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Essential role of the Arg^{112} residue of cytochrome P450cam for electron transfer from reduced putidaredoxin

Hideo Koga[a], Yasuhiro Sagara[a], Tsuyoshi Yaoi[a], Mitsushi Tsujimura[a], Kazuhide Nakamura[a], Kazuhisa Sekimizu[a], Ryu Makino[b], Hideo Shimada[b], Yuzuru Ishimura[b], Kei Yura[c], Mitiko Go[c], Masamichi Ikeguchi[d], Tadao Horiuchi[d,*]

[a]*Department of Microbiology, Faculty of Pharmaceutical Sciences, Kyushu University 62, Higashi-ku, Fukuoka 812, Japan*
[b]*Department of Biochemistry, School of Medicine, Keio University, Tokyo 160, Japan*
[c]*Department of Biology, Faculty of Science, Nagoya University, Nagoya 464-01, Japan*
[d]*Department of Bioengineering, Faculty of Engineering, Soka University, Hachioji, Tokyo 192, Japan*

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Cytochrome P450cam (CYP101) of *Pseudomonas putida* PpG1 in which Arg^{112} is substituted by Cys was isolated by in vitro random mutagenesis of the *camC* gene DNA coding for P450cam. The absorption spectra of the purified mutant enzyme were similar to those of the wild type enzyme, but its substrate-dependent NADH oxidation activity in the presence of putidaredoxin (Pd) and putidaredoxin reductase (PdR) was extremely low. The rate constant of electron transfer from reduced Pd to the heme of the mutant P450cam, measured on an anaerobic stopped flow apparatus, was 1/400 of that of the wild type enzyme and the dissociation constant of the mutant P450cam for oxidized Pd was several fold higher than that of the wild type enzyme. A considerable decrease in mid-point potential of the mutant enzyme was also noted. We conclude that Arg^{112}, which is located on the surface of the P450cam molecule and hydrogen-bonded to one of the heme propionate chains, plays an essential role in the electron transfer from Pd.

Cytochrome P450cam; Random mutagenesis; Amino acid substitution; Putidaredoxin; Electron transfer; Binding of P450cam with putidaredoxin

1. INTRODUCTION

Cytochrome P450cam (P450cam) of *Pseudomonas putida* PpG1 (ATCC 17453) [1,2] is the terminal monooxygenase of the *d*-camphor hydroxylase system catalysing the following reaction [3–6]:

$$d\text{-Camphor} + NADH + O_2 \rightarrow 5\text{-}exo\text{-hydroxycamphor} + NAD^+ + H_2O$$

For this reaction to proceed, it is necessary for P450cam (encoded by the *camC* gene [7,8]) to receive two electrons separately from NADH at different steps of the catalytic cycle. Each of these electron transfers is mediated sequentially by putidaredoxin reductase (PdR, a flavoprotein encoded by the *camA* gene [9]) and putidaredoxin (Pd, an iron-sulfur protein encoded by the *camB* gene [9]). All the *cam* genes are on a plasmid.

In this study, we attempted to isolate P450cam mutants that are defective in *d*-camphor hydroxylation by random mutagenesis of a plasmid DNA fragment containing the *camC* gene using hydroxylamine as a mutagen. Transformation of a $camC^-$ strain of *P. putida* with the mutagenized plasmid DNA led to the isolation of mutants that can grow with 5-*exo*-hydroxycamphor, but not *d*-camphor, as the sole carbon source, even though they can produce a P450cam-like protein (see section 2). From nucleotide sequencing of the plasmid DNAs from the isolated mutants, we found that one mutant contains a *camC* gene encoding a mutant P450cam in which Arg^{112} is replaced by Cys. We overproduced the mutant P450cam in *E. coli* and studied its structure and catalytic activity. Here we report that the Arg^{112}Cys P450cam protein has a drastic defect in the electron transfer from reduced Pd, albeit its structure is similar to that of the wild type enzyme. We conclude that Arg^{112} plays an important role in the electron transfer from reduced Pd to P450cam.

*Corresponding author.

Abbreviations: P450cam, cytochrome P450cam; Pd, putidaredoxin; PdR, putidaredoxin reductase; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; Arg^{112}, arginine residue at the position 112 of P450cam; Arg^{112}Cys, a mutant in which Arg^{112} has been changed by Cys.

