Role of \textsuperscript{112}Arg of Cytochrome P450\textsubscript{cam} in the Electron Transfer from Reduced Putidaredoxin

ANALYSES WITH SITE-DIRECTED MUTANTS*

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The mechanism for the reduction of ferric cytochrome P450\textsubscript{cam}, by reduced putidaredoxin, the physiological electron donor for the cytochrome, has been studied by using site-directed mutants of cytochrome P450\textsubscript{cam} in which Arg\textsubscript{112}, an amino acid residue at the presumed binding site for putidaredoxin, was changed to several other amino acid residues. The affinity of reduced putidaredoxin for ferric cytochrome P450\textsubscript{cam} to form a dимер complex was decreased greatly by changing Arg\textsubscript{112} to a neutral amino acid such as Cys, Met, or Tyr. The rate of intracomplex electron transfer from putidaredoxin to cytochrome P450\textsubscript{cam} also diminished upon replacing the basic residue with neutral ones, being 42, 18, 4.0, 1.3, and 0.16 s\textsuperscript{-1} for Arg (wild type), Lys, Cys, Met, and Tyr enzymes, respectively. Furthermore, the oxidation-reduction potential of cytochrome P450\textsubscript{cam} (Fe\textsuperscript{3+}/Fe\textsuperscript{2+} couple) decreased in a similar way to the decrease in the rate of electron transfer upon amino acid substitution; the values were –138, –162, –182, –200, and –195 mV for Arg (wild type), Lys, Cys, Met, and Tyr enzymes, respectively. These results indicate that the amino acid substitution at position 112 affects the oxidation-reduction potential of the heme iron in cytochrome P450\textsubscript{cam} thereby diminishing the rate of electron transfer between the two metal centers. The rate of electron transfer from putidaredoxin to oxyferrous cytochrome P450\textsubscript{cam}, also diminished upon substitution of Arg\textsubscript{112} with a neutral amino acid.

Cytochrome P450 is a generic name given to a family of protoheme-proteins that metabolize a wide variety of natural and unnatural substances such as steroids, fatty acids, hydrocarbons, and xenobiotics. Among them, cytochrome P450\textsubscript{cam} (P450\textsubscript{cam})\textsuperscript{3} of Pseudomonas putida (CYP101) has been the focus of intense mechanistic studies; its three-dimensional structure has been solved at 1.63-Å resolution by Poulos and co-workers (1, 2), allowing us to make detailed analyses of the structure-function relationship. It catalyzes the hydroxylation of d-camphor to give 5-exo-hydroxycamphor as in the following scheme.

\[
d-\text{Camphor} + \text{NADH} + \text{H}^+ + \text{O}_2 \rightarrow 5-\text{exo-hydroxycamphor} + \text{NAD}^+ + \text{H}_2\text{O}
\]

The monoxygenation reaction requires, in addition to d-camphor and molecular oxygen, two reducing equivalents, which are transferred from NADH to P450\textsubscript{cam} through a specific electron transfer system composed of NADH-putidaredoxin reductase (PdR), a flavoprotein, and putidaredoxin (Pd), an iron-sulfur (Fe\textsubscript{S}\textsubscript{4}) protein. In the reaction, Pd receives electrons from PdR and transfers them to P450\textsubscript{cam}; Pd serves as the direct electron donor for P450\textsubscript{cam}.

