{SP\alpha}$ and (B) $P450_{BS\beta}$ with palmitic acid.

carboxylate group in the substrate is expected to be crucial for the formation of compound I (Scheme 2). This substrate-assisted reaction mechanism also contributes to the high substrate specificity of the enzymes; thus, $P450_{SP\alpha}$ and $P450_{BS\beta}$ never oxidize substrates other than fatty acids. Thus, their substrate specificities are generally high and the catalytic activity toward nonnative substrates is very low. To utilize these P450s for nonnative substrate oxidation, their substrate specificities need to be changed. Nevertheless, we succeeded in oxidizing non-native substrates other than fatty acids by $P450_{SP\alpha}$[18] and $P450_{BS\beta}$[28],[29]and[30] by employing a series of short-alkyl-chain carboxylic acids as decoy molecules (inert dummy substrates). Given that the peroxygenation of non-native substrates proceeds with decoy molecules, these reactions are catalyzed with the assistance of these short-alkyl-chain carboxylic acids. It appears that decoy molecules are misrecognized by $P450_{BS\beta}$ as the native substrate and the resulting salt bridge (between Arg-242 and the carboxyl group of decoy molecule) serves as a general acid–base catalyst. By employing this simple trick of substrate misrecognition by using decoy molecules, non-native substrate oxidations such as the sulfoxidation of thioanisole,[29] one-electron




**Scheme 2.** Proposed catalytic reaction mechanism for the hydroxylation of fatty acid catalysed by $P450_{SP\alpha}$.

oxidation of guaiacol,[28] epoxidation of styrene,[31] hydroxylation of C–H bond of ethylbenzene,[28] and hydroxylation of aromatic C–H bond of 1-methoxynaphthalene[30] were all catalyzed by $P450_{BS\beta}$ and $P450_{SP\alpha}$. Interestingly, the catalytic activity is highly dependent on the alkyl chain length of decoy molecules. Moreover, $P450_{SP\alpha}$ could be used to catalyze epoxidation reactions of styrene by adding a variety of short-alkyl-chain carboxylic acids as decoy molecules.[31]

Very recently, it has been reported that the oxidation of non-native substrates can be catalyzed by $P450_{SP\alpha}$ and $P450_{BS\beta}$ in the presence of a high concentration of the acetate anion, which also serves as a general acid–base catalyst.[32] The epoxidation of styrene catalyzed by $P450_{SP\alpha}$ and $P450_{BS\beta}$ was therefore performed with a high concentration of the acetate anion to investigate whether acetic acid could work as a decoy molecule.[31] It was found that both $P450_{SP\alpha}$ and $P450_{BS\beta}$ catalyzed styrene epoxidation, and the catalytic activity was enhanced by increasing the concentration of acetate anion. It is assumed that the acetate anion interacts with the Arg-242 of $P450_{BS\beta}$ and Arg-241 of $P450_{SP\alpha}$ in the distal side of the heme and serves as a general acid–base catalyst. The initial turnover frequency for the epoxidation of styrene catalyzed by $P450_{BS\beta}$ reached 590 $min^{-1}$ in the presence of 1 M acetic acid.

Although the addition of external carboxylic acids (decoy molecules) is effective for the oxidation of non-native substrates, the use of decoy molecules would not be suitable for the enzyme reaction *in vivo* in *Escherichia coli*. To perform peroxygenation of non-native substrates by $P450_{SP\alpha}$ without the use of external carboxylic acids, we introduced an amino acid bearing a carboxylate side chain to mimic the fatty-acid binding state of $P450_{SP\alpha}$. The crystal structure of $P450_{SP\alpha}$ suggests that Ala-245, in the distal I helix, is a candidate that is suitable for placing a carboxylate group close to the heme by mutagenesis. We have demonstrated that the A245E mutant of $P450_{SP\alpha}$ can oxidize a various of non-native substrates while the $P450_{SP\alpha}$ wild-type without catalytic ability. We suggested that the carboxylate of

Glu-245 involves in the generation of active species and play the key role (Figure 3B).[27] For the further application as biocatalysts, we prepared mutants of $P450_{SP\alpha}$ based on A245E mutant to improve catalytic activity for non-native substrates such as aromatic hydroxylation of indole to generate indigo. While A245E mutant of $P450_{SP\alpha}$ afforded high catalytic ability for styrene catalysis,[31] the hydroxylations of 1-methoxynaphthalene and indole were not efficient. To improve the catalytic activity toward 1-methoxynaphthalene and indole, we decided to examine double mutants of $P450_{SP\alpha}$, L78F/A245E and A245E/F288G. We expected a possible π-π interaction in the case of L78F/A245E. In the case of A245E/F288G, the active site would be much larger compared with A245E which would result in better accommodation of large substrates.

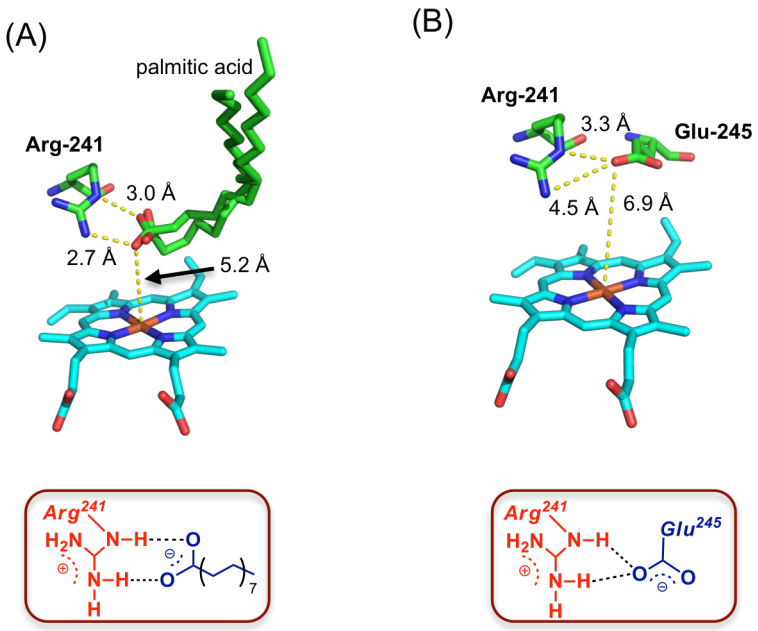



**Figure 3.** The active site structure of $P450_{SP\alpha}$. (A) fatty-acid-bound form, and (B) A245E mutant (PDB ID: 3VOO).

# 2.2 Experimental Method

## 2.2.1 Materials

All chemical reagents were purchased from commercial sources. δ-aminolevulinic acid hydrochloride and DNase I (20 U/μL) were purchased from Cosmo Bio Co., Ltd (Tokyo, Japan) and Roche Diagnostics K. K. (Basel, Switzerland), respectively. Thrombin and myristic acid ($C_{14}$) were purchased from Sigma-Aldrich Co., (St. Louis, MO). $H_2O_2$, dithiothreitol (DTT), ethylene glycol, sodium dodecyl sulfate (SDS), cholic acid, isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin sulfate, and reduced glutathione were obtained from WAKO Pure Chemical Industries, Ltd (Osaka, Japan). The following chemicals were purchased from Nacalai Tesque Inc. (Kyoto, Japan): trimethylchlorosilane (TMCS), glycerol, potassium chloride, tris(hydroxymethyl)aminomethane, urea, hydrochloric acid, phosphoric acid, $K_2HPO_4$, ampicillin sodium salt, lysozyme, and phenylmethylsulfonyl fluoride.

## 2.2.2 Measurement

UV–Visible (UV-Vis) absorption spectra were recorded on a UV-2600 PC spectrophotometers (SHIMADZU CORPORATION) or AGILENT 8453 with a thermal controller 89090A, and the data were collected using quartz cuvettes of 1 cm path length with screw caps. The purification procedures described below were carried out using a Bioassist eZ system (TOSOH CORPORATION). The protein figures were depicted using The PyMOL Molecular Graphics System (DeLano Schrödinger, LLC.), Version 1.8.

## 2.2.3 Protein Expression and Purification

A recombinant enzyme of $P450_{SP\alpha}$[33],[22] was expressed in transformed *Escherichia coli* strain BL21 as previously reported with some modification. For expression of $P450_{SP\alpha}$, *E. Coli* M15 were initially cultivated in LB medium supplemented with 100 μg/ml of ampicillin at 27°C. Cells were grown to an optical density at 600 nm ($OD_{600}$) of 0.7-0.8, and δ-aminolevulinic acid (0.5 mM) was added. While $OD_{600}$ = 1.0-1.2, gene expression was induced by addition of IPTG (0.1 mM), and cultivation followed by induction at 20°C for 20 hours at 80 rpm. Cells were then harvested by centrifugation and lysed in 0.1 M Tris-HCl buffer (pH 7.5) containing 20% (v/v) ethylene glycol, 1 mM DTT, 1% (w/v) cholic acid, 0.1% (w/v) SDS, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride containing 1 mg/ml lysozyme, and 140 U/100 ml DNase I. After sonication of the suspended cells, the supernatant was collected by centrifugation and diluted in two volumes of 0.1 M Tris-HCl buffer (pH 7.5) containing 20% (v/v) ethylene glycol and 1 mM DTT. The diluted solution was loaded on a glutathione–sepharose column (GE Healthcare UK Ltd, Little Chalfont, UK) and the column was washed with 0.1 M Tris-HCl buffer (pH 7.5) with 1 mM DTT, 0.3% (w/v) cholic acid, 20% (v/v) ethylene glycol, and 0.03% (w/v) SDS. Glutathione *S*-transferase (GST)-fusion $P450_{SP\alpha}$ was eluted with 0.1 M Tris-HCl buffer (pH 7.5) with 1 mM DTT, 0.3% (w/v) cholic acid, 20% (v/v) ethylene glycol, 0.03% (w/v) SDS, 1.5 M urea, and 5 mM reduced glutathione. Thrombin cleavage of GST-fusion $P450_{SP\alpha}$ was performed during dialysis against 0.1 M potassium phosphate buffer (pH 7.0) with 20% (v/v) glycerol and 0.3 M KCl and at 4 $^{o}$C for 15 h. The reaction mixture was passed through a glutathione Sepharose column again and a benzamidine Sepharose column (GE Healthcare UK Ltd) to separate GST and thrombin from $P450_{SP\alpha}$, respectively. The fractions were collected and concentrated to less than 5 ml by centrifugation using Amicon Ultra filter units (Millipore, Co.,

Cork, Ireland). The concentrated sample was employing and loaded onto an S-200 Sephacryl HR column (GE Healthcare, Little Chalfont, United Kingdom) for size exclusion chromatography as further purified. The $P450_{SP\alpha}$ purity was estimated using SDS-PAGE, and the $P450_{SP\alpha}$ concentration was determined by using CO difference spectra.[34]and[35] All mutants were prepared according to the procedure used for the WT (Figure 4).