2. MATERIALS AND METHODS

2.1. *Plasmid construction and hydroxylamine mutagenesis*

The DNA fragment, containing the *camC* gene [8,10], was cloned on pTS1210 (carrying the *ori* of pBR322 for *E. coli*, the *ori* of pSa for *P. putida*, and the genes for ampicillin and kanamycin resistance [10]) down-stream of the ampicillin promoter, pHS422 thus obtained was mutagenized with hydroxylamine in vitro (0.1 M hydroxylamine in 50 mM Na-phosphate (pH 7.5) for 30 min at 75°C [11]), and was used

to transform *P. putida* MTC3011 (*camC*$^-$, kanamycin sensitive [10]) which can use 5-*exo*-hydroxycamphor, but not *d*-camphor, as the carbon source. *camC*$^-$ and kanamycin resistant transformants, which did not form a colony on the synthetic media plate [10] containing *d*-camphor as the sole carbon source, were isolated. Plasmid DNAs were extracted and their nucleotide sequences were determined by the method of Sanger et al. [12] to identify the mutational sites in the *camC* gene

2.2 *Overproduction of the enzyme*

The mutant plasmids derived from pHS422 were used to transform *E coli* D1210 [10] and the transformants were cultured at 30°C in L-broth [10]. Protein was determined by the method of Lowry et al. [13] using bovine serum albumin as a standard, and the content of total P450cam protein in *E coli* cells was estimated using SDS-PAGE [14]. In the case of the Arg^{112}Cys mutant, the P450cam protein produced accounted for as much as 5% of the total bacterial protein

2.3. *Purification and assay of the enzymes*

P450cam, Pd, and PdR were purified by conventional methods [4,9] and homogeneity of the enzymes was verified by SDS-PAGE. The specific content of purified Arg^{112}Cys protein was 12–15 nmol/mg protein, a value which is considerably lower than that of the wild type P450cam (21 nmol/mg protein) The activity of each enzyme was assayed by measuring the camphor-dependent NADH oxidation in the presence of excess of Pd and PdR in 50 mM K-phosphate (pH 7.4) at 25°C [2,15]. One enzyme unit is the amount required to oxidize 1 nmol of NADH per second [9]

2.4. *Optical measurements*

Absorption spectra of the enzymes were measured at 20°C on a Hitachi Model U-3210 spectrophotometer, except for the oxygenated form of the mutant protein which was measured at 10°C The enzyme concentration was 8 μM in 50 mM K-phosphate (pH 7.4) containing 50 mM KCl with or without 1 mM *d*-camphor. The enzyme was reduced by the addition of $Na_2S_2O_4$. Oxygenated or carbon monoxide-bound enzyme was obtained by bubbling of oxygen or carbon monoxide, respectively, into reduced P450cam. The autooxidation rate of the oxygenated form was determined by measuring the increase in absorbance at 392 nm due to the formation of the ferric form of P450cam. To determine the specific content of P450cam, the protein concentrations were adjusted to 4 mg/ml. CO difference spectra [16] were recorded, and the P450cam levels were estimated from the absorption difference at 446 and 490 nm (448 and 490 nm in the case of the mutant, see text), using an ε_{mM} of 93 [4,10].

2.5. *Substrate binding*

Dissociation constants (K_d) of *d*-camphor or norcamphor for substrate-free ferric P450cam were calculated from double reciprocal plots of the absorbance decrease at 418 nm vs. substrate concentration. Binding titrations were performed with 1–3 μM P450cam in 20 mM K-phosphate (pH 7.4) containing 5% glycerol and 0.1 M NaCl at 20°C.

2.6 *Measurement of dissociation constant (K_s) of P450cam to oxidized Pd*

The methods of Sligar [17] and Hintz et al. [18] were used. The sample cuvette contained 600 μl of 10 mM K-phosphate (pH 7.4) containing 1 mM *d*-camphor, and 2 μM wild type P450cam. The reference cuvette also contained 600 μl of the same solution except for P450cam. 2 mM Pd was added stepwise to both cuvettes up to 16 μl. Difference spectra (peak-position at 392 nm) were stored in a computer and calculated to correct for dilution effects. The reciprocal of absolute values of the absorbance at 392 nm was then plotted against the reciprocal of free Pd concentrations, and dissociation constant (K_s) was calculated.