When the mechanism for the electron transfer reaction between Pd and P450\textsubscript{cam} was examined, reduced Pd and ferric P450\textsubscript{cam} molecules were found to associate rapidly to form a bimolecular complex, followed by an intracomplex electron transfer giving ferrous P450\textsubscript{cam} and oxidized Pd (3–5). Then, on the basis of results obtained from computer modeling of P450\textsubscript{cam}-cytochrome b\textsubscript{5}, the binding site of Pd on P450\textsubscript{cam} was suggested (6) to be in the proximal surface of P450\textsubscript{cam} which contains four basic amino acid residues (Arg\textsubscript{72}, Arg\textsubscript{112}, Lys\textsubscript{344}, and Arg\textsubscript{864}). Stayton and Sligar (7) showed that neutralization of a positive charge at Arg\textsubscript{112} or Lys\textsubscript{344} by site-directed mutagenesis resulted in a decrease in the binding affinity of P450\textsubscript{cam} to Pd, although the changes in the values observed were rather small (1.5-fold at the maximum). More recently, however, Koga et al. (8) as well as Nakamura et al. (9) demonstrated that charge neutralization at Arg\textsubscript{112}, another basic amino acid among the four basic residues, evoked a dramatic decrease in the catalytic activity of P450\textsubscript{cam}. Catalytic activities of Cys, Glu, and Gln mutants were less than 1% of that of the wild-type enzyme in a reconstituted assay system composed of NADH, PdR, Pd, and a mutant P450\textsubscript{cam}. Thus, the presumed binding site in the proximal surface could be the real site for the binding of Pd to P450\textsubscript{cam} at least in part.

In the present study, we further elucidate the role of Arg\textsubscript{112} in the electron transfer reaction from reduced Pd to ferric P450\textsubscript{cam} by using site-directed mutants of P450\textsubscript{cam} in which the Arg residue was changed to a Lys, Cys, Tyr, or Met residue. Since they have different charges and hydrogen-bonding capacities with one another, we had hoped that their use might give us a clue to identify the role of the basic residue Arg\textsubscript{112} in the Pd-P450\textsubscript{cam} interaction. The results revealed that the oxidation and reduction (redox) potential of the heme iron in P450\textsubscript{cam} was...
greatly affected by the properties of an amino acid at 112-position and that such changes in the redox potential were reflected in the rates of electron transfer between the two metal centers, i.e. the heme iron in P450<sub>cam</sub> and nonheme iron in Pd. Possible mechanisms through which an amino acid substitution affects the redox potential are discussed. Finally, the cationic charge at Arg<sub>112</sub> was found to be important also in the second electron transfer, i.e. in the reduction of the ferrous oxygenated form of P450<sub>cam</sub> (oxy-P450<sub>cam</sub>) by reduced Pd.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparations and Catalytic Activity**—The wild-type P450<sub>cam</sub> and its mutants, prepared by the method of Kramer et al. (10), were expressed in Escherichia coli strain JM109 and purified with the procedures described previously (11). Purified preparations with an RZ value (A392/A280) greater than 1.45 were employed in this study. Pd and PdR were expressed also in E. coli strain JM109 and were purified to a homogeneous state according to the methods described by Gunsalus and Wagner (12). Monooxygenase activity of P450<sub>cam</sub> was measured in a reconstituted assay system containing 1 mM d-camphor, 360 μM NADH, 50 mM KCl, 14 μM Pd, 0.12 μM PdR, and an appropriate amount of P450<sub>cam</sub>, in 50 mM potassium phosphate buffer, pH 7.4, under normal atmospheric conditions at 20°C. The rate of reaction was determined by measuring both oxygen consumption and NADH oxidation rates simultaneously in a special cuvette described elsewhere (13). In most cases, the ratio of the two rates was 1:1, indicating that O<sub>2</sub> was consumed by a two-electron reduction process. When necessary, enzyme activity was determined by measuring the hydroxylated product of d-camphor (11). Only 5-endo-hydroxycamphor was detected as the hydroxylated product of d-camphor throughout this study.

**Spectrophotometry and Stopped Flow Experiments**—All spectrophotometric measurements were carried out in 50 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl and 1 mM d-camphor, hereafter called the standard buffer. Optical absorption spectra of the proteins were recorded with a Shimadzu spectrophotometer, model MPS-2000 (Kyoto, Japan). CD spectra of the enzymes were measured from 190 to 250 nm with an automatic spectropolarimeter (Tokyo, Japan) at an ambient temperature. The buffer used for the CD measurements was 25 mM potassium phosphate buffer, pH 7.4, containing 25 mM KCl and 0.5 mM d-camphor.

