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Significant contribution of arginine-112 and its positive charge of *Pseudomonas putida* cytochrome *P*-450$_{cam}$ in the electron transport from putidaredoxin

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Abstract

Cytochrome *P*-450$_{cam}$ of *Pseudomonas putida* is a prototype of various eukaryotic cytochrome *P*-450 molecules. Arg-112 located on the surface of this protein is highly conserved among various other cytochromes *P*-450. In this study, we constructed mutant genes for *P*-450$_{cam}$ in which Arg-112 was replaced by Gln or Glu, expressed them in *Escherichia coli* and purified the mutant proteins. Their enzymic activities were analyzed in the reconstituted system to determine the function of Arg-112. K_d values for *d*-camphor of Arg^{112}-Gln and Arg^{112}-Glu were much the same as those of the wild-type enzyme, whereas K_d values for the oxidized form of putidaredoxin, which is an acidic protein and is the redox partner of *P*-450$_{cam}$, were 240 and 530 μM, respectively. These values are 8 and 19 times larger than that of the wild-type enzyme (28 μM), thereby indicating lower affinities of the mutant enzymes for the oxidized putidaredoxin. Reaction rate constants for reduction by the reduced form of putidaredoxin, measured using the stopped flow method, were 45.5, $9.0 \cdot 10^{-3}$ and $9.0 \cdot 10^{-4}$ s^{-1} for the wild type, Arg^{112}-Gln and Arg^{112}-Glu, respectively. Thus, Arg-112 of *P*-450$_{cam}$ plays an important role in the interaction with putidaredoxin and in the high efficiency of the electron transfer; the positive charge of the residue seeming to contribute to the process. The yields in *Escherichia coli*, the heme contents in the purified fractions and heat stability of the mutant proteins were lower than those of the wild type enzyme, suggesting that Arg-112 of *P*-450$_{cam}$ is also important for stability of *P*-450$_{cam}$.

Key words: *P*-450$_{cam}$; Arginine; Putidaredoxin; Electron transfer; *(P. putida)*

1. Introduction

Cytochrome *P*-450$_{cam}$ (*P*-450$_{cam}$, encoded by *camC* gene [1,2]) has been purified from the soil bacterium *Pseudomonas putida*, ATCC 17453, which can grow using *d*-camphor as the sole carbon source and this heme protein catalyzes the stereospecific methylene hydroxylation of *d*-camphor to form 5-*exo*-hydroxycamphor as the first step in camphor catabolism [3,4]. *P*-450$_{cam}$ functions as the terminal monooxygenase in the camphor hydroxylating multienzyme system [5] in which the reducing equivalents of NADH are transferred from putidaredoxin reductase (a flavoprotein, encoded by *camA* gene [6]) to *P*-450$_{cam}$ via putidaredoxin (an iron-sulfur protein, encoded by *camB* gene [6]).

The reaction cycle of *P*-450$_{cam}$ consists of the following steps; (i) substrate binding to the oxidized form of *P*-450$_{cam}$ followed by production of the substrate-bound oxidized form, (ii) formation of the reduced form by receiving the first electron from putidaredoxin, (iii) formation of substrate-bound oxygenated form by introduction of molecular oxygen and (iv) reverting to the initial sub-

Abbreviations: *P*-450$_{cam}$, cytochrome *P*-450$_{cam}$; Arg^{112}-Gln, mutant *P*-450$_{cam}$ protein in which Arg-112 was replaced by Gln; Arg^{112}-Glu, mutant *P*-450$_{cam}$ protein in which Arg-112 was replaced by Glu; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

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strate-free oxidized form by receiving the second electron from putidaredoxin and releasing a water molecule and the hydroxylated substrate [5]. The X-ray crystal structure of putidaredoxin has not been determined and details on the mechanism of electron transfer from putidaredoxin to *P*-450_{cam} are still uncertain, although there are reports that the carboxy terminal Trp of putidaredoxin plays an important role in the electron transfer to *P*-450_{cam} [7–9]. For *P*-450_{cam}, even though the X-ray crystal structure of *P*-450_{cam} has been determined [10–12], the amino-acid residue(s) of *P*-450_{cam} which would contact putidaredoxin are uncertain. Such being the case, analyses of amino-acid residues contributing to the interaction with putidaredoxin are important to elucidation the electron transfer mechanism on *P*-450_{cam}. We introduced random mutation with hydroxylamine into the *camC* gene, and screened mutants which could not grow with *d*-camphor but did grow with 5-*exo*-hydroxycamphor. We isolated the Arg^{112}-Cys mutant and found that this mutant *P*-450_{cam} had a significantly low affinity for putidaredoxin [13]. Arg-112 is located on the surface of *P*-450_{cam} (Fig. 1) and the residue is highly conserved among various cytochromes *P*-450 [14,15]. For further characterization of the role of Arg-112, we constructed mutant genes in which Arg-112 was replaced by Gln, which is neutral and of similar size to Arg, or by Glu, which is negatively charged. The mutant *P*-450_{cam} proteins were expressed in *Escherichia coli*, purified, and their enzymic properties were compared using the reconstituted system. In this report, we provide evidence for the notion that the positively charged residue of Arg-112 is important for *P*-450_{cam} to interact with putidaredoxin, and that Arg-112 participates in the stable formation of heme-containing *P*-450_{cam}.