**The sequence of synthetic gene (CYP152B1) optimized for expression in *E. coli.***

atgcctaagacaccacacacgaaaggcccggacgaaaccctttctctcctcgccgatccctatcggttcatctccaggcag
tgccagcgcctaggcgcaaacgcttttgaatcccgtttcctgctgaaaaagaccaactgccttaaggggccaaggccgc
cgaaatcttctacgatacgacccgcttcgagcgcgaaggtgccatgccggtcgctattcagaagacactgctgggccaag
gcggcgttcagggtctggacggcgagacccatcggcaccgcaagcagatgttcatgggcctgatgacaccagagcgcg
tgcgggcgctggcgcaattgttcgaggccgagtggcgcagggcggtgccgggctggacgcgcaaaggagagatcgtc
ttctatgacgagttgcatgagccgctgacgcgagcggtatgcgcttgggcgggagttcctcttccggacgatgaagccgg
caaccgcgcgggcgagctgcgggcgctgttcgacgccgccggctcggcgagcccaaggcatctttggtcacggctgg
cccgtcgtcgcgtcgacgcatgggcgaagcggatcattgaaggcattcgggccgggagcatcggctcgggctcgggg
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aaatgtccttcgcccgaccgtcgcgatcgcagtgtacatcaccttcgtcgcccacgcgctgcaaacctgttcagggatcag
ggcggccttggttcagcagccggactatgccgagctcttcgtccaagaggtccgccgattctatcccttctttcccgctgtg
gtggcgcgcgcgagccaagatttcgagtgggaggggatggcctttccggaagggcgtcaagtggtgctagacctttatg
ggagcaatcacgacgcagcgacctgggccgacccccaggagtttcgccctgagcgtttcagggcttgggacgaagact
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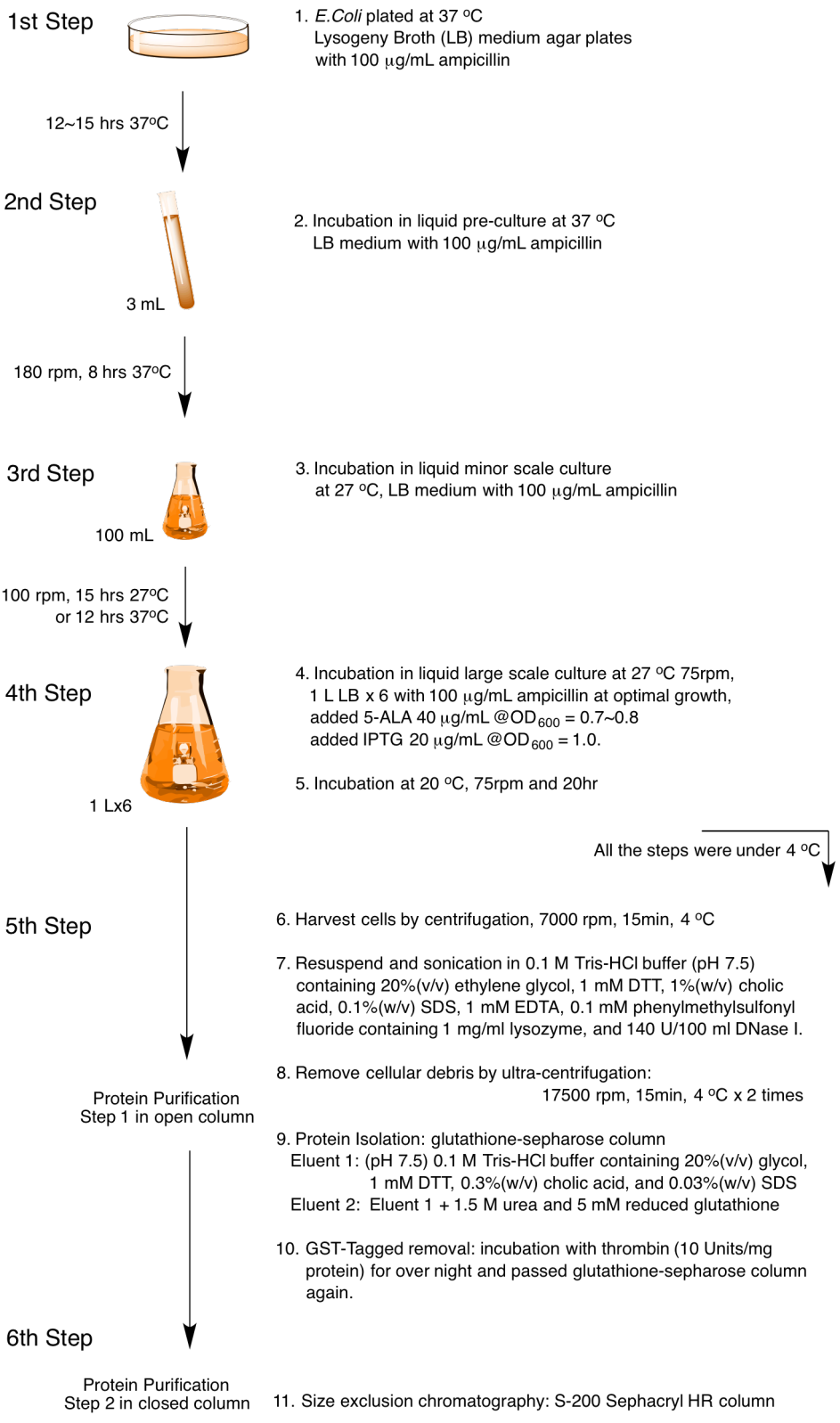


**Figure 4.** Protein preparation protocol for recombinant form $P450_{SP\alpha}$.

## 2.2.4 Preparation of $P450_{SP\alpha}$ mutants

Site-directed mutagenesis of $P450_{SP\alpha}$ was performed according to the instruction manual of the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) using pGEX-AX2 plasmids containing the gene of $P450_{SP\alpha}$ WT as templates.[22] The primers of one-point mutagenesis were (mutated codons are underlined and capitalized) 5'-tcgcccgaccgtcGAGatcgcagtgtacatc-3' (sense) and 5'-gatgtacactgcgatCTCgacggtcgggcga -3' (antisense) for $P450_{SP\alpha}$ A245E. The primers of double-point mutagenesis were containing the gene of $P450_{SP\alpha}$ A245E as templates, and the sequences were (mutated codons are underlined and capitalized) 5'-cagaagacactgTTTggccaaggcgg-3' (sense) and 5'-cgccgccttggccAAAcagtgtcttc-3' (antisense) for $P450_{SP\alpha}$ L78F/A245E, 5'-attctatcccttcGGTcccgctgtggt-3' (sense) and 5'-caccacagcgggACCgaagggatagaa-3' (antisense) for $P450_{SP\alpha}$ A245E/F288G. Expression and purification of the $P450_{SP\alpha}$ mutants were performed as described

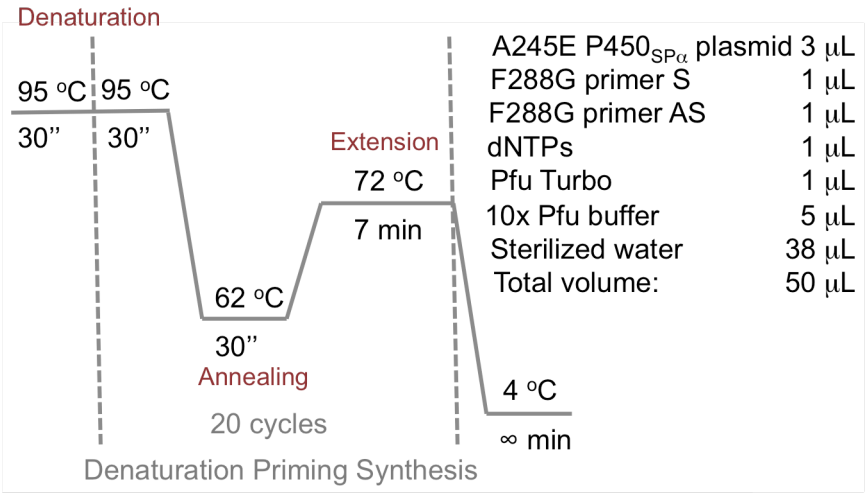


**Figure 5.** Schematic diagram of the PCR process for $P450_{SP\alpha}$ A245E/F288G mutant. $P450_{SP\alpha}$ L78F/A245E mutant was under the same condition.

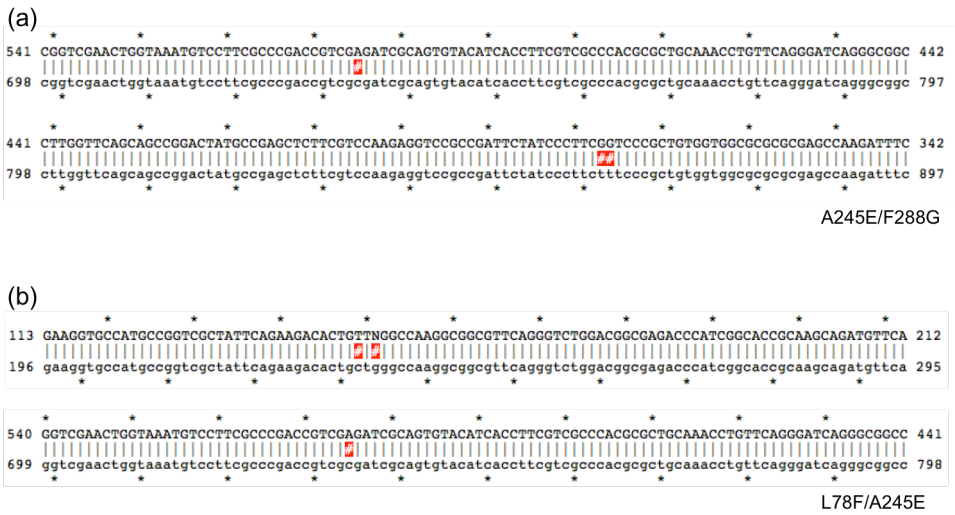


**Figure 6.** Part of DNA sequencing chromatogram for $P450_{SP\alpha}$ mutant (a) A245E/F288G and (b) L78F/A245E.

above.

## 2.2.5 Determinations of P450 Concentration

Recombinant $P450_{SP\alpha}$ was measured by reduced CO-difference spectroscopy.[35]

## 2.2.6 1-Methoxynaphthalene oxidation

The reaction was carried out in 100 mM potassium phosphate buffer (pH 7.0) with 0.5 μM of A245E mutant of $P450_{SP\alpha}$, 0.5 mM of 1-methoxynaphthalene (dissolved in ethanol, the final mixture includes 5% of ethanol), and 0.5mM of $H_2O_2$ for 1 min at 25 °C. The molarities were all shown in final concentrations. The catalytic experiment was observed definitely by color from colorless to blue. The reaction mixture was extracted with chloroform with the equal volume of chloroform

to extract Russig's blue from aqueous phase. The extract was filtrated and diluted to 5 mL. By monitoring the absorption at 634.5 nm (ε=18,900 $M^{-1}cm^{-1}$ in chloroform [30]) the catalytic activities of hydroxylation of 1-methoxynaphthalene were determined. The turnover rates are averages of at least three measurements.

## 2.2.7 Indole Oxidation

The reaction was carried out in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM indole (dissolved in ethanol, the final mixture includes 5% of ethanol), 0.5 μM of A245E mutant of $P450_{SP\alpha}$, 0.5mM of $H_2O_2$ for 1 min at 25 °C. The molarities were all shown in final concentrations. The catalytic experiment was observed definitely by color from colorless to blue. The mixture is extracted with chloroform to extract indigo from aqueous phase. The catalytic activity was determined by the absorbance of indigo at 605 nm with a molar absorption coefficient of 11,000 $M^{-1}cm^{-1}$ (determined by commercially available indigo) and the turnover numbers were averaged of least three measurements. Indole and 1-methoxynaphthalene were very useful substrates because the oxidation products show blue color (Figure 7).

## 2.2.8 Extinction Coefficient (ε) of Indigo in $CHCl_3$

According to Beer's Law: A = εbc, the absorbance (A) of indigo in chloroform was determined by the UV-Visible absorbance spectra in a 1-cm cell. The wavelength of maximum absorbance intensity, $\lambda_{max}$ = 603 nm was recorded. Solution of known concentration of commercially available indigo was prepared in chloroform by volumetric flask. UV-Visible spectra of further diluted solutions were obtained to

determine the $\lambda_{max}$. Absorbance of solutions of different concentrations was also obtained respectively. From the collected absorbance values, ε was calculated by determining the slope of a plot of absorbance Vs concentration. The ε of indigo in chloroform was measured and gave an extinction coefficient of 11,000 $M^{-1}$ $cm^{-1}$.

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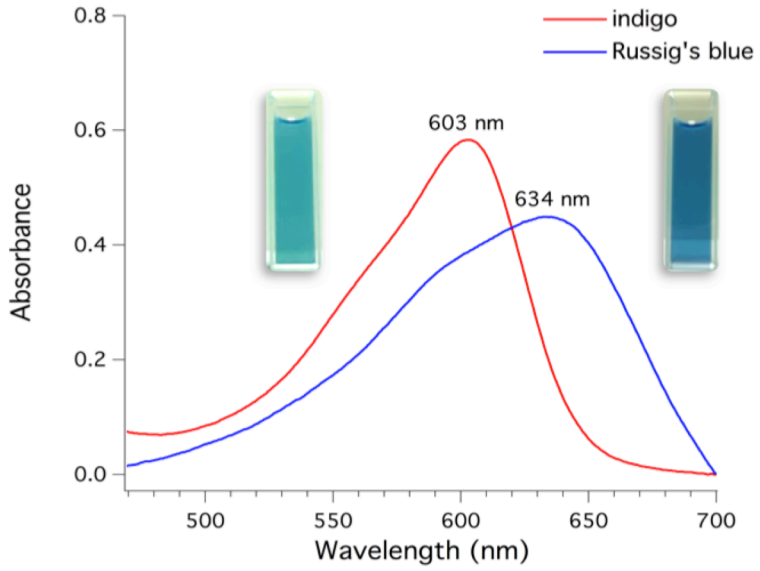



**Figure 7.** UV-Visible absorption spectra of catalytic products by $P450_{SP\alpha}$ mutants in chloroform. $\varepsilon_{603\ nm@CHCl3}$ of indigo is 1.1 X $10^4$ $M^{-1}$ $cm^{-1}$, and $\varepsilon_{634\ nm@CHCl3}$ of Russig's blue is 1.89 X $10^4$ $M^{-1}$ $cm^{-1}$.