The rate of reduction of ferric P450<sub>cam</sub> by reduced Pd, i.e., the first electron transfer, was measured with a UNISOKU stopped flow spectrophotometer, model RSP-601 (Osaka, Japan). Usually, one reservoir of the stopped flow apparatus contained 2 mM P450<sub>cam</sub> in the standard buffer under anaerobic conditions; the other contained 3-54 μM reduced Pd in the standard buffer equilibrated with 1.0 atmosphere CO. The reaction was started by mixing the two solutions and followed by measuring the formation of the ferrous CO form of P450<sub>cam</sub> at 446 nm (4). Changes in absorbance were digitized on a Yokogawa DL1200 oscilloscope (Tokyo, Japan). Reduced Pd was prepared by the addition of 360 μM NADH and a catalytic amount of PdR (about 0.1 μM) to the solution of oxidized Pd under anaerobic conditions. An oxygen-scavenging system composed of glucose (60 mM), glucose oxidase (0.1 mg/ml; Sigma), and catalase (3000 units in 1 mM; Sigma) was included in the medium to ensure the anaerobiosis (4, 5). All measurements were done at 20°C.

Reduction of oxy-P450<sub>cam</sub>, by reduced Pd, i.e., the second electron transfer, was assessed by following the spectral changes of oxy-P450<sub>cam</sub> (380-540 nm) after mixing the oxy form (5 μM) with reduced Pd (18 μM) in the stopped flow apparatus at 4°C. Oxy-P450<sub>cam</sub> was prepared by mixing the ferrous P450<sub>cam</sub> solution with an equal amount of O<sub>2</sub>-saturated buffer at 4°C and was immediately transferred to a reservoir of the stopped flow apparatus. The ferrous P450<sub>cam</sub> was prepared by adding a minimum volume of 20 mM sodium dithionite solution to ferric P450<sub>cam</sub> under anaerobic conditions. The reduced Pd was prepared as described above. The buffer system employed in these experiments was the same as that used in the measurements of the first electron transfer rate.

**Kinetic Modeling of the First Electron Transfer**—The reduction of ferric P450<sub>cam</sub> by reduced Pd has been shown to be represented by a two-step model presented in Equation 1 (4, 14).

\[
\begin{align*}
-\frac{d[A]}{dt} &= -k_1 [B] - k_{-1} [C] \\
-\frac{d[B]}{dt} &= k_2 [A] - k_{-2} [C] \\
-\frac{d[C]}{dt} &= k_3 [A] - k_{-3} [B] \\
-\frac{d[D]}{dt} &= k_{5} [C] - k_{-5} [D]
\end{align*}
\]

(Eq. 2)

In the present experiment, the associating species A and B correspond to ferric P450<sub>cam</sub> and reduced Pd, respectively, and the species C is an equimolar complex of ferric P450<sub>cam</sub> and reduced Pd. Then D represents the product of the reaction, i.e., oxidized Pd-ferrous P450<sub>cam</sub> complex. The complex further dissociates into oxidized Pd and ferrous P450<sub>cam</sub>, but this reaction is not considered here; the ferrous P450<sub>cam</sub> formed is immediately trapped as its CO adduct in the presence of an excess amount of CO either in the complexed or dissociated state (3, 4). Thus, the formation of the product D could be monitored by measuring the amount of the ferrous CO complex of P450<sub>cam</sub> at 446 nm. Under such conditions, the dissociation of the CO-ferrous adduct is very slow, and hence we can assume $k_5 = 0$ for a kinetic modeling.