2. Materials and methods

2.1. Construction of mutant genes by site-directed mutagenesis

General DNA techniques used were according to Sambrook et al. [16]. Genes for mutant *P*-450_{cam} proteins were constructed by the method of Kunkel [17]. The *Pst*I-*Hin*dIII fragment containing the *camC* gene from pHS422 [18] was ligated to the *Pst*I-*Hin*dIII site of M13mp18 RF DNA [19], and used for the transformation of *E. coli* CJ236 [17]. Then, the single-stranded phage DNA was isolated from the transformed cells harboring the phage and was used as the template. A mixture of two synthetic oligonucleotides, 5′-GCGCCAGTTT(C/G)AGGCCTTG-

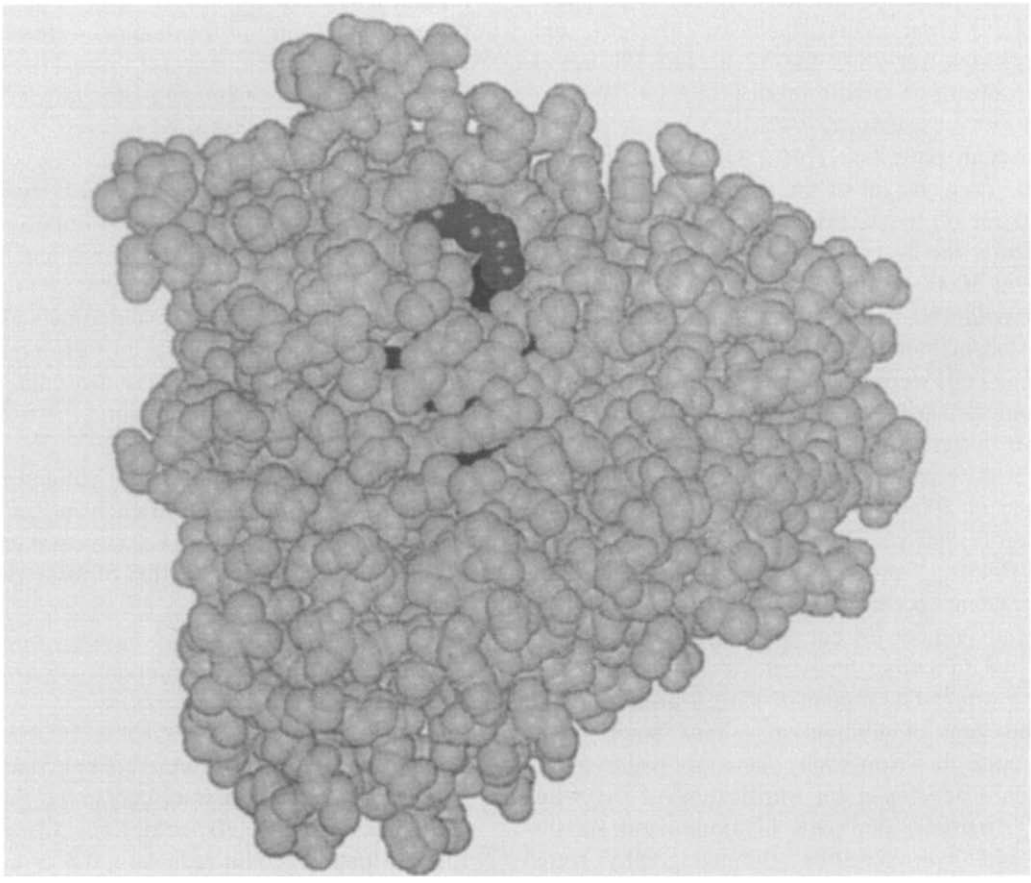

Fig. 1. View of *P*-450_{cam} from the heme-proximal side. The presentation is based on atomic coordinates determined by X-ray crystallography [12] and which were provided by Brookhaven National Laboratory [33]. Arg-112 is highlighted and the heme molecule is shown in black.

GCCAACCA-3', was used as the probe for change Arg-112 to Gln or Glu. The underlined area in the above sequence shows the position of mismatches. Screening of the mutants was carried out as follows; the single-stranded DNA of a candidate clone was hybridized with the *Pst*I-*Hin*dIII fragment containing the wild type *camC* gene which was cloned in M13mp18 phage [19]. The hybridized DNA was digested with S1 nuclease to cleave the mismatched position, and the sample was analyzed on agarose gel electrophoresis. Introduction of the base changes was confirmed by identifying the small-size DNA fragment. Next, replacement of Arg-112 with Gln or Glu was confirmed by sequencing the *camC* gene, using a DNA sequencer (Applied BioSystems Japan, Model 373A).