## 2.3 Result and Discussions

Based on the crystal structure analysis, an $P450_{SP\alpha}$ A245E mutant was prepared to place a carboxylate group close to the distal side of the heme (Figure 8A). The UV-Vis spectrum of A245E mutant showed absorption maximum at 417 nm that was identical to that observed for the wild-type enzyme. The CO adduct of A245E mutant showed the absorption at 444 nm. In contrast to the wild-type $P450_{SP\alpha}$, which does

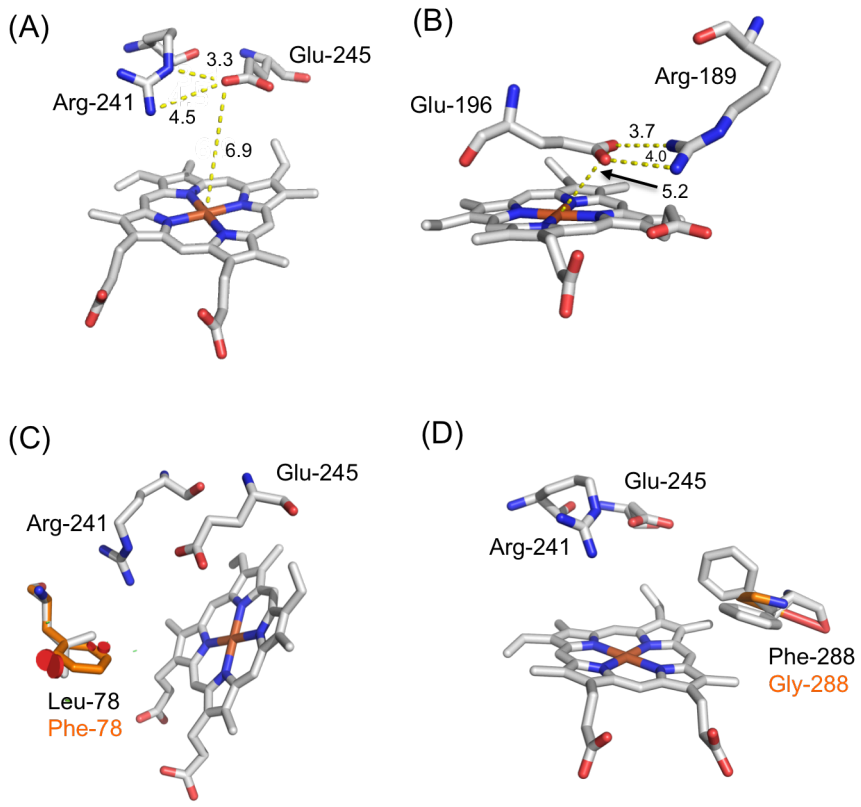
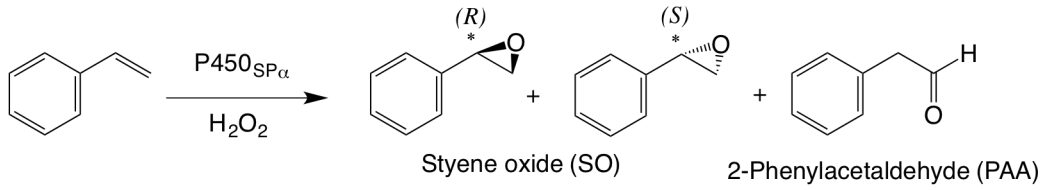



**Figure 8.** The active site structures of $P450_{SP\alpha}$ mutants and *Aae*APO. (A) A245E (PDB ID: 3VOO), (B) *Aae*APO (PDB ID: 2YP1), (C) L78F/A245E, and (D) A245E/F288G. Simulated conformations are shown as orange sticks. All distances are given in Å.

**Table 1.** Catalytic activity ($k_{cat}$) for styrene oxidation by $P450_{SP\alpha}$ and mutants evaluated by Michaelis–Menten kinetics[a]




| | $K_{cat}$/min$^{-1}$ | $K_m$/mM | $K_{cat}$ / $K_m$ /M$^{-1}$S$^{-1}$ | SO : PAA[b] | % ee[b] |
|---|---|---|---|---|---|
| Wild-type | - | - | - | - | - |
| A245E | 280 ± 40 | 1.5 ± 0.4 | 190 | 70:30 | 20 (*S*) |

[a] Reaction conditions: 0.5–3 mM styrene, 4 mM $H_2O_2$ and 1 μM $P450_{SP\alpha}$ in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C for 1 min.
[b] The values under the conditions of 3 mM styrene. "-" = Not detected.

not catalyze styrene oxidation, A245E catalyzes this reaction with $k_{cat}$ and $K_m$ estimated to be 280 min$^{-1}$ and 1.5 mM, respectively, based on Michaelis–Menten kinetics analysis (Table 1). The A245E mutant of $P450_{SP\alpha}$, which mimics a substrate-binding state by placing a carboxylate group at the active site, facilitates oxidation of nonnative substrates. We also examined the hydroxylation of indole to give indigo[36] as well as the hydroxylation of 1-methoxynaphthalene to give Russig's blue[37] and found that the A245E mutant also catalyzes these reactions (Figure 9). The catalytic activities for the hydroxylation of indole and 1-methoxynaphthalene were estimated to be 21 and 75 min$^{-1}$, respectively, indicating that the A245E mutant catalyzes the hydroxylation of various substrates bearing structures that differ from those of fatty acids. X-ray crystal structure analysis of A245E (PDB ID: 3VOO) allows us to evaluate the location and orientation of the mutated amino acids in their active sites. The overall structures of the mutants, which were obtained in substrate-free forms, were found to be essentially the same as that of the wild type (Figure 3). The crystal structure of A245E showed that the carboxylate

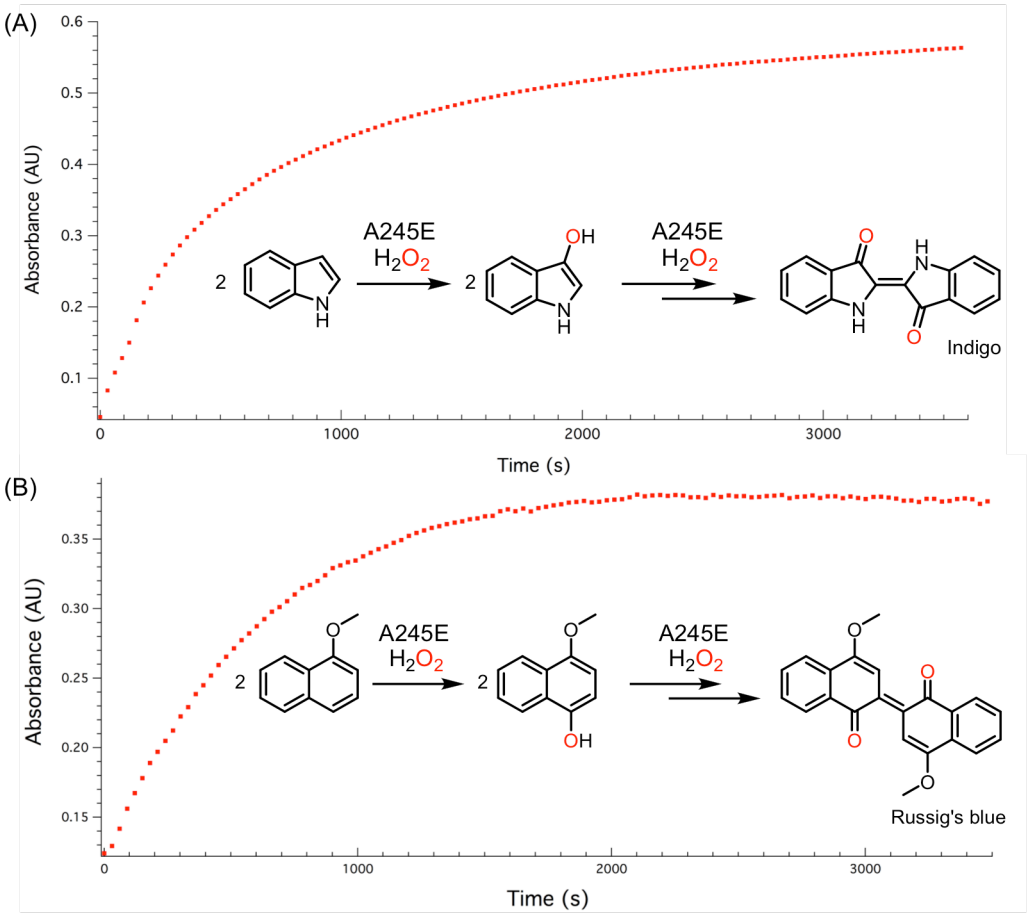



**Figure 9.** Time courses of catalytic reactions by mutants of $P450_{SP\alpha}$ monitored by absorbance changed at (A) 603 nm for indigo, and (B) 634 nm for Russig's blue.

group of Glu-245 is located above the heme (Figure 8A). The distance between the nearest carboxylate oxygen atom of Glu-245 and the heme iron is estimated to be 6.9 Å, which is 1.7 Å longer than that of palmitic acid (5.2 Å). A triad structure (Arg–Glu–Heme) in the A245E mutant is similar to that of the first aromatic peroxygenases (APOs), AaeAPO, which was discovered in the widely cultivated agaric basidiomycete *Agrocybe aegerita* (Figure 8B).[38] Because A245E mutant of $P450_{SP\alpha}$ can oxidize various non-native substrates, the carboxylate of Glu-245 involves in the generation of active species. While styrene was effectively oxidized

by A245E mutant, larger substrates, 1-methoxynaphthalene and indole, were not hydroxylated efficiently. To improve catalytic activity, we prepared double mutants of A245E mutant, L78F/A245E (Figure 8C) and A245E/F288G (Figure 8D).

By replacing Leu-78 with Phe, π-π interaction betweeen phenyl ring of Phe-78 and the substrates would be expected. By replacing Phe-288 with Gly, the size of active site would be increase to help accomodation of the substrates into the

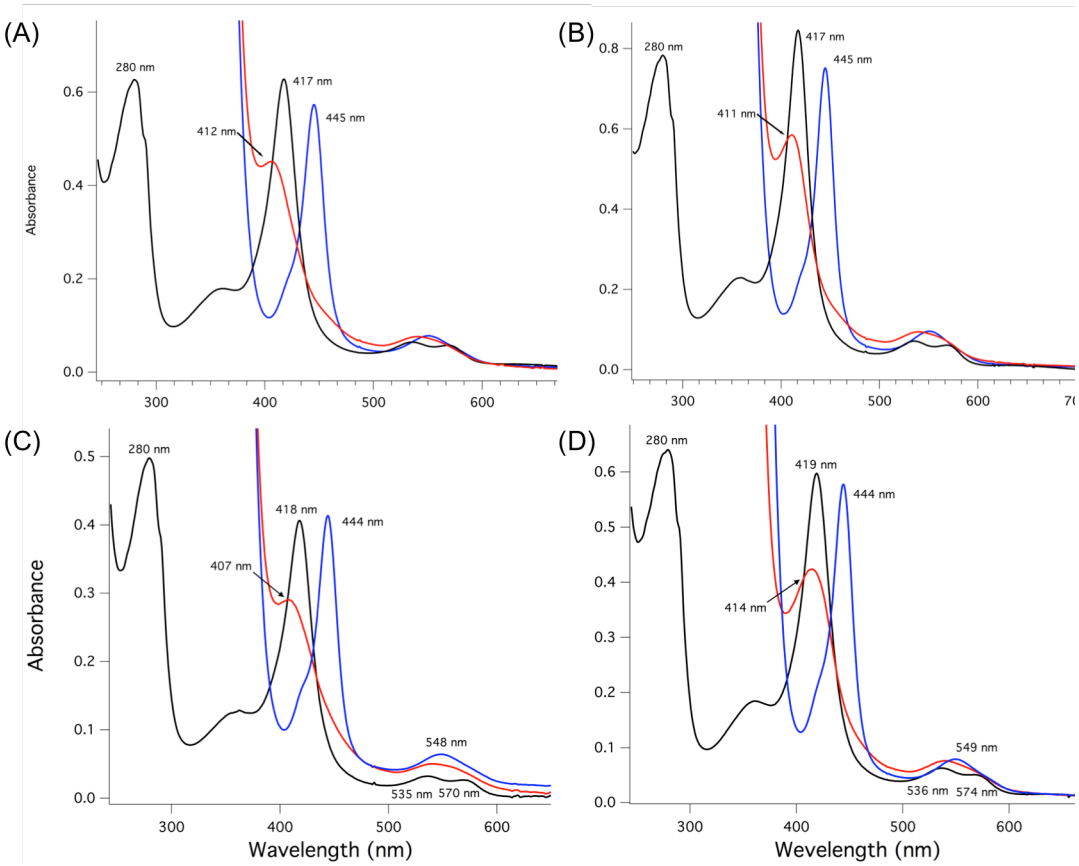



**Figure 10.** UV-Visible absorption spectra of $P450_{SP\alpha}$ mutants (a) F288G (b) L78F (c) A245E/F288G and (d) L78F/A245E in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M KCl and 20% (v/v) glycerol. The resting state (black line), the reduced $Fe^{2+}$ state (red line), and the $Fe^{2+}$–CO state (blue line).

activesite. The CO adducts of L78F/A245E and A245E/F288G showed peaks at 444 nm, indicating correct folding of these mutants (Figure 10). The catalytic activities for indole hydroxylation catalyzed by L78F/A245E and A245E/F288G were higher than A245E at pH 7.4. The catalytic activity of 1-methoxynaphthalene hydroxylation by L78F/A245E and A245E/F288G were also higher than A245E at pH 7.0. The additional mutations (L78F and F288G) were effective to enhance the hydroxylation catalytic activity of A245E mutant. We presume that the increased pocket size of A245E/F288G would stabilize the binding of 1-methoxynaphthalene and indole. Presumably, the π-π interaction between L78F and substrates contributes better a stable binding of substrates.

**Table 2.** Catalytic activity (TOF) of substrates oxidation by P450s and mutants

| Enzyme | Substrate | Concentration | pH | TOF ($min^{-1}$) |
|---|---|---|---|---|
| $P450_{BS\beta}$ WT[1] | 1-methoxynaphthalene | 0.67 mM | 7.0 | 112 ± 2[a]* |
| $P450_{SP\alpha}$ WT | 1-methoxynaphthalene | 0.5 mM | 7.0 | 21.3 ± 0.7* |
| $P450_{SP\alpha}$ A245E | 1-methoxynaphthalene | 0.5 mM | 7.0 | 75.0 ± 0.6 |
| $P450_{SP\alpha}$ L78F/A245E | 1-methoxynaphthalene | 0.5 mM | 7.0 | 93.6 ± 3.4 |
| $P450_{SP\alpha}$ A245E/F288G | 1-methoxynaphthalene | 0.5 mM | 7.0 | 133.6 ± 0.7 |
| $P450_{SP\alpha}$ A245E | Indole | 0.5 mM | 7.4 | 21.4 ± 2.3 |
| $P450_{SP\alpha}$ L78F/A245E | Indole | 0.5 mM | 7.4 | 52.6 ± 1.9 |
| $P450_{SP\alpha}$ A245E/F288G | Indole | 0.5 mM | 7.4 | 53.7 ± 2.2 |

[a] The monooxygenase-activity of 1-methoxynaphthalene by $P450_{BS\beta}$ is adopted from *J. Biol. Inorg. Chem.* **2010,** *15,* 1109-1115.
*Carboxylic acid, heptanoic acid, is added as the decoy molecular to assist the reactions.