The differential equations of Equations 2 and 3 were iteratively solved with a digital computer by using the fourth-order Runge-Kutta numerical method at 2 μs intervals. The initial concentrations of P450<sub>cam</sub> and Pd were determined by measuring absorption spectra of both proteins, and parameters $k_1$, $k_2$, and $k_3$ were set variable to obtain the best fit to the experimental data. The dissociation constant of reduced Pd-ferric P450<sub>cam</sub> complex can be obtained by dividing $k_{-1}$ by $k_2$.

Oxidation and Reduction Potentials—The redox potential of P450<sub>cam</sub> was measured under anaerobic conditions by the method of Makino et al. (15) with minor modifications. In brief, the reaction mixture for the measurements contained about 46 μM of the wild-type or a mutant P450<sub>cam</sub>, and the following components in 50 mM potassium phosphate buffer, pH 7.4: 50 mM KCl, 500 μM d-camphor, protoporphyrin IX, EDTA, α-hydroxyphenazine, pyocyanine, phenosafranine, and safranine T. Ferric P450<sub>cam</sub> was reduced by illuminating the sample with white tungsten light (150 W) under anaerobic conditions at 20°C. A micro-combination electrode from Ingold (Pt-4800-M5), which was calibrated by measuring the potential of phenosafranine, was used to monitor the potential of the Fe<sup>2+</sup>/Fe<sup>3+</sup> couple in P450<sub>cam</sub>.

**RESULTS**

Spectra and Activities of P450<sub>cam</sub> Mutants—Fig. 1 shows absorption spectra of ferric, ferrous, and ferrous CO forms of
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Table I

| Protein | O\textsubscript{2} consumption rate | Product/O\textsubscript{2} consumed | Redox potential (E\textsubscript{m} versus NHE) |
|---------|----------------------------------|----------------------------------|----------------------------------|
| Arg^{112} | 1300 | 100 | 1 | -138 |
| Lys^{112} | 200 | 88 | 12 | -162 |
| Cys^{112} | 1.3 | 69 | 16 | -182 |
| Met^{112} | 1.7 | 86 | 15 | -200 |
| Tyr^{112} | 0.8 | 50 | 30 | -195 |

Met^{112} mutant of P450 cam in the presence of 1 mM d-camphor. The spectra were almost indistinguishable from those of the wild-type enzyme (11, 12) and also from those of other 112-position mutants such as Lys, Cys, and Tyr enzymes; the peak positions of the enzymes agreed well within plus or minus 0.5 nm, and their extinction coefficients were not greatly different from one another. CD spectra (190–250 nm) of the mutant enzymes were also indistinguishable from that of the wild-type enzyme (data not shown). These results indicate that the mutation at position 112 had little influence on the structure of heme and its vicinity of P450 cam as well as on the polypeptide backbone folding. The finding was in conformity with the results of a computer simulation study on the structure of the Cys mutant described by Koga et al. (8), where little conformational change was expected as compared with that of the wild-type enzyme.

The catalytic activity of the enzyme was dramatically affected by these mutations. As shown in Table I, the rates of oxygen consumption by Cys, Met, and Tyr mutants were 1/1,000, 1/764, and 1/1,625 of that by the wild-type (Arg) enzyme, respectively. Meanwhile, the replacement of Arg by Lys, which has a cationic charge, did not result in such a big decrease in the rate of oxygen consumption; the oxygen consumption by the Lys mutant was reduced to less than 0.01% of that by wild-type enzyme.

Effects of Mutation on the First Electron Transfer—Fig. 2A and B show time courses of the reaction, in which 1 μM ferric Lys mutant of P450 cam was reduced to a ferrous state by either 15 or 27 μM of reduced Pd. The open circles represent experimental data points, and the lines through the points are simulated curves obtained by assuming the mechanism in Equation 1. Both the experimental and simulated curves fitted well to a single-exponential curve in accordance with that for the wild-type P450 cam (4). Here we show only the time courses for the Lys mutant, but the reaction of all other P450 cam with reduced Pd proceeded in a similar way; they apparently followed first-order kinetics. Then we plotted apparent first-order rate constants (k\textsubscript{obs}) as a function of reduced Pd concentration (Fig. 3). As seen, all mutant enzymes exhibited significantly smaller k\textsubscript{obs} values than that of the wild-type enzyme, and changes in k\textsubscript{obs} reached to a plateau at high concentrations of Pd. Such a nonlinear behavior is consistent with a two-step mechanism in Equation 1 consisting of a rapid diprotein complex formation (second-order reaction) and following intracomplex electron transfer (first-order reaction), where the latter becomes the rate-limiting step at higher concentrations of Pd.