2 7. *Kinetic measurements*

The rate of reduction of the wild-type and mutant enzymes was measured by the method of Hintz and Peterson [19] on a Union Giken stopped flow apparatus, Model RA-403. One reservoir contained the wild-type or mutant P450cam (3.0 μM) in CO-saturated 50 mM K-phosphate (pH 7.4) containing 50 mM KCl and 1 mM *d*-camphor. The other reservoir contained Pd (3.0–34.0 μM) in the above CO-saturated buffer. Pd in the reservoir was reduced by NADH in the presence of a catalytic amount of PdR. Reduction of the wild type and mutant P450cam was monitored at 446 nm. Experiments were done at 20°C.

2.8. *Oxidation–reduction potential measurements*

The potential of the wild type and mutant enzymes was determined at pH 7.4. About 50 μM of the enzyme in 50 mM K-phosphate (pH 7.4) containing 50 mM KCl and 1 mM *d*-camphor was used for the measurements.

2.9. *Reagents and other enzymes*

7-DEAZA Sequencing kit and restriction endonucleases were purchased from Takara Shuzo Co. All other reagents were of a guaranteed grade and were purchased from commercial suppliers

2.10. *Computer simulated mutation*

The mutated structure was built by locating Cβ and Sγ of Cys to the positions of Cβ and Cγ of Arg^{112}, respectively. Energy minimization was performed by the method of a conjugate gradient search with hydrogen atoms that had the capacity to form hydrogen bonds to other atoms until the root mean square force reached 0 1 kcal/mol/Å or less. The criteria was reached by 574 cycles. Atoms within the sphere of a 10 Å radius from the mutated Cys Cα were relaxed and other atoms were fixed. The list of atoms within the sphere was updated every 50 steps. Every procedure was carried out on BIOGRAF [20].

3. RESULTS AND DISCUSSION

3.1. *Effect of the mutation on the structure of P-450cam*

As shown in Table I, the absorption maxima of the Arg^{112}Cys mutant of P450cam in different states are practically the same with those of the wild type enzyme, suggesting that the mutation does not significantly affect the structure around the heme. The observation that the addition of *d*-camphor to the oxidized mutant enzyme converts the absorption spectrum to the high-spin type indicates that the *d*-camphor binding ability is not impaired by the mutation. In fact, spectrophotometric titration showed that the wild type and mutant P450cam have the same dissociation constants

Table I

Peak positions of absorption spectra for wild type and Arg^{112}Cys cytochromes P450cam in oxidized, camphor-bound oxidized, camphor-bound reduced, oxygen-bound reduced, and carbon monoxide-bound reduced forms

Form	Wild type			Arg^{112}Cys		
Oxidized	417	537	570	417	536	567
Camphor-bound oxidized	392	508	641	392	507	642
Camphor-bound reduced	409	542		408	544	
O_2-bound reduced	355*	418	553	419	552	
CO-bound reduced	446	552		448	552	

The assay conditions are described in Section 2 The wavelength of each peak-position is given in nm. *Shoulder

(K_d) for *d*-camphor (3 μM) and norcamphor (20 μM). A difference noticed between the two proteins was the instability of the oxygenated form of the mutant P450cam, which underwent autoxidation at rates 4.4×10^{-3}/s at 9.9°C, 6.2×10^{-3}/s at 13.0°C, and 8.2×10^{-3}/s at 15.0°C. These values are almost 4 times higher than those determined for the wild-type oxygenated form (9.8×10^{-4}/s at 9.8°C, 2.1×10^{-3}/s at 15.0°C, and 3.5×10^{-3}/s at 19.5°C). On the other hand, the CO-ligated form of the reduced mutant enzyme was as stable as the wild-type counterpart.

The effect of Arg^{112} substitution by Cys on the conformation of P450cam was examined by a computer simulated mutation as described in section 2. We found little conformational change and there were slight shifts in the position of the atoms after the energy refinement. A root mean square deviation of all the Cα atoms between the wild-type and mutated structures was only 0.13 Å. It can thus be concluded that the amino acid substitution does not lead to serious steric effects on the conformation of P450cam.

3.2. *Defects in electron transfer from Pd to P-450cam*

In a reconstituted system containing Pd and PdR, the Arg^{112}Cys enzyme showed an extremely low *d*-camphor-dependent NADH oxidation activity (<2 units/mg protein) whereas the activity of the wild type enzyme was 460 units/mg protein. As described above, this low activity does not seem to be due to a defect of the mutant enzyme to bind the substrate, *d*-camphor.