2.2. Overproduction and purification of $P\text{-}450_{cam}$ proteins

The *Pst*I-*Hin*dIII fragment containing the wild-type or mutant *camC* gene was inserted into the *Pst*I-*Hin*dIII site of pUC18 [19], and used for transformation of *E. coli* RRI [20]. The operon fusion of the *lacZ* gene and the *camC* gene containing ribosome binding site was constructed so that transcription would be initiated by the *lac* promoter but the original ribosome binding site was effective for the translation of $P\text{-}450_{cam}$. The *E. coli* cells harboring the expression plasmid for the wild-type or mutant $P\text{-}450_{cam}$ were cultured in 3 ml of PY-medium (1% (w/v) polypeptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, and 0.1% (w/v) glucose) at 28°C for 12 h, and 1% (v/v) inoculated into 60 ml of terrific broth (1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, and 100 mM potassium phosphate (pH 7.4)) and cultured at 28°C for 12 h. Then, 60 ml of the preculture was inoculated into 600 ml of the terrific broth, and culture was continued at 28°C for 24 h. The cells were harvested by centrifugation at $5000 \times g$ for 5 min at 4°C and washed once with ice-cold P-0 buffer (20 mM potassium phosphate (pH 7.4) containing 5% (v/v) glycerol and 1 mM *d*-camphor). The cells were resuspended into 5 vols. of P-0 buffer, and sonicated using a Branson Cell Disruptor 200 in ice-cold P-0 buffer using twelve 30 s pulses at 1 min intervals and at 40% of maximum power. The suspension was centrifuged at $5000 \times g$ for 5 min at 4°C, then the supernatant was further centrifuged at $100\,000 \times g$ for 60 min at 4°C. $P\text{-}450_{cam}$ proteins were purified from the resultant supernatant, according to the method described by Gunsalus and Wagner [5] but with modifications [21], and used for the following analyses. We found that the Arg^{112}-Gln and Arg^{112}-Glu mutant $P\text{-}450_{cam}$ proteins were unstable in the steps of ammonium sulfate fractionation and hydroxyapatite chromatography. Thus, we had to modify the procedure developed for purification of the wild type $P\text{-}450_{cam}$, namely, the steps of ammonium sulfate fractionation and hydroxyapatite chromatography were omitted and rechromatography on anion-exchange column was done after the gel filtration. The concentration of purified $P\text{-}450_{cam}$ (substrate-bound oxidized form) was determined by measuring the optical density at 391 nm ($\varepsilon = 102.0\ mM^{-1}\ cm^{-1}$) [5] and purity of the $P\text{-}450_{cam}$ was also analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) which was carried out according to the method of Laemmli [22]. Protein concentration was determined by the method of Lowry et al. [23], using bovine serum albumin as a standard.

2.3. Spectral analysis of $P\text{-}450_{cam}$ corresponding to each step of the reaction cycle

Absorption spectra were measured on each form of the wild-type and mutant $P\text{-}450_{cam}$ proteins at 2.5 μM in P-100 buffer (P-0 buffer containing 100 mM KCl), except for the substrate-free oxidized form which was in P-0 buffer without *d*-camphor. The substrate-free oxidized form was prepared by removing the substrate from the fraction by gel filtration through a Sephadex G-15 column (0.5×13 cm). Reduced forms were prepared by adding a few grains of solid sodium dithionite to the sample. Oxygen-bound forms were prepared by bubbling oxygen gas for 20 s after the reduction by sodium dithionite. CO-bound forms were prepared by bubbling CO gas for 30 s after the reduction by sodium dithionite. Spectra were measured at 20°C using a Hitachi U-3210 spectrophotometer, and analyses of the oxygen-bound form of the mutants were carried out at 8°C.

2.4. Measurement of camphor-dependent NADH oxidation activities in the reconstituted system

To the reaction mixture (920 μl) containing 50 mM potassium phosphate (pH 7.4), 1 nmol of putidaredoxin reductase, 5 nmol of putidaredoxin and 50 pmol of $P\text{-}450_{cam}$, 50 μl of 8 mM *d*-camphor solution in water was added, then the reaction was started by adding 30 μl of 10 mM NADH. The sample was mixed immediately and the initial rate of the reaction was determined at 20°C from decrease in absorbance at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) [6].

Putidaredoxin reductase and putidaredoxin were expressed in *E. coli* and purified from the cell lysates, as described elsewhere [5,24]. Construction and expression of the plasmids for overproduction of these enzymes has been documented [21].

2.5. Measurement of CO-difference spectrum in the reconstituted system

After the addition of NADH (final concentration of 250 μm) to the reaction mixture containing 50 mM potassium phosphate (pH 7.4), 100 mM KCl, 300 μM *d*-camphor, 1.0 μM putidaredoxin reductase, 1.5 or 15 μM putidaredoxin, and 1.5 μM $P\text{-}450_{cam}$, the background absorbance from 400 nm to 500 nm was immediately determined,

then, CO gas was bubbled into the mixture for 30 s and absorbance from 400 nm to 500 nm was measured.