To determine the rate constants, k\textsubscript{1}, k\textsubscript{2}, and k\textsubscript{3} in Equation 1, we performed two-step model fitting onto the time course data obtained for each P450 cam species. The best-fit values thus obtained for the rate constants in the reaction of each P450 cam species at 20 °C were summarized in Table II, and the time course curves were simulated using such values for the Lys mutant as have been illustrated already in Fig. 2, (A and B) as examples. The results revealed that both the dissociation constant K\textsubscript{d} and the electron transfer rate k\textsubscript{1} for Cys, Met, and Tyr mutants were 500–2500-fold and 1/10–1/200 that of the wild-type enzyme, respectively. Thus, the charge neutralization at position 112 caused significant decreases in the affinity of reduced Pd for ferric P450 cam together with the decreases in the rate of intracomplex electron transfer. On the other hand, K\textsubscript{d} and k\textsubscript{2} of the Lys mutant were not greatly different from those of the wild-type enzyme, indicating that the presence of a positive...
The reaction employing Pd concentration below 2 μM, which is the concentration after mixing on the stopped flow apparatus, was slow to observe whole kinetic traces by the data acquisition system of our instruments. Thus, the kinetic traces at Pd concentrations higher than 2 μM were employed for the analysis.

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Effects of Mutation on the Second Electron Transfer—The reduction of oxy-P450_{cam}, a ternary complex of oxygen and d-camphor bound to ferric P450_{cam}, is a slow process. Subsequent release of the hydroxycamphor from the enzyme was followed by the binding of another d-camphor molecule to regenerate the d-camphor-bound ferric P450_{cam}. The rate of second electron transfer was therefore assessed by monitoring the formation of ferric P450_{cam} after mixing oxy-P450_{cam}, with reduced Pd. Fig. 5A shows changes in absorbance at 404 nm due to conversion of the oxy form of the wild-type P450_{cam} to the ferric form as a function of time. Since 404 nm is an isosbestic point of substrate-free and bound forms of ferric enzyme, changes in absorbance at this wavelength can represent changes in both substrate-free and bound forms of enzyme.

Table II

Best fit parameters for the electron transfer reaction from reduced putidaredoxin to various ferric cytochromes P450_{cam}. The parameters were obtained by fitting Equations 2 and 3 (see "Experimental Procedures") to the observed kinetic traces as described in the text. The values of the dissociation constant were calculated to the observed kinetic traces as described in the text. The values of the dissociation constant were calculated

\[
K_d = k_f/k_i
\]

charge at 112-position stabilizes the P450_{cam}-Pd complex and promotes an efficient intracomplex electron transfer. The values obtained for the wild-type enzyme were in good agreement with the values reported previously by Pederson et al. (3) and Hintz et al. (17).

Effects of Mutation on the Redox Potential—Ferric forms of all P450_{cam} species employed in this study showed a typical behavior of a one-electron carrier during the reductive titration in the presence of 1 mM d-camphor. Midpoint potentials (E_m in mV) of five P450_{cam} species thus obtained are also listed in Table I. The value of E_m = −138 mV for the wild-type P450_{cam} was lowered to −162, −182, −200, and −195 mV upon substitution of Lys, Cys, Met, and Tyr for Arg, respectively. It should be noted that the values for the latter three mutants are close

to the value of −215 mV, the midpoint potential of their electron donor, Pd. Then the decrease in E_m (24–62 mV) appeared not correlative with a single property of amino acid such as volume, length, and charge of the side chain, but with their combinations.