## 2.4 Conclusion

The A245E mutant of $P450_{SP\alpha}$ can oxidize non-native substrates suggesting that the carboxylate of Glu-245 involves in the generation of active species and play the key role in the oxidation reaction. The A245E mutant of $P450_{SP\alpha}$ essentially mimics the substrate-bound state and facilitates the generation of active species. It is important to mention that, to our knowledge, this is the first example of a P450 mutant having a carboxylate in the distal side of the heme that accelerates $H_2O_2$-dependent oxidation. The effect of additional mutations of the residues around Glu-245 of A245E mutant upon the catalytic activities of non-native substrates oxidation was examined. The L78F/A245E and A245E/F288G mutants showed improved catalytic activity of the hydroxylation of 1-methoxynaphthalene and indole. These mutations are anticipated to become one of the key mutations that can lead to further development of biocatalysts by point mutagenesis as well as through directed evolution. These findings also further contribute to our understanding of the reaction mechanism of $H_2O_2$- dependent monooxygenation.

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# CHAPTER 3.

# Use of Apomyoglobin to Gently Remove the Heme from $H_2O_2$-Dependent Cytochrome P450 and its Reconstitution

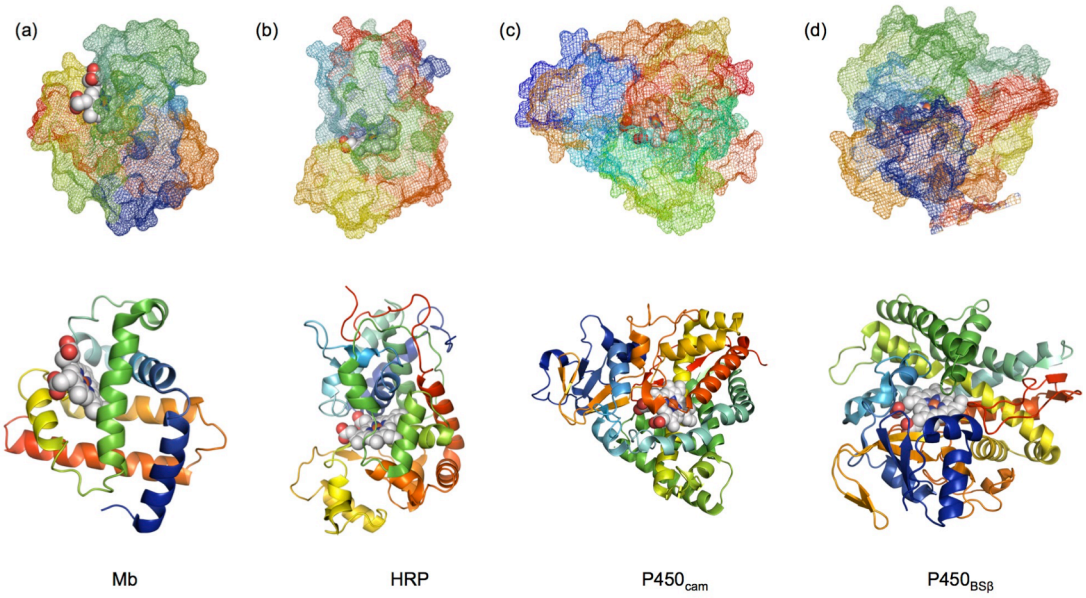
## 3.1 Introduction

Heme, iron-protoporphyrin IX (FePPIX), is one of the most significant metal complexes in nature, serving as the prosthetic group for hemoproteins to perform the diverse functions, including oxygen transport and storage, gas sensing, electron transfer, and catalysis.[1]–[2] The functions of hemoproteins depend on the nature of heme; therefore, replacement of the native cofactor, heme, to heme analogues are expected to drastically change properties of hemoproteins.[3] To remove the heme followed by the reconstitution with artificial cofactors has been reported as the effective tool.[3] A variety of reconstituted hemoproteins containing heme analogues, heme derivatives including different metal ions, chemically modified hemes, and synthetic metal complexes have been constructed with the aim of developing synthetic proteins with (improved) catalytic activity or entirely different functions.[4]–[5]

To allow reconstitution of hemoproteins with synthetic metal complexes, the tightly bound heme must be removed to give apoproteins. Noncovalently bound *b*-type hemes could be extracted from hemoproteins using organic solvents such as acetone and 2-butanone under acidic conditions (pH 2–4). Teale *et al.* first reported the removal of heme from hemoproteins utilizing acid–butanol (pH = 2) to prepare apo forms of horse heart and skeletal muscle myoglobin (Mb).[6] Several methods based on slight modification of this acid–butanol method have been reported for the preparation of apo forms of hemoproteins such as HRP and $P450_{cam}$ (see Figures 1 for these different heme enzyme structures).[7]–[8] However, these methods are not suitable if the hemoproteins as well as their apo-forms are not sufficiently stable in organic solvents and/or under acidic conditions. Although preparation of apoproteins in bacterial cells has been developed as an alternative, their reconstitution was limited to metal complexes possessing a protoporphyrin IX framework. To avoid the use of harsh chemical treatments in the preparation of apoproteins, and to expand the range of applicable metal complexes to include various synthetic metal complexes, it is important to develop a simple strategy for removing the heme of hemoproteins

without the need for organic solvents and acidic conditions.

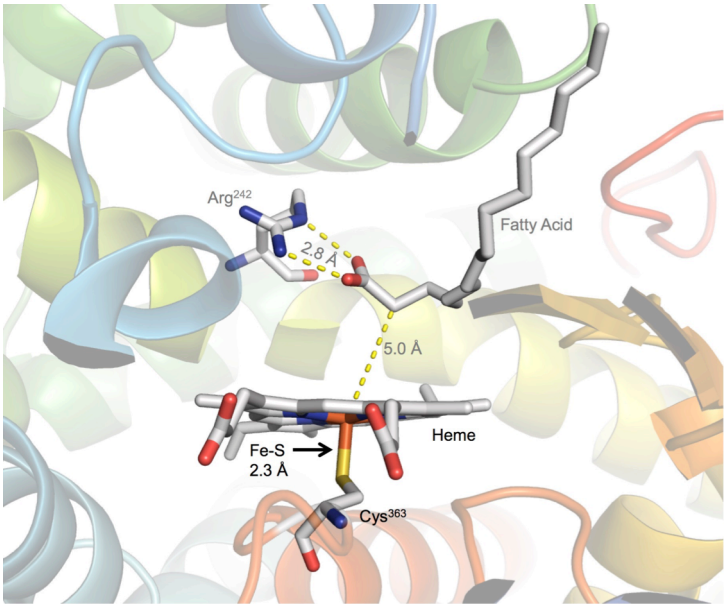
We decided to employ apo-Mb as a heme scavenger because it binds strongly to heme and can be readily prepared by the aforementioned acid–organic solvent-methods; the dissociation constant of apo-Mb for heme is reported to be $10^{-14}$ $M^{-1}$.[9] Cytochrome $P450_{BS\beta}$ ($P450_{BS\beta}$) is one of hemoproteins that is irreversibly denatured during the preparation of apoprotein under the methods described above. Therefore, it has been believed impossible to prepare reconstituted $P450_{BS\beta}$. However, $P450_{BS\beta}$ is regarded as a promising candidate for the construction of a biocatalyst




**Figures 1.** Structures of four different heme enzymes. The hemes in the enzymes are represented as sphere models. (a) Mb (PDB ID: 1MBN), (b) HRP (PDB ID: 1W4W), (c) P450cam (PDB ID: 3WRH), and (d) $P450_{BS\beta}$ (PDB ID: 1IZO). The heme of Mb is positioned close to the surface of protein, while the heme of HRP is partially buried. The heme pockets of P450cam as well as $P450_{BS\beta}$ are located deep inside of the protein.

because $P450_{BS\beta}$ and its unique family, the hydrogen peroxide-dependent P450s, efficiently utilize hydrogen peroxide to catalyze various C–H bond hydroxylation and some other types of oxidation reactions.[10]–[11] If the heme of $P450_{BS\beta}$ could be replaced with synthetic metal complexes, the properties of the enzymatic reaction catalyzed by $P450_{BS\beta}$ could be altered to allow the development of a versatile biocatalyst (Figure 2).

In this Chapter, a simple and useful method for the preparation of apo-$P450_{BS\beta}$ using apo-Mb under very mild conditions and its reconstitution with hemin or $Mn^{III}$-protoporphyrin IX (Mn-PPIX) is described (Scheme 1). A completely pure apo-$P450_{BS\beta}$ can be obtained by employing this method. It is also reported here that the resulting apo-$P450_{BS\beta}$ can be reconstituted with manganese protoporphyrin IX (MnPPIX) and that the resulting reconstituted $P450_{BS\beta}$ with MnPPIX exhibits improved one-electron oxidation activity when *m*CPBA was employed as an oxidant.




**Figures 2.** The active site of $P450_{BS\beta}$ with fatty acid. The heme, $Arg^{242}$, $Cys^{363}$, and fatty acid are show as stick models. The distances between two atoms are also shown.

This is the first report of preparation of apo-form $H_2O_2$-dependent P450 under mild conditions retaining its secondary structures.

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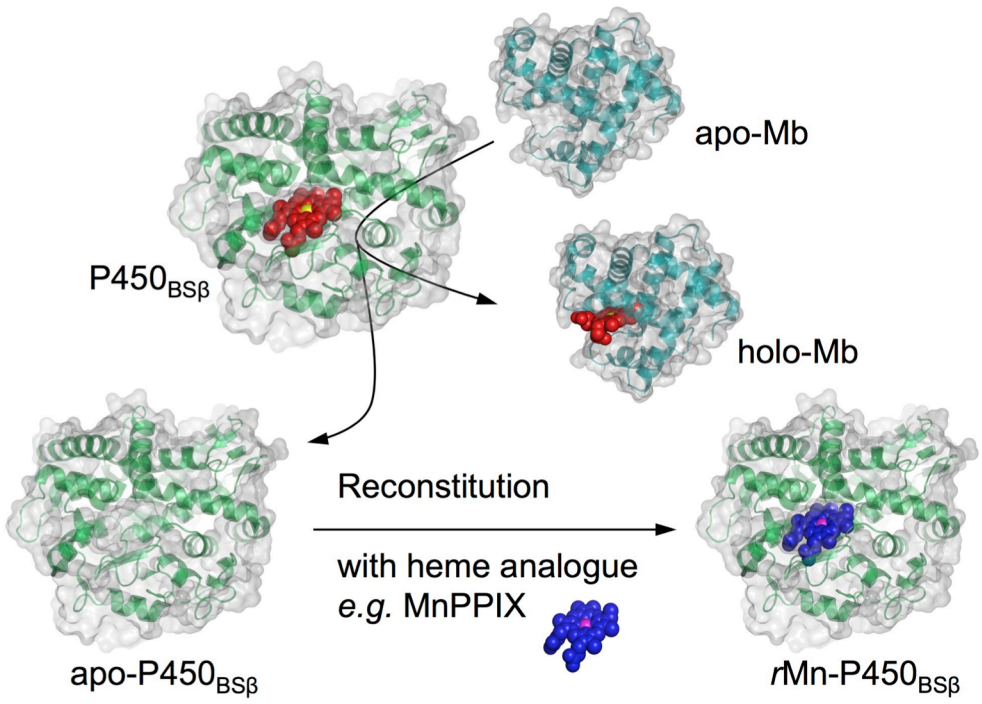



**Scheme 1.** Schematic illustration of apo-Mb capturing native cofactor heme (red with iron atom in yellow) and subsequently reconstituted with an artificial cofactor (blue with manganese atom in magenta) to generate a functional enzyme from holo-form $H_2O_2$-dependent $P450_{BS\beta}$.

## 3.2 Experimental Methods

### 3.2.1 Materials

All chemicals were purchased from commercial sources. Mn(III) protoporphyrin IX chloride was obtained from Frontier Scientific, Inc. (Logan, UT, USA) and used after exchanging its $Cl^-$ for $BF_4^-$ by treating with $AgBF_4$. Hydrogen peroxide, dithiothreitol (DTT), ethylene glycol, sodium dodecyl sulfate (SDS), isopropyl-β-D-1-thiogalactopyranoside (IPTG), and kanamycin sulfate were obtained from WAKO Pure Chemical Industries, Ltd. (Osaka, Japan). Glycerol, potassium chloride, urea, hydrochloric acid, potassium phosphate, ampicillin sodium salt and phenylmethylsulfonyl fluoride were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Horse skeletal muscle Mb was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Concentrations of chemicals described in procedures are the final concentrations.