2.6. Determination of dissociation constants for the binding of the P-450$_{cam}$ mutant proteins to oxidized putidaredoxin and d-camphor

The methods of Sligar [25] and Hintz et al. [26] were used for determination of the dissociation constants to oxidized putidaredoxin. We measured the putidaredoxin-induced spectral change of P-450$_{cam}$ in the presence of d-camphor as follows. A sample cuvette contained 2.0 μM P-450$_{cam}$ in 10 mM potassium phosphate (pH 7.4) containing 1 mM d-camphor. A reference cuvette contained the same solution but without the P-450$_{cam}$. The same amount of oxidized putidaredoxin was added to both cuvettes, and the difference of absorbance at 392 nm was measured. The data were stored in a computer and the effect of dilution was corrected by calculation. At first, ΔA_{max} was determined by least squares linear regression analysis on double reciprocal plotting of ΔA_{392} (decrease of absorbance at 392 nm) and [Pd$_{total}$] (concentration of total putidaredoxin), and [Pd$_{free}$] (concentration of free putidaredoxin) was determined using the following formula;

$$[Pd_{free}] = [Pd_{total}] - (\Delta A_{392}/\Delta A\max) \times [P-450_{cam}]$$

Then, ΔA_{max} was recalculated by replotting ΔA_{392} and [Pd$_{free}$]. The K_d value was determined by repeating this calculation three times.

Dissociation constants for the binding of the P-450$_{cam}$ proteins to d-camphor were determined as described by Peterson [27] using 2.0 μM P-450$_{cam}$ proteins.

2.7. Measurement of rate constants for the reduction of P-450$_{cam}$ by reduced putidaredoxin

The reduction rates of the wild type and mutant P-450$_{cam}$ proteins by reduced putidaredoxin were measured according to the method of Hintz and Peterson [28] using a Hitachi 557 spectrophotometer equipped with a stopped flow apparatus (Hitachi 557–0814; gas drive unit, C-812754; mixing unit, C-783396) under anaerobic conditions. Solution A consisted of 1.5 μM P-450$_{cam}$ in 5 ml of 20 mM potassium phosphate (pH 7.4) containing 1 mM d-camphor and 120 mM glucose, and solution B consisted of 300 μM NADH, 1.0 μM putidaredoxin reductase, and 9.0 μM putidaredoxin in 5 ml of the same buffer. The solutions placed in the sample chamber of the stopped-flow apparatus were degassed by aspiration and bubbled with nitrogen gas for 30 s, then 15 μl each of 30 mg/ml catalase and 30 mg/ml glucose oxidase were added to the solutions, using needles. The degassing and bubbling with nitrogen gas were repeated three more times to obtain an anaerobic condition, then bubbling of CO gas and degassing was repeated twice. Finally, CO gas was bubbled into the solutions for 30 s. Reduction of the P-450$_{cam}$ was initiated by mixing the solutions, for which the piston of the syringes of the stopped-flow apparatus was driven with the pressure of 4 kg/cm^2. The reaction was monitored on formation of the CO-bound reduced form of the P-450$_{cam}$ by measuring the difference of the absorbance between 446 nm and 490 nm ($\varepsilon = 92.8$ mM^{-1} cm^{-1}) [5]. The temperature in the sample chamber was kept at 20°C.

2.8. Chemicals

NADH was purchased from Sigma, d-camphor from Nacalai Tesque (Kyoto), Sephadex G-15 from Pharmacia and glucose oxidase (grade I) from Boehringer-Mannheim. All other reagents used were of analytical or biochemical grade.

3. Results

3.1. Expression and purification of Arg^{112}-Gln and Arg^{112}-Glu mutants of P-450$_{cam}$

We constructed mutant cytochrome P-450$_{cam}$ genes by site-directed mutagenesis followed by cloning into an expression vector, pUC18, after which the mutant genes were expressed in *E. coli*. Judging from the analysis on SDS-PAGE (Fig. 2), Arg^{112}-Gln and Arg^{112}-Glu mutant pro-

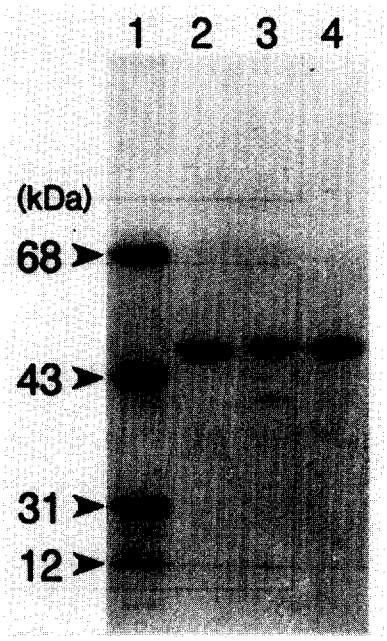

Fig. 2. SDS-PAGE analysis of purified cytochrome P-450$_{cam}$ proteins. The samples (5 μg each) were loaded onto an SDS-10% (w/v) polyacrylamide gel. Proteins were visualized by staining with Coomassie brilliant blue R-250. Lane 1, molecular mass standards (bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa); lane 2, wild type P-450$_{cam}$; lane 3, Arg^{112}-Glu mutant; lane 4, Arg^{112}-Gln mutant.