Redox Potential and Electron Transfer Rate (k_2)—Fig. 4 shows a plot of intracomplex electron transfer (k_2) against the changes in the redox potential of P450_{cam}. In addition to the data obtained in the present study (closed circle), those from a study by Fisher and Sligar (18) who examined changes in the redox potential of the wild-type enzyme upon combination with various substrate analogs (open circle) were employed in this plot. A trend is clearly seen; the decrease in the redox potential accompanies the decrease in the electron transfer rate, which is expressed in a logarithmic scale. On the basis of these observations and theoretical considerations previously reported (19), we have concluded that the decrease in the rate of intracomplex electron transfer observed in this study is mainly due to the lowered redox potential of the heme iron.
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and the oxy form of the Lys enzyme showed a similar but slower decay of the oxy form (t\textsubscript{1/2} = 130 ms) as compared with that of the wild-type enzyme.

A dramatic effect on the kinetics was found when Arg\textsuperscript{112} was replaced with Cys, Met, or Tyr. The trace with the Tyr mutant was shown in Fig. 5B, where changes in absorption at 404 nm were not detected even at 400 ms after the mixing. The situation was the same for the Cys and Met enzymes. These results clearly indicated that the removal of a positive charge at position 112 reduced the rate of electron transfer from reduced Pd to oxy-P450\textsubscript{cam}. The dotted line through the points represents a single-exponential fit of the data.

Fig. 5. Stopped flow analysis of the reaction between oxy-P450\textsubscript{cam} and reduced Pd. The reaction was followed by monitoring the changes in absorbance at 404 nm of P450\textsubscript{cam}, after mixing oxy-P450\textsubscript{cam} with reduced Pd. Panel A presents the data for the wild-type P450\textsubscript{cam}, and panel B shows the data for the Tyr\textsuperscript{112} mutant. Final concentrations after mixing in the stopped flow apparatus were as follows: oxy-P450\textsubscript{cam}, 2.5 μM; reduced Pd, 9.0 μM. The reaction was performed at 4 °C in the standard buffer system, pH 7.4, containing 1 mM d-camphor. The dotted line through the points represents a single-exponential fit of the data.

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**DISCUSSION**

We found in this study that charge neutralization at Arg\textsuperscript{112}, a surface amino acid residue of P450\textsubscript{cam}, affected the redox potential of the heme iron, which is buried in the interior of the protein; the redox potential of the wild-type P450\textsubscript{cam} was lowered to −182 to −200 mV in the mutant enzymes having a neutral amino acid residue such as Cys, Met, or Tyr at the 112-position. It should be noted that the redox potential of Pd, the electron donor for P450\textsubscript{cam}, is −215 mV under comparable conditions (15). The mutation also affected the rate of electron transfer from reduced Pd to either ferric or oxyferrous P450\textsubscript{cam} and hence overall catalytic activity. It has been shown that the electron transfer process, especially that to oxy-P450\textsubscript{cam}, is the rate-limiting step in the overall catalytic reaction catalyzed by P450\textsubscript{cam}. The effect of amino acid substitution on both electron transfer and catalytic rates can be regarded as the secondary to the effect on the redox potential, since redox potential has been known to be an important determinant of the rate of outer sphere electron transfer between two redox centers (19, 20). A question thus arises as to the mechanisms by which mutation causes the changes in the redox potential.