### 3.2.2 Measurements

UV-Visible (UV-Vis) absorption spectra were recorded on a UV-2600 PC spectrophotometer (Shimadzu Corporation) or Agilent 8453 with an 89090A thermal controller (Agilent Technologies, Inc.), and the data were collected using screw-capped quartz cuvettes of 1-cm path length. Circular dichroism (CD) spectra were recorded with quartz cuvettes of 0.1 cm path length using a J-720WI spectropolarimeter (Jasco Corporation) equipped with a temperature controller, and the data were recorded from 200 to 260 nm at 24 °C, 20 nm/min scan speed, and an average spectrum was produced from four independent consecutive scans. High-performance liquid chromatography (HPLC) measurement was performed with SHIMADZU CTO-10A and LC-10A and through by a reverse-phase HPLC column in 5μM, 4.6 × 250 mm (Inertsil ODS-3, GL Sciences Inc.) Size distributions were recorded by dynamic light scattering (DLS) using a Nano ZS (Malvern Instruments Ltd.) with disposable plastic cuvettes and caps. Inductively coupled plasma–optical

emission spectrometer (ICP-OES) measurements were performed on a Varian Vista-Pro ICP Spectrometer (Varian Inc.). The purification procedures described below were carried out using a Bioassist eZ system (Tosoh Corporation). All scientific graphics were prepared using Igor Pro (WaveMetrics, Inc.), version 6.37. The protein figures were generated using the PyMOL Molecular Graphics System (version 1.8; DeLano Schrödinger, LLC.)

### 3.2.3 Protein expression and purification

For the expression of recombinant $P450_{BS\beta}$, 6× histidine-tagged (6×His-tagged) $P450_{BS\beta}$ was expressed in the transformed *Escherichia coli* strain M15. Cells were cultivated at 27 °C in Luria–Bertani medium supplemented with 100 μg/mL of ampicillin and 25 μg/mL of kanamycin. Once the culture reached an optical density at 600 nm ($OD_{600}$) of 0.7–0.8, heme precursor δ-aminolevulinic acid (0.5 mM) was added, and the culture was incubated for an additional 30 min. At $OD_{600}$ = 1.0–1.2, gene expression was induced by addition of IPTG (0.1 mM), and cultivation was followed by culture at 20 °C for 20 h rotating at 80 rpm. Cells were then harvested by centrifugation and lysed in 0.1 M potassium phosphate buffer (pH 7.0) with 0.3 M potassium chloride, 50 mM imidazole, and 20% (v/v) glycerol. Purification of 6×His-tagged $P450_{BS\beta}$ was performed by Ni-chelate affinity chromatography (GE Healthcare, Little Chalfont, UK). The protein was further purified using S-200 Sephacryl (GE Healthcare) size exclusion chromatography. An $A_{Soret}/A_{280}$ absorbance ratio ≥ 1 indicated the high purity of the protein, which was also confirmed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE). Reduced CO-difference spectra were used to determine the concentration of P450 by subjecting the purified recombinant protein to sodium dithionite and CO gas.[12] Protein samples were stored at −80 °C for further use.

### 3.2.4 Preparation of apo-Mb

A heme moiety was extracted from horse skeletal muscle Mb by the butan-2-one

extraction according to a published procedure[6] with slight modifications. The pH of a Mb solution (100 mg Mb in 40 mL Milli-Q water) was adjusted to 2.5 in an ice-water bath using chilled 0.1 M $HCl_{(aq)}$. The solution was transferred to a separation funnel, and an equal volume of 2-butanone was added. The mixture was gently shaken for 30 s and then allowed to stand for 10 min at 4°C. The colorless aqueous phase was separated and dialyzed for 24 h at 4 °C against 5 L of Milli-Q water, and then for 24 h against PBS buffer (pH 7.3). Next, the solution was passed through a 0.22 μm filter and stored at 4 °C for further use. The concentration of prepared apo-Mb was spectrophotometrically determined using its extinction coefficient of 15,700 $M^{-1}cm^{-1}$ at 280 nm.[13]

### 3.2.5 Apo-$P450_{BS\beta}$: Heme Transfer Reaction

The removal of the heme from $P450_{BS\beta}$ to prepare apo-$P450_{BS\beta}$ was performed by treating $P450_{BS\beta}$ with apo-Mb. The reaction was carried out in 0.1 M potassium phosphate buffer (pH 7.0) with 0.3 M potassium chloride and 20% (v/v) glycerol with $P450_{BS\beta}$ (25 mL, 8.5 μM). Solid $Na_2S_2O_4$ was mixed into the solution (1 mg/mL) at 25 °C to generate reduced $P450_{BS\beta}$. Five equivalents of apo-Mb (25 mL, 42.5 μM) was added to the reaction buffer, and the final concentration of glycerol adjusted to 5% by additional PBS buffer. The reaction was maintained in a gas-tight flask for 24 h without stirring. Before purification, 0.02% of Triton X-100 was added to aid the separation of $P450_{BS\beta}$ and Mb. Purification of 6×His-tagged apo-$P450_{BS\beta}$ was performed by Ni-chelate affinity chromatography to remove holo-Mb and excess apo-Mb. This is the same purification procedure used for recombinant $P450_{BS\beta}$. Sample purity was checked by SDS–PAGE. Pure apo-$P450_{BS\beta}$ was stored at 4 °C for further use or stored at −80 °C for long-term preservation.

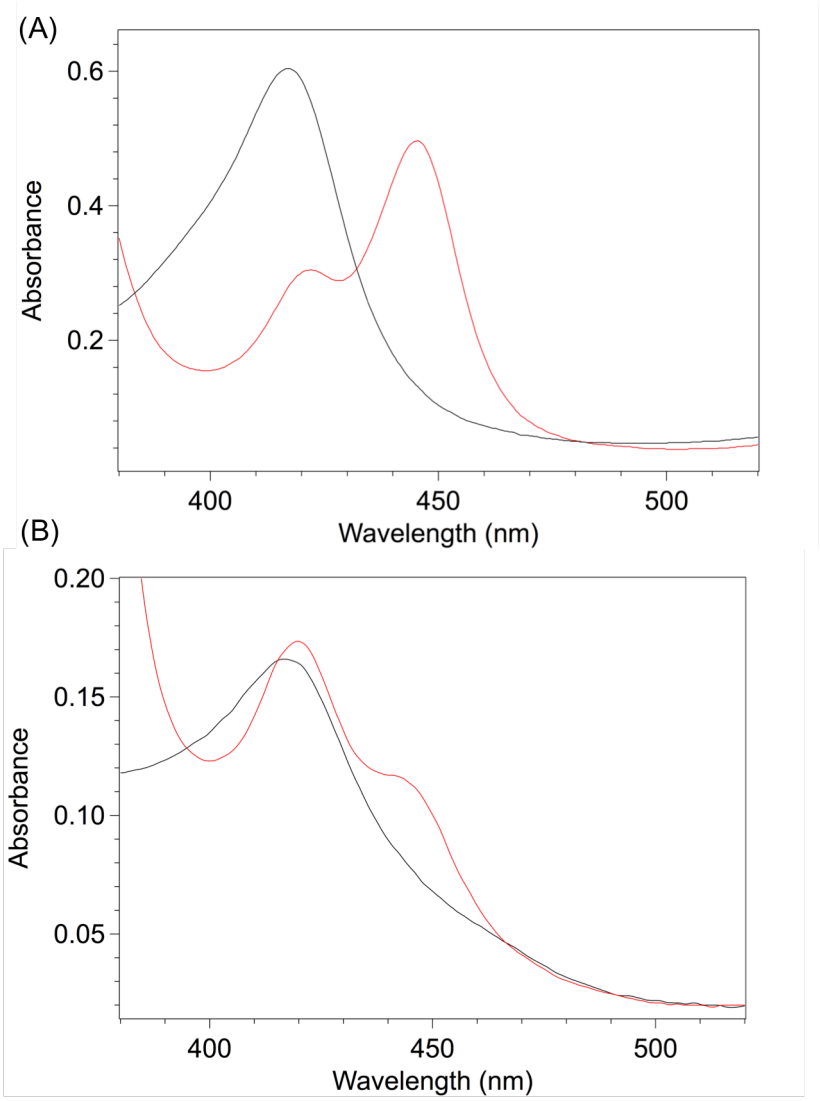
### 3.2.6 Reconstitution of apo-$P450_{BS\beta}$

Reconstitution of apo-$P450_{BS\beta}$ was performed by adapting the procedure for

apo-$P450_{cam}$[14] at 25 °C as well as subsequent protein purification at 4 °C. After the preparation of protoporphyrin complex solution, it should be added into apo-$P450_{BS\beta}$ buffer immediately. Any delay in addition of protoporphyrin complex solution to apo-$P450_{BS\beta}$ led to incomplete recombination due to its low solubility in water solution.[13]

***r*Fe-P450$_{BS\beta}$.** A 7 mL apo-$P450_{BS\beta}$ (6 μM) solution at pH 7.0 under an argon atmosphere was combined with urea (2 M), DTT (10 mM), histidine (10 mM), and Triton X-100 (0.02%). The solution was then left to stand a further 15 min at room temperature. A 50 μL KOH solution (0.1 M) containing hemin (2.5 eq., 2.1 mM) and myristic acid (0.2 mM), diluted tenfold with Milli-Q water, was added dropwise to the solution of apoprotein. The whole reaction was performed in a gas-tight flask for 24 h without stirring. Before purification, an Amicon ultra centrifugal filter was used to remove excess hemin and other chemicals. Purification of 6×His-tagged *r*Fe-$P450_{BS\beta}$ was performed by Ni-chelate affinity chromatography to remove free hemin by the same procedure used for preparation of recombinant $P450_{BS\beta}$. The final purified *r*Fe-$P450_{BS\beta}$ gave a single band on SDS–PAGE.

***r*Mn-P450$_{BS\beta}$.** The insertion of MnPPIX into the apoprotein was carried out using a 7 mL apo-$P450_{BS\beta}$ (6 μM) solution at pH 7.0 under an argon atmosphere. The apoprotein solution was combined with urea (2 M), DTT (10 mM), histidine (10 mM), and Triton™ X-100 (0.02%), and then left to stand for 15 min at room temperature. A 210-μL KOH solution (0.1 M) containing MnPPIX($BF_4$) (2.5 eq., 0.5 mM, the concentration determined using a $\varepsilon_{462nm}$ of 25,000 $M^{-1}cm^{-1}$),[15] diluted tenfold with Milli-Q water, was added dropwise to the solution of apoprotein. The whole reaction was then incubated in a gas-tight flask wrapped in aluminum foil for 24 h without stirring. Before purification, an Amicon ultra centrifugal filter was used to remove excess MnPPIX and other chemicals. Purification of 6×His-tagged *r*Mn-$P450_{BS\beta}$ was performed by Ni-chelate affinity chromatography to remove free MnPPIX by the




**Figures 3.** UV-Visible absorption spectra of ferric and ferrous-CO bound form of (A) recombinant $P450_{BS\beta}$ and (B) *r*Fe-$P450_{BS\beta}$ in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M KCl and 20% (v/v) glycerol. The resting state of P450 (black line) and the $Fe^{2+}$–CO state (red line). *r*Fe-P450 led to a higher content of the P420 isoform to 60% ($Abs_{420nm}$ = 0.174 and $Abs_{445nm}$ = 0.117) than recombinant $P450_{BS\beta}$.

same procedure as that used for recombinant $P450_{BS\beta}$. The final purified *r*Mn-$P450_{BS}$ gave a single band on SDS–PAGE, and the $A_{Soret(381)}/A_{280}$ absorbance ratio was 0.7.

### 3.2.7 Determinations of P450 concentration

The concentration of the recombinant $P450_{BS\beta}$ was measured by reduced CO-difference spectroscopy.[12] In the case of *r*Fe-$P450_{BS\beta}$ with a higher content of P420 isoform (Figure 3), the concentration was measured by reduced pyridine–hemochrome spectroscopy.[16] The concentrations of holo- and apo-P450 for CD spectroscopy measurements were determined by the Bradford protein assay.[17] *r*Mn-$P450_{BS\beta}$ was quantitated from the Bradford protein assay and multiplied by values of $A_{Soret}/A_{280}$.

### 3.2.8 Guaiacol oxidation

**Using $H_2O_2$ as an oxidant.** For each catalytic reaction, the total volume of the mixture was maintained at 1 mL. The reaction was carried out in a 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C in the presence of 50 nM P450, 10 mM guaiacol (30 μL of 0.4 M ethanol solution), and 20 mM *n*-heptanoic acid (50 μL of 0.4 M ethanol solution). Reactions were initiated by the addition of 4 mM hydrogen peroxide (300 μL of 14 mM aqueous solution). Initial oxidation rates were determined by monitoring the absorption change at 470 nm relative to the tetramer of guaiacol with an extinction coefficient of 26,600 $M^{-1}cm^{-1}$.[18] All initial turnover numbers (TON) are the average of at least three measurements, and are expressed as nmol product/nmol P450/min) ± standard deviation.

**Using *m*CPBA as an oxidant.** The reaction conditions were similar to those described above, except that the oxidant was changed to *m*CPBA and the reaction mixture does not contain *n*-heptanoic acid. The reactions were started by adding 4 mM *m*CPBA (70 μL of 60 mM ethanol solution) and monitored by the absorption changed at 470 nm. The oxidation products were also determined quantitatively, as described previously.