teins with a molecular mass of 45 000 were overexpressed and the levels were fairly equivalent with those of the wild type. Judging from the CO-difference spectra with the cell extracts, however, the amount of heme-containing Arg^{112}-Gln and Arg^{112}-Glu was 36% and 11% of that of the wild type $P\text{-}450_{cam}$, respectively. Using cell extracts, specific activities of the camphor-dependent NADH oxidation reaction based on the heme content of these mutant enzymes were less than 2% of that of the wild type (data not shown). We purified these $P\text{-}450_{cam}$ proteins by monitoring the absorbance at 392 nm, the Soret peak of the substrate-bound oxidized form of $P\text{-}450_{cam}$. The final fractions of $P\text{-}450_{cam}$ proteins were essentially homogeneous, as determined by analysis on SDS-PAGE (Fig. 2). The specific heme content of the preparations was 20.6, 12.9 and 6.0 nmol/mg protein for the wild type, Arg^{112}-Gln and Arg^{112}-Glu, respectively. Although the value for the wild type was as expected, those for the mutant

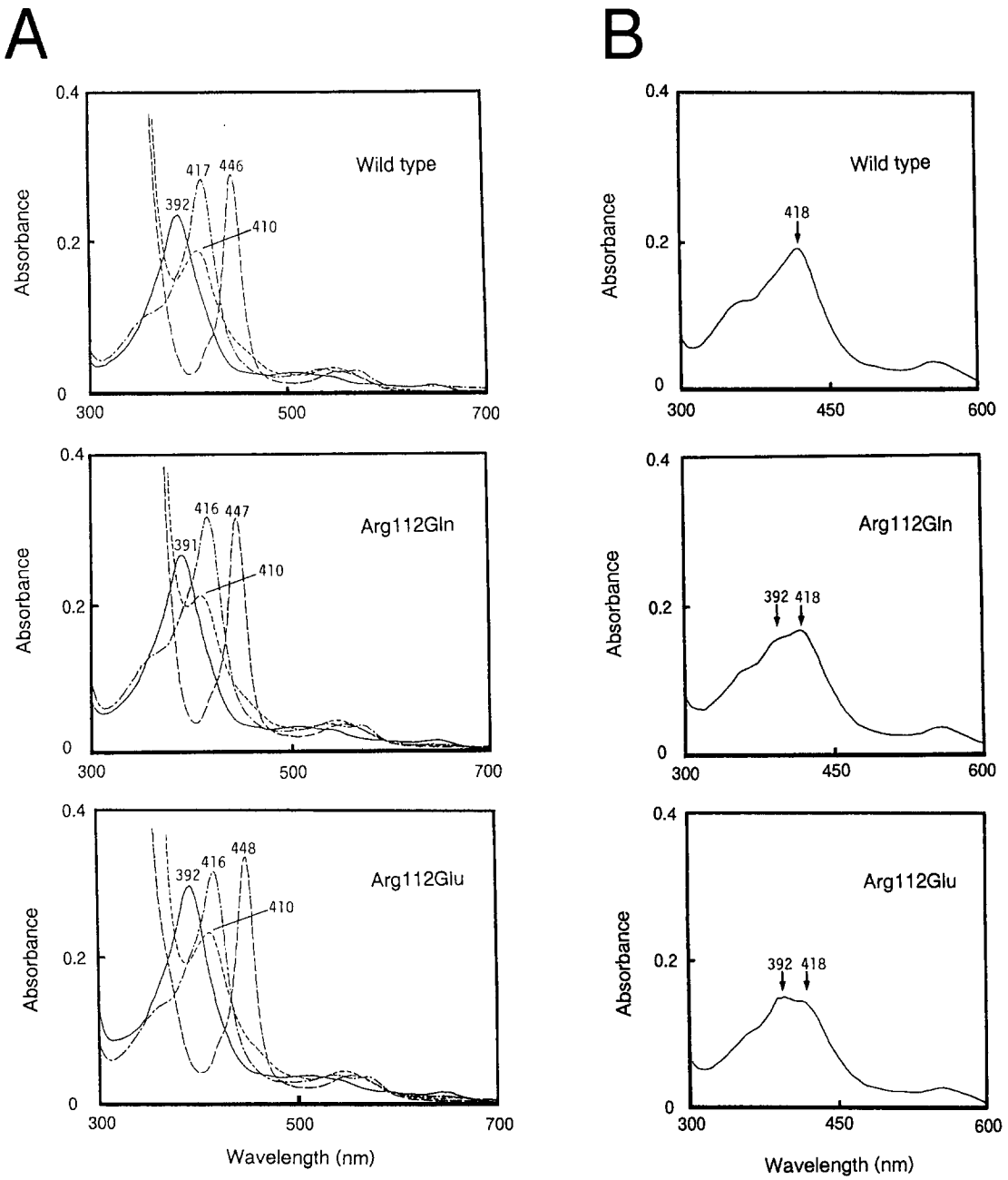

Fig. 3. Absorption spectra of wild-type and Arg-112 mutant $P\text{-}450_{cam}$ proteins. (A): Spectra were obtained at pH 7.4 and 20°C in 20 mM potassium phosphate containing 100 mM KCl, 5% (v/v) glycerol and 1 mM *d*-camphor, except that the spectrum of the enzyme in the substrate-free ferric state (broken line with dot; peak at 417, 416 nm) was measured without *d*-camphor. Solid line (peak at 391, 392 nm), substrate-bound ferric form; dashed line (peak at 410 nm), ferrous form; broken line (peak at 446, 447, 448 nm), CO-ligated ferrous form. Concentration of the $P\text{-}450_{cam}$ was 2.5 μM, respectively. (B): Spectrum of the oxygen-bound form. Temperature was kept at 20°C for wild type and at 8°C for mutants. Other conditions were the same as those in (A).

enzymes were low, suggesting that the latter preparations contained apo-protein. The lower values in the mutant enzymes are probably due to their lack of stability during purification processes.

3.2. Comparison of spectral properties between the mutants and the wild-type P-450$_{cam}$ proteins

To obtain information on environmental conditions around the heme molecule in the purified P-450$_{cam}$ proteins, we measured the absolute spectrum corresponding to each step in the reaction cycle. All the spectra of oxidized, substrate-bound oxidized, substrate-bound reduced, and CO-bound reduced forms were similar between the wild type and the mutants (Fig. 3A). Hence, there is probably