We also observed that, in the reconstituted system, the Soret peak at 392 nm of the substrate-bound ferric form of the mutant P450cam did not change even 20 min after addition of NADH. Under the same conditions, substrate-bound ferric form of the wild type enzyme was readily reduced and bound to dioxygen. However, the mutant enzyme as well as wild type enzyme can readily be reduced with $Na_2S_2O_4$ (data not shown). Therefore, the mutant enzyme seems to have a defect in the transfer of the first electron from reduced Pd.

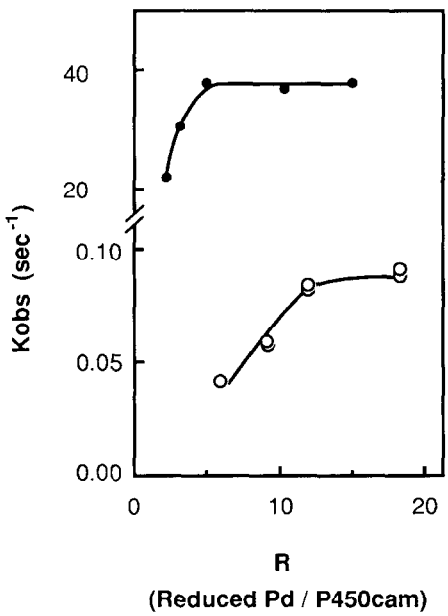

Fig. 1. Reduction of cytochromes P450cam by reduced Pd. Details are described in section 2. Closed circle, wild type enzyme; open circle, Arg^{112}Cys enzyme.

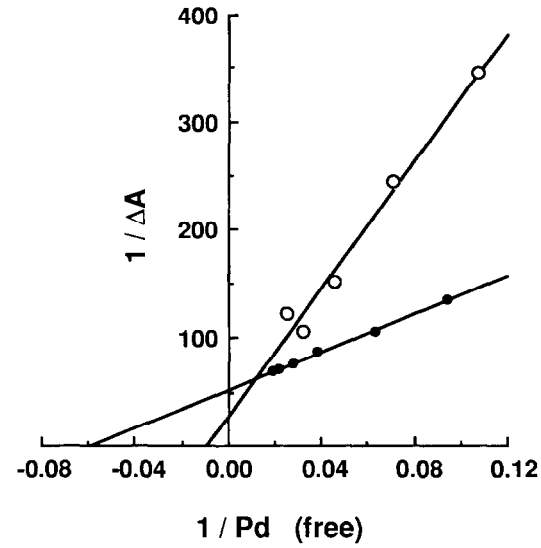

Fig. 2. Double-reciprocal plot of titration of cytochromes P450cam with Pd. Details are described in section 2. Closed circle, wild type enzyme (K_s = 16.8 μM); open circle, Arg^{112}Cys enzyme (K_s = 106 μM)

This was substantiated by measuring reduction kinetics of the mutant P450cam by reduced Pd using anaerobic stopped flow spectrophotometry [19] at 20°C under a CO atmosphere [16]. As shown in Fig. 1, the first order rate constant (k_{obs}) for the mutant enzyme was as low as 0.1/s even when the molar ratio (R) of reduced Pd to P450cam was >15, whereas k_{obs} was 40/s for the wild type enzyme when R was >5. These results show that the mutant P450cam has a markedly decreased affinity for Pd and its reduction by reduced Pd is extremely slow. It is thus clear that Arg^{112} of P450cam plays a crucial role in binding to and electron transfer from reduced Pd to the heme.

It has been reported that the binding of oxidized Pd to camphor-bound ferric P450cam is accompanied by a decrease in the Soret absorption band at 392 nm [17,18,21]. We therefore compared this spectral decrease between the wild type and mutant P450cam. As depicted in Fig. 2, in both cases, the reciprocal of the absorbance decrease at 392 nm ($1/\Delta 392$ nm) is linearly correlated with the reciprocal of the concentration of free Pd. However, the spectroscopic dissociation constant (K_s) of oxidized P450cam to oxidized Pd was 106 μM for the mutant enzyme, a value which is 6 times higher than that (16.8 μM) for the wild type enzyme, as in the case for mutant P450scc and adrenodoxin [22,23]. Although the affinities of oxidized P450cam for oxi-

dized Pd and for reduced Pd are markedly different [18], this finding is consistent with the notion that the mutant P450cam interacts with Pd more weakly than the wild type P450cam.