### 3.2.9 ICP-OES

The iron and manganese content in *r*P450$_{BS\beta}$ were determined by ICP-OES. Concentrated samples of purified *r*Fe-P450$_{BS\beta}$ and *r*Mn-P450$_{BS\beta}$ protein were diluted with 0.2 M $HNO_3$ to 2 mL of 1.5, 3.0, 4.5 and 6.0 μM for the determinations. The solution was placed into a new glass vial and submitted to the ICP-OES system via a peristaltic pump. The sample was aspirated with argon, passed over plasma, and analyzed for iron and manganese. A control experiment was performed using Milli-Q water as a sample. Commercial calibration standards and multi-element standards (Wako Pure Chemical Industries, Ltd.) were used.

## 3.3 Results and Discussion

The removal of the heme from $P450_{BS\beta}$ to prepare apo-$P450_{BS\beta}$ was performed by treating $P450_{BS\beta}$ with apo-Mb. When five equivalents of apo-Mb were added to a solution of histidine-tagged $P450_{BS\beta}$ at 25 °C, apparent shifts of the Soret absorption band and Q-band were observed. The absorption peak of $P450_{BS\beta}$ at 417 nm shifted to 409 nm, which is readily attributed to the formation of holo-Mb (Figure 4A). After 24 h incubation, the resulting apo-$P450_{BS\beta}$ was purified by Ni-chelate affinity chromatography, but a small amount of holo-$P450_{BS\beta}$ was still observed. To bring the heme transportation to completion, $P450_{BS\beta}$ was reduced using $Na_2S_2O_4$ because the dissociation rate constant of reduced heme ($Fe^{II}$) coordinated with cysteine is rapid compared with that of heme ($Fe^{III}$).[19] DFT calculations also showed a weaker binding of cysteine to ferroheme than to ferriheme. A clear shift of the absorption maxima of heme was again observed after addition of apo-Mb, and no remarkable change was observed after 24 h (Figure 4B). It is worth noting that the absorption spectral change was very slow when the reaction of preparing apo-$P450_{BS\beta}$ was under 4 °C after 24 hr incubation (Figure 5).

Apo-$P450_{BS\beta}$ purified by Ni-chelate affinity chromatography did not show any absorption assignable to heme, indicating that the heme of $P450_{BS\beta}$ can be removed completely (Figure 6 and 7). Interestingly, the CD spectrum of apo-$P450_{BS\beta}$ was essentially identical to that of the holo form, indicating a small structural change upon the removal of the heme (Figure 8), while the CD spectra of apoproteins produced by acid–organic solvent methods were generally significantly different from those of the holo form.[20] For example, apo-$P450_{cam}$ prepared by the acid–butanone method gave a weaker CD signal, indicative of partial denaturation. The hydrodynamic diameters of holo- and apo-$P450_{BS\beta}$ determined by DLS analysis were 4.36 and 5.05 nm, respectively (Figure 9), which are consistent with the size of holo-$P450_{BS\beta}$ (PDB ID: 1IZO), indicating that there are no appreciable denaturation or aggregation. These results clearly showed that apo-$P450_{BS\beta}$ could be prepared using apo-Mb and the resulting apo-$P450_{BS\beta}$ retained almost the same conformation as its holo form without denaturation.

With pure apo-$P450_{BS\beta}$ in hand, we reconstituted it according to the reported method for preparing reconstituted $P450_{cam}$.[14] Initially, apo-$P450_{BS\beta}$ was

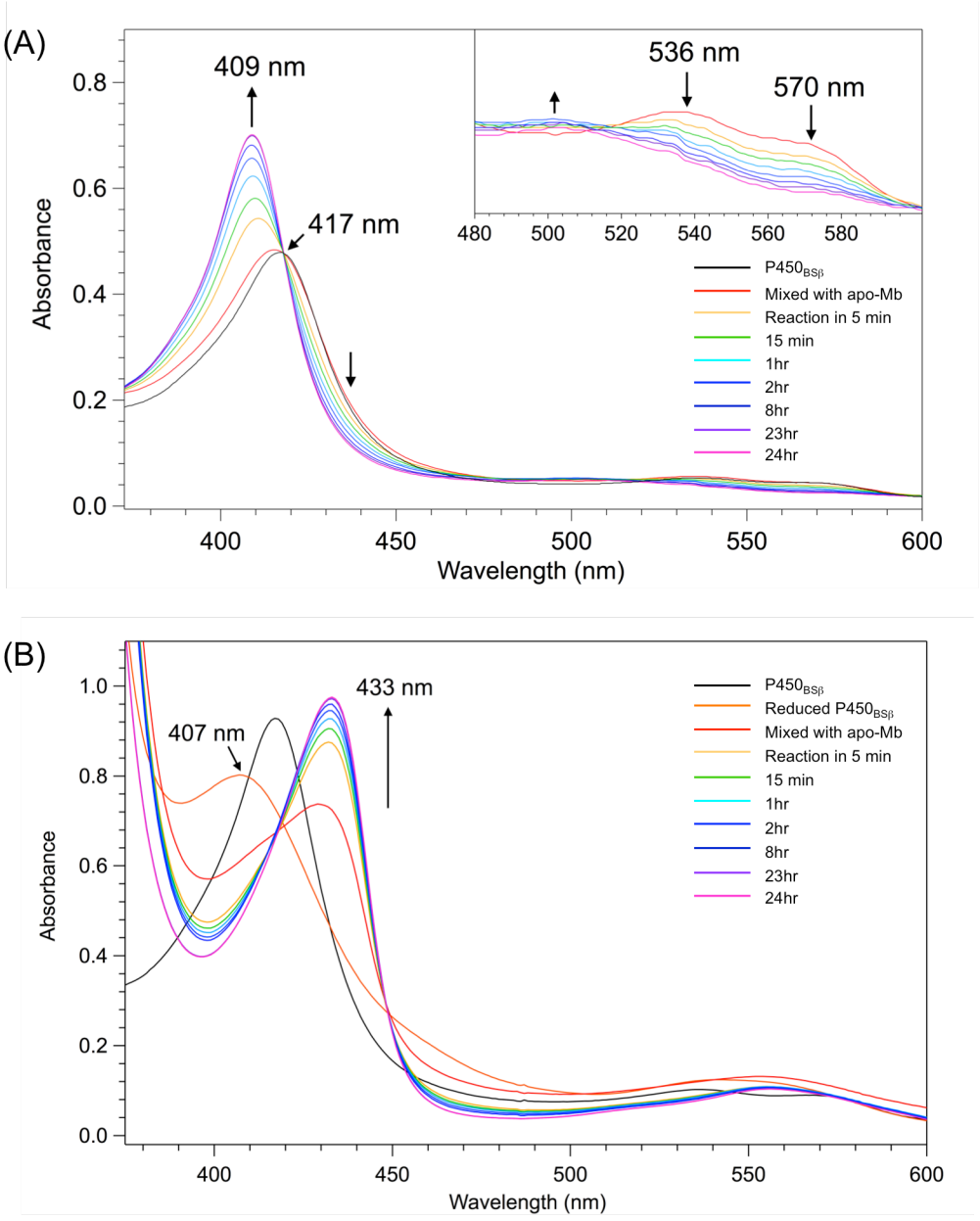
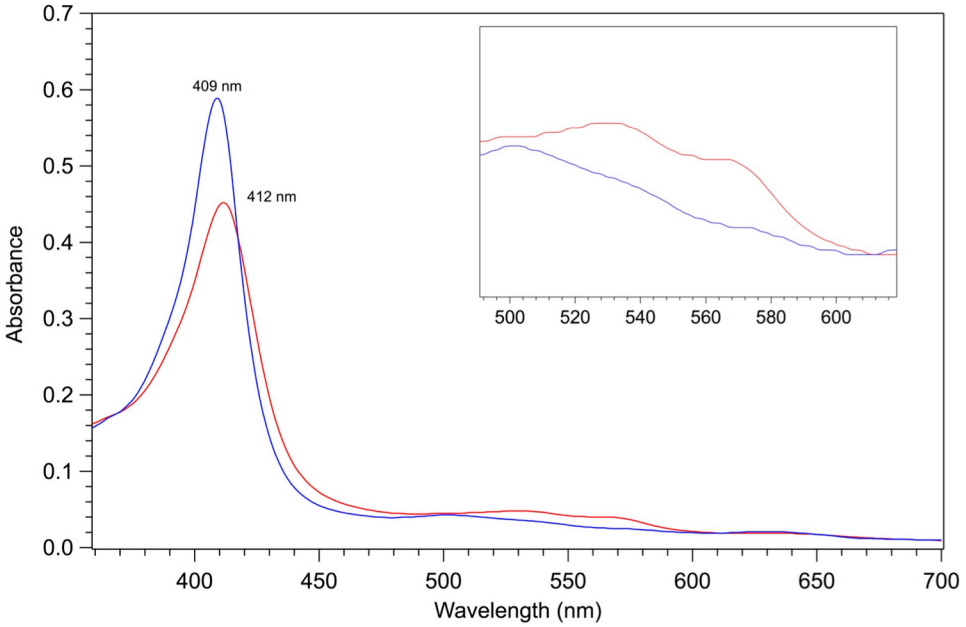



**Figure 4** Time course of UV-Vis absorption spectra changes during the reactions at 25 °C for 24 h. (A) $P450_{BS\beta}$ with apo-Mb; (B) reduced $P450_{BS\beta}$ with apo-Mb.




**Figures 5.** UV-Vis absorption spectra of $P450_{BS\beta}$ with apo-Mb at 4 °C vs. 25 ˚C after 24 hours. Comparison result between 25 °C experiment (blue line) was observed with the Soret band from heme of Mb at 409 nm, and 4 °C experiment (red line) was observed the Soret band shifted to 412 nm only with an apparent Q band of heme of P450 (expanded part). The result showed that the rate of heme transfer at 4 °C was slower then that at 25 °C.

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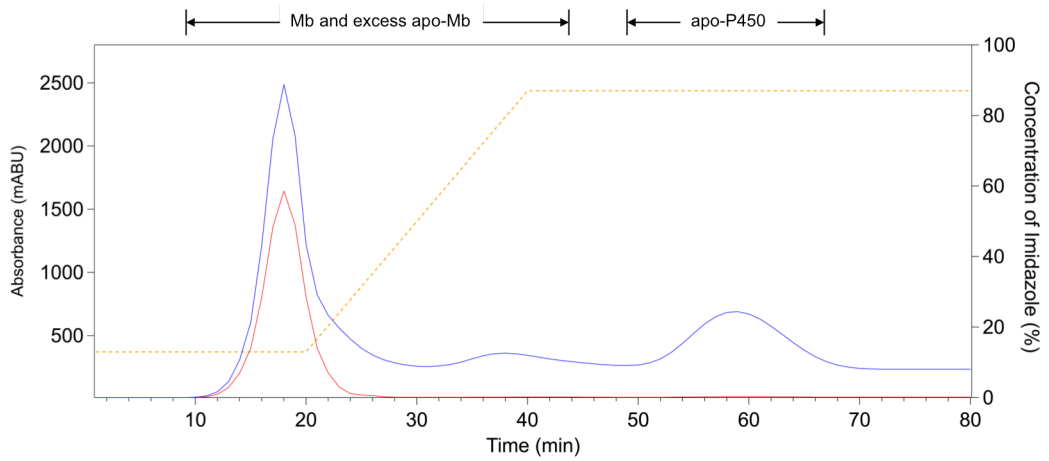
reconstituted with heme to confirm that it is possible to reinsert heme into the appropriate position of $P450_{BS\beta}$ and to fully recover its catalytic activity. A solution of apo-$P450_{BS\beta}$ was added with 2.5 equivalents of hemin in the presence of 10 mM DTT and 10 mM histidine, and then incubated for 24 h under argon. Reconstituted $P450_{BS\beta}$ (*r*Fe-$P450_{BS\beta}$) could be purified by Ni-chelate affinity chromatography. The UV-Vis absorption spectrum showed a Soret peak at 417 nm. The CD spectrum of reconstituted $P450_{BS\beta}$ remained similar to that of recombinant $P450_{BS\beta}$ and the structure of α-helical proteins (Figure 10).

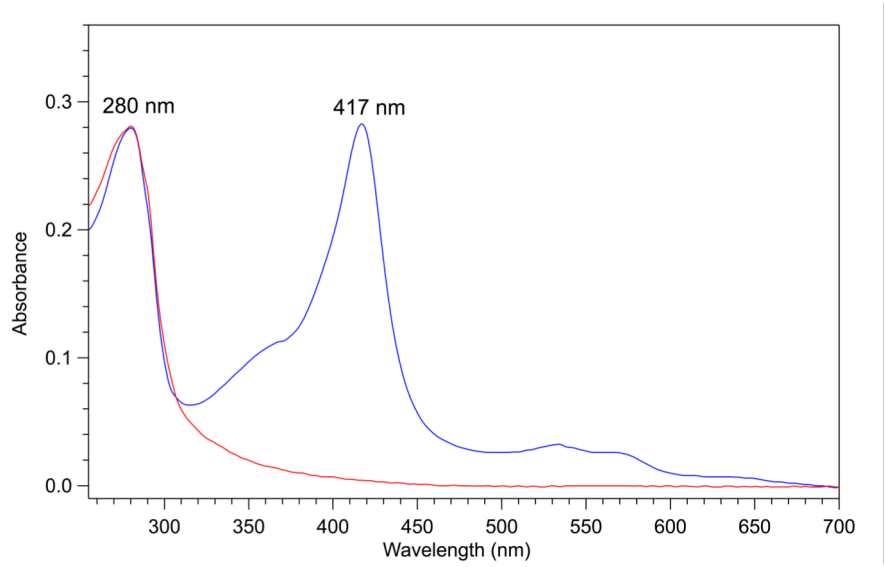
*r*Fe-$P450_{BS\beta}$ exhibited catalytic activity similar to that of native $P450_{BS\beta}$ for the one-electron oxidation of guaiacol with the assistance of *n*-heptanoic acid, which

accelerates the generation of active species of $P450_{BS\beta}$ because the carboxylate of *n*-heptanoic acid serves as an acid–base catalyst.[21] The initial TOF (nmol product/nmol P450/min) of one-electron oxidation of guaiacol by *r*Fe-$P450_{BS\beta}$ with heptanoic acid was estimated to be 3150, which is almost the same as that of $P450_{BS\beta}$ under similar conditions (Table 1).[22] These results indicate that apo-$P450_{BS\beta}$ prepared using apo-Mb can be reconstituted with heme and that the reconstituted *r*Fe-$P450_{BS\beta}$ has a catalytic activity comparable to that of recombinant $P450_{BS\beta}$.