no drastic change in the structure of the mutant enzymes. At 20°C, we observed a typical spectrum of the oxygen-bound form with a peak at 418 nm for the wild-type P-450$_{cam}$ but not in the mutant enzymes. At 8°C, Arg^{112}-Gln and Arg^{112}-Glu showed a small peak and a shoulder at 418 nm, respectively, with a shoulder at 392 nm suggesting the coexistence of the substrate-bound oxidized form (Fig. 3B). This is probably due to a lack of stability of the oxygen-bound form of the mutant enzymes and auto-oxidation of the mutant enzymes leading to the substrate-bound oxidized forms.

3.3. Comparison of enzymic properties between the mutants and the wild-type P-450$_{cam}$ proteins

Using the reconstituted system, we measured the camphor-dependent NADH oxidation activities of the purified enzymes. The mutant enzymes showed little activity (less than 1% of that of the wild type, data not shown), indicating that at least one of the steps in the reaction cycle of P-450$_{cam}$ is defective in the mutant enzymes. To elucidate the interaction of P-450$_{cam}$ proteins and d-camphor, the first step of the reaction cycle, we measured the dissociation constants by analysis of the substrate binding spectrum. The results showed that the affinities of the mutant enzymes for the substrate was similar to that for the wild-type enzyme (Table 1). The concentration of d-camphor in the standard assay mixture for camphor-dependent NADH oxidation reaction was 400 μM, a value much higher than the K_d value, indicating that the lower activity of the mutant enzymes cannot be explained by lower affinities for the substrate.

Table 1
Spectroscopic dissociation constants for the binding of the P-450$_{cam}$ proteins to d-camphor. Assay was carried out as described in Section 2

P-450$_{cam}$	K_d ±S.D.(μM)
Wild-type	2.4 ± 0.06
Arg^{112}-Gln	1.9 ± 0.04
Arg^{112}-Glu	1.8 ± 0.05

Next, we examined the efficiency of the P-450$_{cam}$ proteins at the second step of the reaction cycle, transfer of the first electron from the reduced putidaredoxin to the substrate-bound oxidized P-450$_{cam}$. The efficiency of P-450$_{cam}$ reduction by the reconstituted system was compared with that by sodium dithionite. When CO gas is dissolved in the reaction mixture, the reduced P-450$_{cam}$ which received the first electron from putidaredoxin immediately produces the stable CO-bound reduced form with an absorbance maximum at 446 nm. We reduced Arg^{112}-Gln and Arg^{112}-Glu using sodium dithionite or the reconstituted system with 1.5 or 15 μM putidaredoxin, and