First of all, an electrostatic interaction of the redox centers with charged groups of the protein must be considered (21). For example, Caffrey and Cusanovich (22) replaced some surface Lys residues of cytochrome c with negatively charged Asp or Glu and showed that the redox potentials (Fe\textsuperscript{3+}/Fe\textsuperscript{2+} couple) of the mutants decreased by 11–14 mV. The decrease in redox potential can be due to an increase in the stability of the ferric state as a result of interaction between the positively charged ferric heme iron and a more negative (less positive) electrostatic field of the protein. The second possibility is the changes in hydrogen-bonding interactions of the heme propionate with amino acid residues in the apoprotein moiety. In their crystallographic studies on P450\textsubscript{cam}, Poulos et al. (1) have shown that the guanidino group of Arg\textsuperscript{112} is hydrogen-bonded to the oxygen atom of 6-propionate of the iron protoporphyrin. Thus, the substitution of Arg\textsuperscript{112} with Lys, Tyr, Met, and Cys leads to the modification or elimination of the hydrogen-bonding interactions between the heme propionate and an amino acid residue at position 112. The interactions through the hydrogen bond will withdraw a negative charge from the propionate group, thereby destabilizing the positive charge on the heme iron. Hence, a weaker hydrogen bond or elimination of the hydrogen bond in the mutant enzymes reduces the degree of charge withdrawal from the propionate and results in the stabilization of the Fe\textsuperscript{3+} heme iron. Such an idea was supported by a recent study of Davies et al. (23) on cytochrome c. They stated that the loss of a single hydrogen bond resulted in a −20 mV drop in the redox potential.

Third, effects on the thiolate axial ligand must also be taken into consideration. Above mentioned propionate is also hydrogen-bonded to the imidazole nitrogen of His\textsuperscript{355}, which is the second from the thiolate axial ligand, Cys\textsuperscript{357}. Lys, Cys, Met, or Tyr incorporated to the 112-position in place of Arg possibly alters the electrostatic and/or conformational states of the charged carboxyl group of the heme propionate. Such changes occurring at the propionate side chain may induce more or less perturbation of the thiolate ligand through a hydrogen bond between the propionate and His\textsuperscript{355} and/or through peptide bonds linking this His and the axial ligand.

In addition to the changes in redox potential, we have also shown that the charge neutralization at the 112-position of P450\textsubscript{cam} reduces the affinity of ferric P450\textsubscript{cam} to Pd (Table I). Electrostatic forces have long been considered to contribute to the interaction between Pd and P450\textsubscript{cam}; positive charges on the surface of P450\textsubscript{cam} have been considered to interact with corresponding negative charges on Pd (4, 5, 6, 24). On the other hand, charge neutralization at the T2- and 344-positions resulted in only a slight increase in the affinity (1.5-fold at the maximum) (7). Furthermore, we recently found that substitution of Arg\textsuperscript{112} with Met perturbed the enzyme activity only sluggishly. Thus, the role of Arg\textsuperscript{112} in the P450\textsubscript{cam}–Pd interaction is unique among the surface amino acid residues so far examined. The reason for this uniqueness of Arg\textsuperscript{112} is unknown at present, but it is possible that only Arg\textsuperscript{112} can form a specific ion pair with an anionic surface residue of Pd, while the others do not. Since a number of negatively charged groups are located on the surface of the protein, including Asp\textsuperscript{9}, Asp\textsuperscript{34}, Asp\textsuperscript{38}, Glu\textsuperscript{77}, Asp\textsuperscript{109}, and Asp\textsuperscript{103} (25, 26), we suggest that one of these residues interact(s) electrostatically with Arg\textsuperscript{112} of P450\textsubscript{cam}.