Given that apo-$P450_{BS\beta}$ could be reconstituted with heme, we have attempted to reconstitute apo-$P450_{BS\beta}$ with MnPPIX in a similar manner, and obtained $P450_{BS\beta}$ reconstituted with MnPPIX (*r*Mn-$P450_{BS\beta}$). The CD spectrum of *r*Mn-$P450_{BS\beta}$ confirmed that *r*Mn-$P450_{BS\beta}$ had the same protein structure as $P450_{BS\beta}$ (Figure 10 and




**Figures 6.** Ni-chelate affinity column chromatography to separate Mb and 6 × His-tagged apo-$P450_{BS\beta}$. The blue line represented absorption at 280 nm and the red line was the absorption at 417 nm. The first peak and weak second peak contained holo-Mb and excess apo-Mb.




**Figures 7.** UV-Vis absorption spectra of holo- and apo-P450$_{BS\beta}$ in 0.1 M potassium phosphate buffer (pH 7.0) with 20% (v/v) glycerol and 0.3 M KCl. Holo-P450$_{BS\beta}$ is shown as a blue solid line and apo-P450$_{BS\beta}$ as a red solid line.

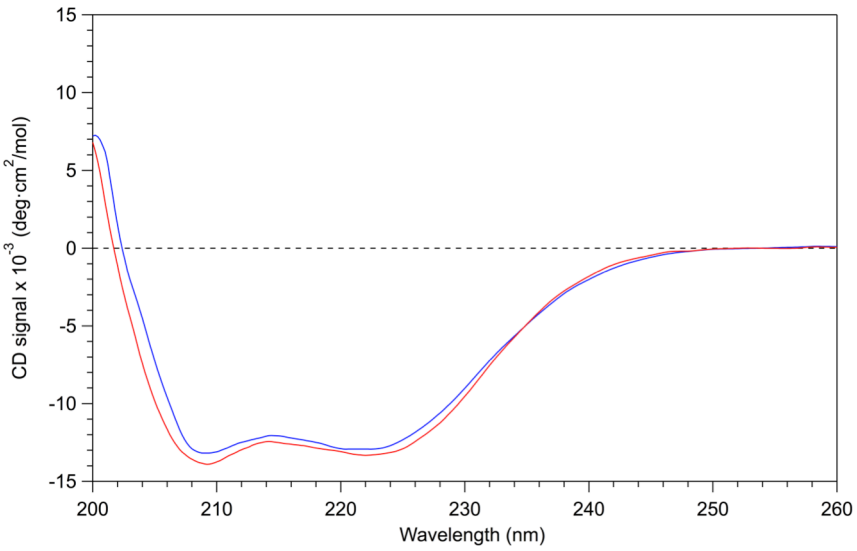
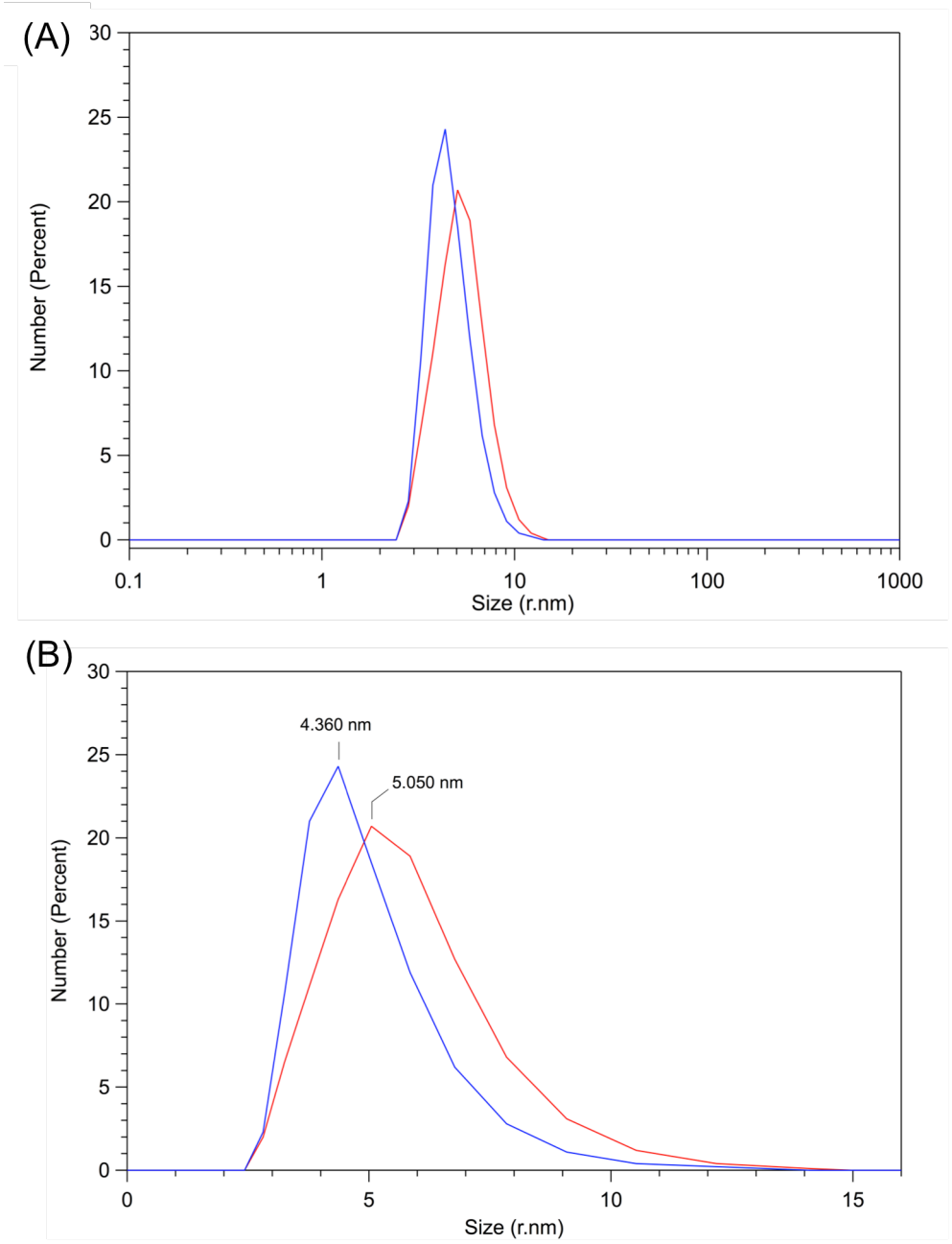
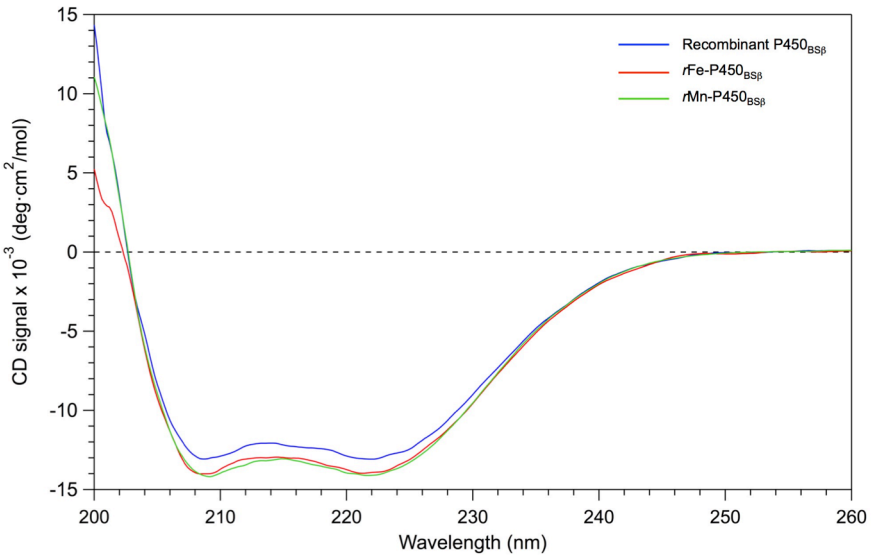



**Figure 8.** CD spectra (0.0025 M) of holo- and apo-P450$_{BS\beta}$ in 0.1 M potassium phosphate buffer (pH 7.0) with 20% (v/v) glycerol and 0.3 M KCl and. The concentrations of protein were calculated using the Bradford method. Holo-P450$_{BS\beta}$ is shown as a blue solid line and apo-P450$_{BS\beta}$ as a red solid line.




**Figures 9.** DLS spectra of holo- and apo-P450$_{BS\beta}$ in different scales, (A) log scale and (B) normal scale. Holo-P450$_{BS\beta}$ is shown as a blue line and apo-P450$_{BS\beta}$ as a red line.




**Figures 10.** CD spectra (0.0025 M) of recombinant (blue line), *r*Fe- (red line) and *r*Mn-P450$_{BS\beta}$ (green line) in 0.1 M potassium phosphate buffer (pH 7.0) with 20% (v/v) glycerol and 0.3 M KCl. The spectra showed that two of reconstituted P450$_{BS\beta}$ were stayed in analogous α-helical structures as the recombinant P450$_{BS\beta}$.

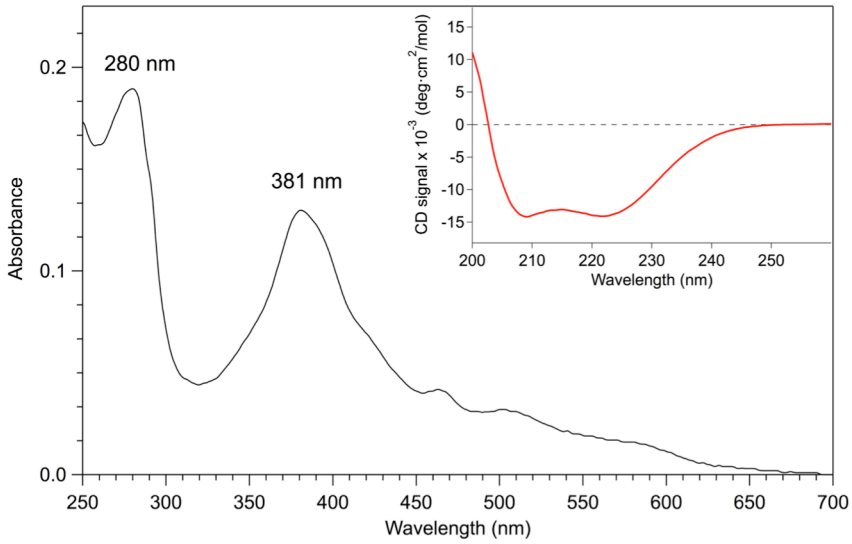
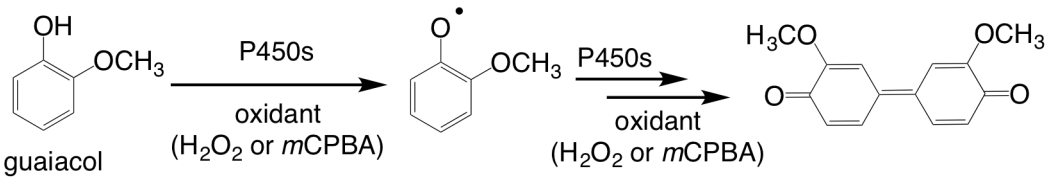



**Figure 11.** UV-Vis absorption (black line) and CD (red line) spectra of *r*Mn-P450$_{BS\beta}$ in 0.1 M potassium phosphate buffer (pH 7.0) with 20% (v/v) glycerol and 0.3 M KCl. The Soret band from MnPPIX in the deep pocket of the active site of P450$_{BS\beta}$ was at 381 nm, and there was no signal of free MnPPIX or adherent (noncovalently bound) MnPPIX at the surface of P450$_{BS\beta}$ in the UV-Vis spectrum. The $A_{Soret(381)}/A_{280}$ absorbance ratio was 70%. The CD spectrum showed that *r*Mn-P450$_{BS\beta}$ remained in the same α-helix structure as the recombinant P450$_{BS\beta}$.

**Table 1.** Catalytic activity for one-electron oxidation of guaiacol by different oxidants with recombinant and reconstituted $P450_{BS\beta}$. The units of catalytic activity are described as TOF (all values are the average of five measurements and are expressed in terms of nmol product/nmol P450/min ± standard deviation).