We next examined if the very low rate of electron transfer is due to a decreased redox potential of the mutant enzyme. The mid-point potential (E_m) of the mutant protein in the presence of *d*-camphor was determined to be −182 mV by using phenosafranin (E_m = −225 mV) as a control. The determined E_m is considerably lower than that of the wild type enzyme (−138 mV). However, since the E_m of reduced Pd is −233 mV [24], the electron transfer from reduced Pd to the mutant P450cam should be possible. In fact, the $Thr^{252}Pro$ mutant of P450cam [25] retaining the Pd binding ability, showed an E_m of −175 mV, but still possessed a k_{obs} value of 4/s (the k_{obs} value for the $Arg^{112}Cys$ mutant is 0.1/s as described above). More work is needed to elucidate the relationship between the E_m and the low electron transfer rate in this system.

3.3. *Role of Arg^{112} in electron transfer*

Taking advantage of the fact that cytochrome b_5 and Pd interact competitively with P450cam at nearly the same site, Stayton et al. [26] computer-graphically studied the binding of cytochrome b_5 to the P450cam and suggested that the binding involves ionic/electrostatic interactions. They also suggested that Arg^{72}, Arg^{112}, and Lys^{344} of P450cam are important basic residues involved in the binding with the acidic protein cytochrome b_5 and also with Pd. In a later study [27], they replaced these amino acid residues, except for Arg^{112}, by Gln or Glu by site-specific mutagenesis. The $Lys^{344}Gln$, $Lys^{344}Glu$, and $Arg^{72}Gln$ mutant proteins thus prepared showed similar V_{max} values and only 20–60% increased K_m values as compared with the wild-type enzymes in the camphor-dependent NADH oxidation activity. Moreover, our newly isolated $His^{347}Ala$ mutant, showed a significant camphor-dependent NADH oxidation activity (about 30% of that of the wild type enzyme, unpublished observation), even though His^{347} is located on the same surface of the molecule and near the region of Arg^{112} and Lys^{344}. These results suggest that the roles of Arg^{72}, Lys^{344}, and His^{347} in the Pd binding are, if any, not essential. On the contrary, our results indicate an essential role of Arg^{112} in the binding and electron transfer from reduced Pd to P450cam.

Based on the X-ray crystallographic structure of P450cam, Poulos et al. [28] reported that the $-NH_2$ (η_1) of the guanidino-group of Arg^{112} is hydrogen-bonded to the oxygen atom at 1D position of the carboxylic acid group of the heme propionate chains in the region of the heme-proximal side. Therefore, it is expected that replacement of the large basic side chain of Arg^{112} by the small, thiolic side chain of Cys has a profound effect on the heme. This effect may explain the lowered redox potential of the $Arg^{112}Cys$ mutant protein and the decreased stability of its oxygenated form. However, the extremely low electron transfer activity of the mutant enzyme cannot be entirely explained by the small size of Cys side chain. Our preliminary study showed that the $Arg^{112}Gln$ and $Arg^{112}Glu$ mutants, which have side chains that are considerably larger than that of Cys at position 112, exhibited much the same electron transfer activities as the $Arg^{112}Cys$ mutant (unpublished observation). We therefore suggest that the hydrogen bond between the guanidino-group of Arg^{112} and the heme propionate chain and the positive charge of the guanidino group are important for the electron transfer from reduced Pd to the heme. In fact, a computer-graphic study showed that the $Arg^{112}Cys$ protein lacks the hydrogen bond between Arg^{112} and the heme (unpublished).

Stopped flow kinetic analysis by Hintz and Peterson [19] has shown that a temperature-dependent phase precedes the temperature-independent electron transfer and they suggested that a conformational change of P450cam is required before the electron transfer takes place. Such a conformational change could also be rate limiting and function as a control point for the electron transfer. A likely possibility is that hydrogen-bonded Arg^{112} is essential for the conformational change of P450cam after binding to reduced Pd.

Finally, it is worth mentioning that Arg^{112} is one of the most conserved amino acid residues in the P450 family [29,30], as compared with Arg^{72}, Lys^{344}, and His^{347} which are also present on the surface of the P450cam molecule.

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