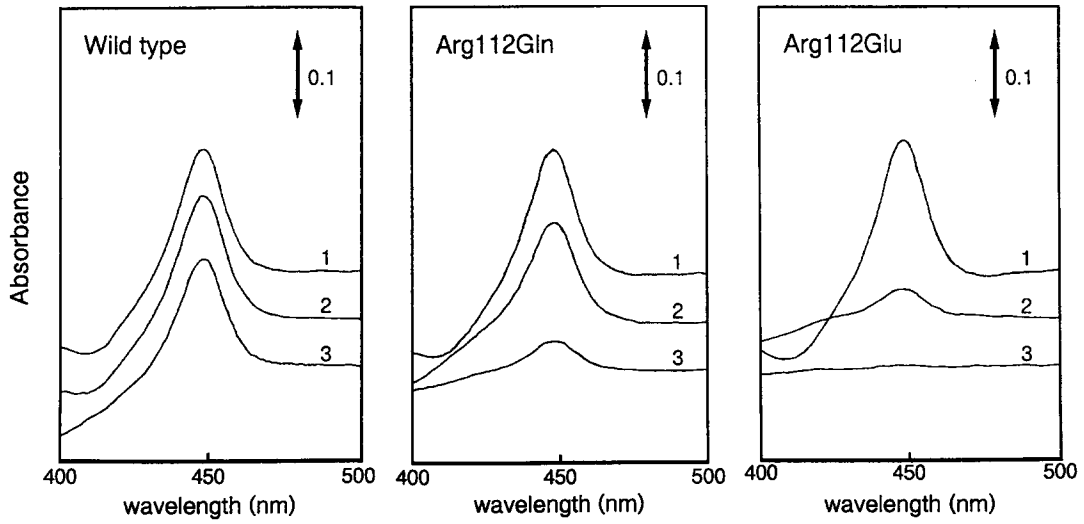

Fig. 4. Low efficiency of reduction of Arg-112 mutants by putidaredoxin. Wild-type and Arg-112 mutant P-450$_{cam}$ proteins (1.5 μM each) were reduced by sodium dithionite (line 1) or the reconstituted system, then the CO-difference spectrum was measured immediately. The reconstituted system contained 1.0 μM putidaredoxin reductase, 15 μM putidaredoxin (line 2) or 1.5 μM putidaredoxin (line 3), 300 μM d-camphor and 250 μM NADH.

measured the CO-difference spectra, the results being shown in Fig. 4. The intensity of the CO-difference spectra of the mutant P-450_{cam} proteins reduced by the reconstituted system with 1.5 μM putidaredoxin was much less than in the case of reduction by sodium dithionite. When the concentration of putidaredoxin was increased to 15 μM, the intensity of the CO-difference spectrum of Arg^{112}-Gln increased to the level seen in case of reduction by sodium dithionite, whereas that of Arg^{112}-Glu showed the level of Arg^{112}-Gln reduced by the system reconstituted with 1.5 μM putidaredoxin. The peak of the Soret band by the wild-type enzyme was seen at 396 nm just after the reduction, the peak shifted from 396 nm to 411 nm after 7 min of the reduction and remained there for 10 min. On the contrary, in the case of Arg^{112}-Gln and Arg^{112}-Glu, the peak was seen at 392 nm and 393 nm, respectively, and did not change for 10 min (data not shown). Thus, these P-450_{cam} mutants remained in the substrate-bound oxidized forms. It would appear that the affinities of Arg^{112}-Gln and Arg^{112}-Glu for putidaredoxin were less than that of the wild-type enzyme and that the transfer efficiency of the first electron from putidaredoxin to Arg^{112}-Glu seems to be less than that to Arg^{112}-Gln.

Heme iron of P-450_{cam} changes from the high spin to the low spin form by adding oxidized putidaredoxin in the presence of camphor [29]. Hintz et al. noted that the value of the association constant of P-450_{cam} to putidaredoxin could be determined from the extrapolated intercept of a double reciprocal plot of $1/\Delta A$ against $1/[Pd]$, using the putidaredoxin-induced change in the absorbance spectrum [26]. A similar phenomenon occurred on cytochrome P-450_{scc} with adrenodoxin [30,31]. We reconfirmed that the decrease of absorbance peak at 392 nm in the spectrum of substrate-bound oxidized P-450_{cam} coupled with the addition of oxidized putidaredoxin, and we determined the dissociation constants of the wild-type and mutant P-450_{cam} proteins for oxidized putidaredoxin in the presence of d-camphor (Fig. 5). The K_d value was 28, 240 and 530 μM for the wild type, Arg^{112}-Gln and Arg^{112}-Glu, respectively. This indicates that the positive charge of Arg-112 is probably important for the affinity of P-450_{cam} to putidaredoxin.

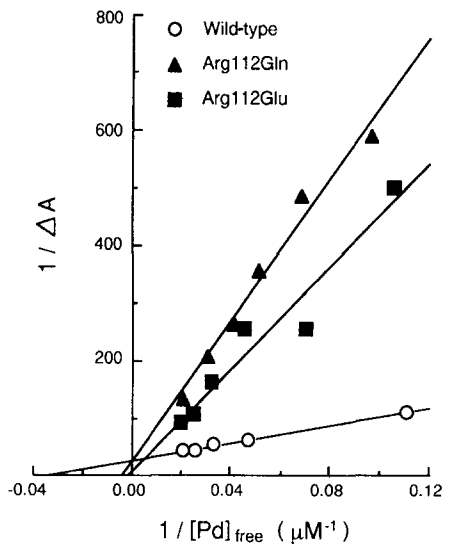

Fig. 5. Double reciprocal plots of changes in absorbance spectra for the titration of oxidized P-450_{cam} proteins by oxidized putidaredoxin. Spectra were obtained at pH 7.4 and room temperature in 20 mM potassium phosphate containing 1 mM d-camphor. In the sample cuvette, oxidized P-450_{cam} protein (2.0 μM each) was also present. Oxidized putidaredoxin in a range of 0–100 μM was added to both the reference and the sample cuvettes and the change in absorbance at 392 nm was measured. The value of the dissociation constant was determined from the extrapolated intercept of the plot.