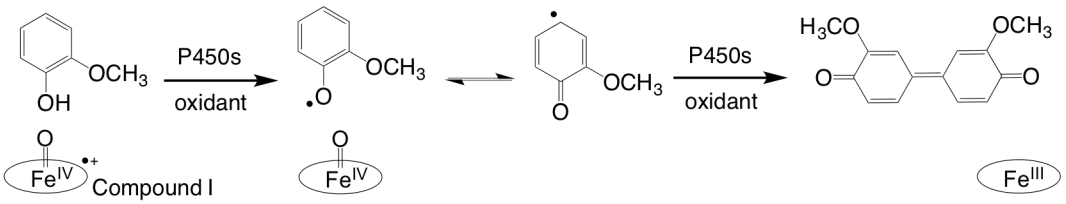



| Enzyme | TOF ($min^{-1}$) | |
|---|---|---|
| Protein | $H_2O_2$[a] | *m*CPBA |
| $P450_{BS\beta}$ | 3,750[b] | 113 ± 5 |
| *r*Fe-$P450_{BS\beta}$ | 3,150 ± 50 | - |
| *r*Mn-$P450_{BS\beta}$ | 40 ± 8 | 2,860 ± 40 |

[a]Heptanoic acid assisted these catalytic experiments. [b]The value of TON is adapted from *Agnew. Chem. Int. Ed.,* 2007, **46**, 3656–3659. [C]The proposed oxidation mechanism of guaiacol with P450 see Scheme 2.

Figure 11). The Soret absorption band of *r*Mn-$P450_{BS\beta}$ was observed at 381 nm, which corresponded to that of $P450_{cam}$ reconstituted with MnPPIX (Figure 11).[23]–[24] ICP-OES showed that 70% of the apo-$P450_{BS\beta}$ was reconstituted with MnPPIX. The UV-Vis absorbance ratio ($A_{Soret(381)}/A_{280}$) of *r*Mn-$P450_{BS\beta}$ was consistent with 70% reconstitution.

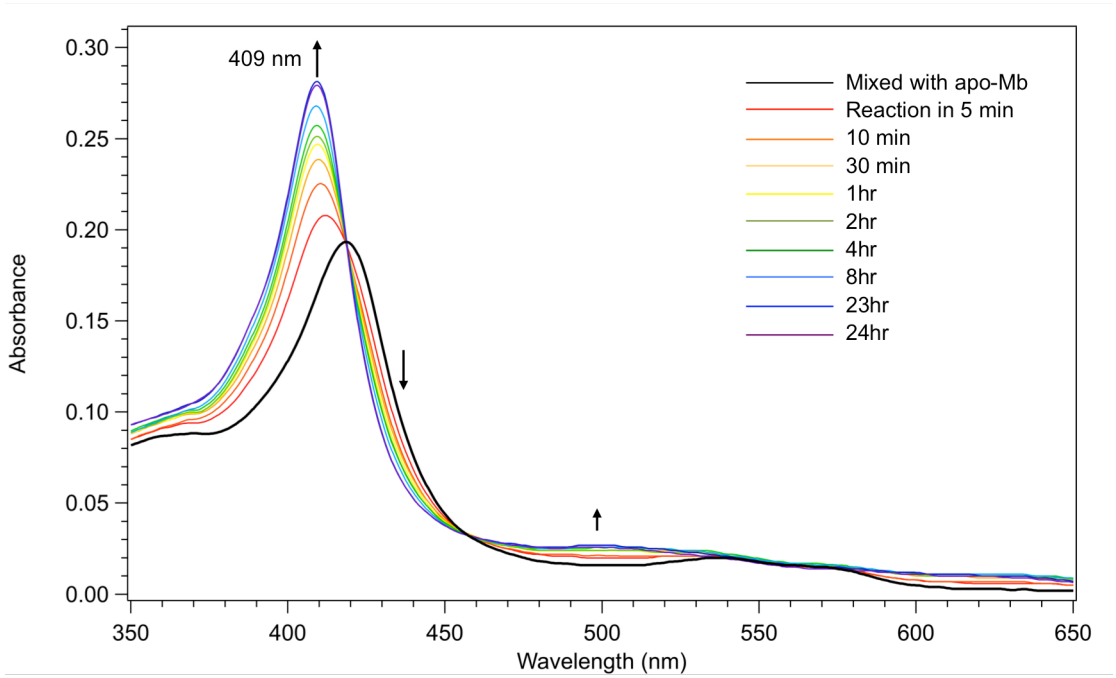
The catalytic activity of *r*Mn-$P450_{BS\beta}$ for one-electron oxidation of guaiacol using hydrogen peroxide as an oxidant was examined, but the TOF was very low at 40 $min^{-1}$. In contrast, when *m*CPBA was used as the oxidant, the catalytic activity was improved (2850 $min^{-1}$) compared with that of recombinant $P450_{BS\beta}$ (Table 1). On the other hand, hydroxylation of cyclohexane was not catalyzed by neither recombinant $P450_{BS\beta}$ nor *r*Fe-$P450_{BS\beta}$. We also examined the hydroxylation of cyclohexane by *r*Mn-$P450_{BS\beta}$ with *m*CPBA, but no product was detected, suggesting either the




**Scheme 2.** Proposed oxidation mechanism of guaiacol with P450.

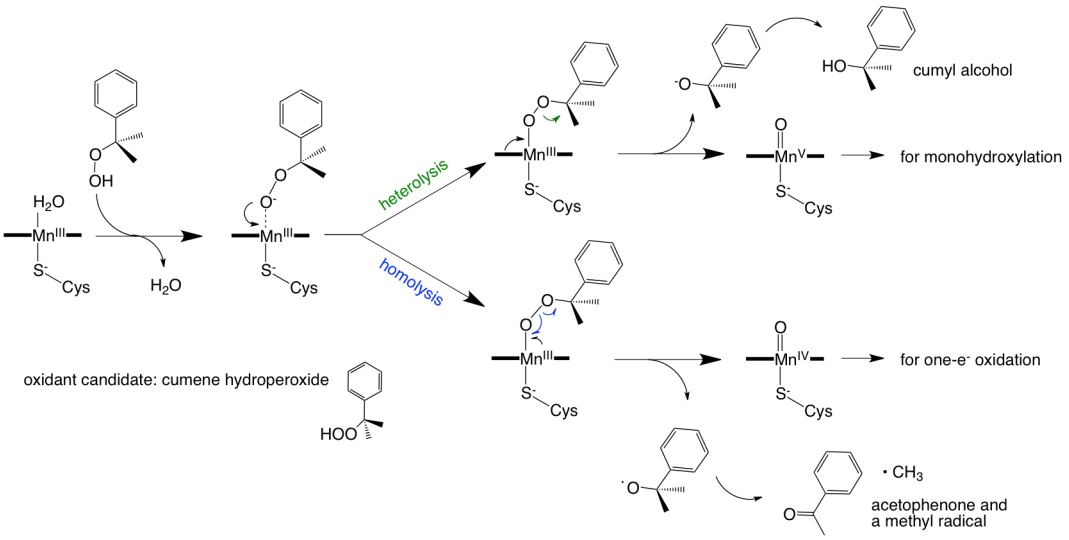
$Mn^{IV}$=O, not the $Mn^{V}$=O, species generated by homolytic cleavage of $Mn^{III}$-OOR or very rapid reduction of $Mn^{V}$=O under the condtions and eventually $Mn^{IV}$=O is responsible for one-electron oxidation by *r*Mn-$P450_{BS\beta}$ in the presence of *m*CPBA (Scheme 3).[25]

Based on apo-$P450_{BS\beta}$ (CYP152A1) can be prepared by using apo-Mb as a heme scavenger without subjecting the proteins to harsh and denaturing conditions. CYP152G1, which is in the same $H_2O_2$-dependent subfamily, was the next candidate




**Figures 12.** Time course of UV-Vis absorption spectra changes during the reaction of CYP152G1 with apo-Mb at 25 °C for 24 h.

under the same condition for heme removal. As the Figure 12, the clear blue shift of the absorption peak from 418 nm to 409 nm had been observed as the same result of $P450_{BS\beta}$ with apo-Mb.




**Scheme 3.** Proposed mechanisms in Mn-$P450_{BS\beta}$ to active species by heterolytic or homolytic O-O bond cleavages.

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## 3.4 Conclusions

We have demonstrated that apo-P450$_{BS\beta}$ can be prepared using apo-Mb as a heme scavenger without subjecting the proteins to harsh and denaturing conditions. Although the preparation of apo-P450s and their reconstitution is generally difficult, over the past decades, the harsh conditions are still the required methods to generate apoproteins. The heme of P450$_{BS\beta}$ was completely removed by apo-Mb to give pure apo-P450$_{BS\beta}$ that could be reconstituted with MnPPIX. The holo-, apo-, *r*Fe- and *r*Mn-P450$_{BS\beta}$ remained as analogous α-helical structures, in contrast to other reported reconstituted P450s and their apo-P450s prepared using the acid–butanone method or incubation with hydrogen peroxide. Although we focused on the preparation of apo-P450, other P450s whose apo-forms are difficult to prepare by conventional acid–organic-solvent methods may also be potent targets for our methodology. Indeed, we showed that the heme of CYP152G1 can be removed by the same approach. Furthermore, we believe that this method is useful to selectively remove the heme of hemoproteins containing other cofactors such as flavin derivatives or iron–sulfur clusters.

## 3.5 Reference


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# CHAPTER 4. CONCLUSIONS

# CONCLUSIONS

In this thesis, the author aimed to develop novel strategies for protein engineering of hydrogen peroxide-dependent P450s. The authore have demonstrated that rationally designed mutants of $P450_{SP\alpha}$ based on its crystal structure efficently catalyze the hydroxylation of indole and 1-methoxynaphthalen, while wild-type $P450_{SP\alpha}$ never catalyzes the hydroxylation of non-native substrates. Furthermore, the author have developted a novel method to prepare apo-$P450_{BS\beta}$ by which the heme of $P450_{BS\beta}$ can be removed without subjecting the protein to harsh conditions.

Chapter 2 describes the hydroxylation reactions of aromatic substrates by $P450_{SP\alpha}$ mutants. The A245E mutant of $P450_{SP\alpha}$ which mimics the substrate-binding-state of $P450_{SP\alpha}$ catalyzes the $H_2O_2$-dependent hydroxylation of non-native substrates. The double-site mutants of $P450_{SP\alpha}$, A245E/F288G and L78F/A245E, afforded better catalytic activity for the hydroxylation of indole and 1-methoxynaphthalene. Both a larger substrate binding space of A245E/F288G and a possible π-π interaction of L78F/A245E with aromatic substrates would contribute to improve catalytic activity. The additional mutations around Glu-245 were effective to enhance the hydroxylation catalytic activity of A245E. The author believes that these findings contribute to further develop new site-directed variants for enhancing catalytic ability toward other non-natural substrates.

Chapter 3 describes a gentle removal of heme from $P450_{BS\beta}$ by utilizing apomyoglobin. The first preparation of apo-form $H_2O_2$-dependent P450 under mild conditions followed by reconstitution with heme analogues is described in this chapter. The heme of $P450_{BS\beta}$ is completely removed by apomyoglobin, because the heme is efficiently transferred from $P450_{BS\beta}$ to myoglobin. The size and secondary structure of apo-$P450_{BS\beta}$ is essentially identical to that of holo-$P450_{BS\beta}$, showing a slight structural due to the removal of heme. The apo-$P450_{BS\beta}$ is reconstituted with hemin

and the resulting reconstituted Fe-P450$_{BS\beta}$ catalyzed the one-electron oxidation of guaiacol. The catalytic activity of reconstituted Fe-P450$_{BS\beta}$ was not much different from that of the recombinant P450$_{BS\beta}$. The recombinant P450$_{BS\beta}$ with MnPPIX showed a higher catalytic activity when meta-chloroperoxybenzoic acid (*m*CPBA) was used as the oxidant. The result obtained in this chapter would lead to preparation of apo-hemoproteins other than P450$_{BS\beta}$. Our method is definitely suitable if the hemoproteins or their apo-forms are not sufficiently stable in organic solvents. The author also believes that the efficiency to prepare reconstituted hemoproteins by employing our method would be much imprved after further optimization of conditions.

Through the research described in this thesis, the author proved that the artificial P450s could be rationally designed based on crystal structures of the wild-type proteins. The development of a novel method for preparation of apo-form protein under mild conditions was also demonstrated. While the author focused on site-directed mutagenesis and heme replacement for engineering of hemoproteins in this thesis, the author believes that our methods are compatible with reported protein-engineering techniques such as chemical modification.

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*March 2017*

*Shih-Cheng Chien*

# List of Publications

Shoji, O.; Fujishiro, T.; Nishio, K.; Kano, Y.; Kimoto, H.; Chien, S.-C.; Onoda, H.; Muramatsu, A.; Tanaka, S.; Hori, A.; Sugimoto, H.; Shirob, Y.; Watanabe, Y., A Substrate-Binding-State Mimic of $H_2O_2$-Dependent Cytochrome P450 Produced by One-Point Mutagenesis and Peroxygenation of Non-Native Substrates. *Catal. Sci. Technol.* **2016,** *6* (15), 5806-5811.

Chien, S.-C.; Shoji, O.; Morimoto, Y.; Watanabe, Y., Use of Apomyoglobin to Gently Rmove Heme from a $H_2O_2$-Dependent Cytochrome P450 and Allow its Reconstitution. *New J. Chem.* **2017,** *41* (1), 302-307.