Table 2
First-order rate constants for the reduction of cytochromes P-450_{cam}. The assay conditions are described in Section 2

P-450_{cam}	k_{obs} (s^{-1})
Wild-type	45.5
Arg^{112}-Gln	$9.0 \cdot 10^{-3}$
Arg^{112}-Glu	$9.0 \cdot 10^{-4}$

The results described above suggest that the efficiency of transfer of the first electron from putidaredoxin was decreased in the mutant enzymes. We measured the rate constants of the reduction reaction of the P-450_{cam} proteins using the stopped-flow method. As shown in Table 2, the rate constant of the reduction by putidaredoxin for Arg^{112}-Gln and Arg^{112}-Glu was 1/5000 and 1/50 000 of that for the wild type, respectively. The results in Fig. 5 and Table 2 strongly suggest that the positive charge of Arg-112 greatly contributes to the interaction of P-450_{cam} with putidaredoxin and the electron transfer reaction between them.

4. Discussion

The amount of holoenzymes of Arg^{112}-Gln and Arg^{112}-Glu expressed in *E. coli* was much less than that of the wild-type enzyme and the remaining activity of the wild type, Arg^{112}-Gln and Arg^{112}-Glu after heat treatment at 50°C for 120 min was 70, 40 and 13%, respectively, indicating that these mutant P-450_{cam} proteins are more heat-labile than the wild-type enzyme. Analyses of the absolute spectrum revealed that the oxygen-bound forms of mutant enzymes were less stable than that of the wild-type enzyme. Poulos et al. reported that Arg-112 together with Gln-108 and His-355 forms hydrogen bonding with the heme-propionate and that this bonding contributes to stability of the heme in the enzyme molecule [12]. When this hydrogen bonding is lost by replacement of Arg-112 by Gln or Glu, release of the heme from the protein might readily occur or binding of the heme origi-

nally might not occur so readily. Structural change around the heme by size change of the residue or by the loss of the hydrogen bonding might also increase the rate of auto-oxidation reaction in the mutant P-450$_{cam}$ proteins. In our previous study, the auto-oxidation rate of Arg^{112}-Cys was 4.5-times larger than that of the wild type [13]. It is likely that the auto-oxidation rates of Arg^{112}-Gln and Arg^{112}-Glu are also faster than that of the wild type, and we found that the oxygen-bound form of the mutant proteins was unstable. Although we did not measure oxygen consumption, reduction of the oxidized forms of the mutant P-450$_{cam}$ proteins would yield superoxide anion and would become the substrate-bound oxidized form.

The intensities of CO-difference spectrum in the reconstituted system and the dissociation constants with putidaredoxin revealed that the affinity of P-450$_{cam}$ proteins to putidaredoxin was decreased, in the following manner; Arg (wild type) $\gg$ Gln $>$ Glu, at the residue-112, respectively. The rate constants of the reduction of P-450$_{cam}$ by reduced putidaredoxin showed that reduction efficiencies were also decreased in the same manner, namely, k_{obs} of the P-450$_{cam}$ reduction by reduced putidaredoxin was 1/5000 and 1/50000 to that of the wild-type enzyme in Arg^{112}-Gln and Arg^{112}-Glu, respectively. These results mean that Arg-112 plays a very important role in electron transfer from putidaredoxin, and the positive charge of this residue seems to be important. We observed camphor-dependent NADH oxidation in the reconstituted system with Arg^{112}-Gln or Arg^{112}-Glu mutant enzyme, although the values were very small. The apparent NADH consumption may be due to a leak of the reducing equivalent from putidaredoxin to oxygen.

When the three-dimensional structure of P-450$_{cam}$ protein is observed from the heme-proximal side, a number of basic amino-acid residues form a circle around the heme. Arg-112, on which we focused our attention in this study, Arg-72, Lys-344 and Arg-364 are such basic amino-acid residues. Stayton and Sligar analyzed Arg-72 and Lys-344 by site-directed mutagenesis [32] and their results are consistent with ours in showing that these basic residues relate to the interaction with putidaredoxin, albeit the effect of the mutants being small. These basic residues probably form a cationic environment around the region near the surface of the enzyme, and contribute for the interaction with putidaredoxin, an acidic protein. However, the K_m value for putidaredoxin in the reconstituted system with Arg^{72}-Gln, Lys^{344}-Gln or Lys^{344}-Glu mutant P-450$_{cam}$ was only 20–60% larger than that of the wild- type enzyme [32]. Therefore, it is likely that, among a number of basic residues, Arg-112 mainly contributes to the electron transfer from putidaredoxin. In conclusion, the hydrogen-bonding of -NH_2 (η_1) of the guanidino-group of Arg-112 to the oxygen atom of the carboxylic acid group of the heme propionate [12] is important not only for stable maintenance of the heme in the P-450$_{cam}$ but also for the electron transfer from putidaredoxin. Presumably -NH_2 (η_2) and $=NH$ (ε) of the guanidino-group of Arg-112 located on the surface of P-450$_{cam}$ are also important for the electron transfer from putidaredoxin.

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