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We have also demonstrated that the second electron transfer was greatly affected by the mutation at the 112-position of \( \text{P}450_{\text{cam}} \). In this connection, the following differences has been noted between the reduction of ferric \( \text{P}450_{\text{cam}} \) and that of oxy-\( \text{P}450_{\text{cam}} \), i.e. the first and the second reduction of \( \text{P}450_{\text{cam}} \) in the reaction cycle. Certain reductants such as spinach ferredoxin, adrenodoxin, and dithionite, which have lower redox potentials than Pd, can reduce ferric \( \text{P}450_{\text{cam}} \) but do not reduce oxy-\( \text{P}450_{\text{cam}} \), to give the hydroxylated product, d-camphor (27). Later, further studies done by Peterson and co-workers (4, 5) revealed that the effect of KCl upon the rates of electron transfer (\( k_{\text{obs}} \)) was different between the two processes; in the first electron transfer, KCl concentrations had no effect on \( k_{\text{obs}} \) until it reached about 0.5 M, above which \( k_{\text{obs}} \) declined with the increase in KCl concentration. For the second electron transfer, on the other hand, there was a large decrease in \( k_{\text{obs}} \) before KCl concentration reached to 0.5 M. Studies on the active site mutants of \( \text{P}450_{\text{cam}} \) by us (16, 28) and by Gerber and Sligar (29, 30) have also shown a dissimilarity between the two processes. In these studies, it has been demonstrated that the mutation of Asp\(^{251} \) to Gly, Ala, or Asn does not reduce the rate of the first process, but dramatically slows down the second reduction rate. While proton transfer also needs to be considered in the process, but dramatically slows down the second reduction.

In the latter process (28, 30, 31), the observation with the Asp251 to Gly, Ala, or Asn does not reduce the rate of the first process. However, our results in Table II and Fig. 5 have clearly shown that the replacement of Arg\(^{112} \) with a neutral amino acid (i.e. Met, Cys, or Tyr) inhibits not only the first electron transfer but also the second process. This implies that Arg\(^{112} \) also contributes to the reduction of oxyferrous \( \text{P}450_{\text{cam}} \). Further experiments are obviously necessary to solve these and other problems in the mechanism(s) of electron transfer reaction from Pd to \( \text{P}450_{\text{cam}} \).

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REFERENCES
1. Poulos, T. L., Finzel, B. C., and Howard, A. J. (1987) J. Mol. Biol. 215, 687–700.
2. Raag, R., and Poulos, T. L. (1989) Biochemistry 28, 7586–7592.
3. Pederson, T. C., Austin, R. H., and Gunsalus, I. C. (1977) in Micromes and Drug Oxidations (Ullrich, V., ed) pp. 275–283, Pergamon Press Ltd., Oxford.
4. Hinth, M. J., and Peterson, J. A. (1981) J. Biol. Chem. 256, 6721–6728.
5. Brewer, C. B., and Peterson, J. A. (1989) J. Biol. Chem. 264, 701–706.
6. Stayton, P. S., Poulos, T. L., and Sligar, S. G. (1989) Biochemistry 28, 8201–8205.
7. Stayton, P. S., and Sligar, S. G. (1990) Biochemistry 29, 7381–7386.
8. Koga, H., Sagara, Y., Yaa, T., Tsujimura, N., Nakamura, K., Sekimizu, K., Makino, R., Shimada, H., Ishimura, Y., Yura, K., Go, M., Ikeguchi, M., and Horiiuchi, T. (1993) FEBS Lett. 311, 109–113.
9. Nakamura, K., Horiuchi, T., Yasukochi, T., Sekimizu, K., Hara, T., and Sligar, Y. (1994) Biochim. Biophys. Acta 1207, 40–48.
10. Kramer, W., Drusta, V., and Poulos, T. L. (1991) Nucleic Acids Res. 19, 9411–9456.
11. Imai, M., Shimada, H., Watanabe, Y., Matsushita-Hibiya, Y., Makino, R., Koga, H., Horiuchi, T., and Ishimura, Y. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 7823–7827.
12. Gunsalus, I. C., and Wagner, G. C. (1978) Methods Enzymol. 52, 166–188.
13. Makino, R., Tanaka, T., Iizuka, T., Ishimura, Y., and Kanegasaki, S. (1986) J. Biol. Chem. 261, 11444–11447.
14. Davies, M. D., and Sligar, S. G. (1992) Biochemistry 31, 11383–11389.
15. Makino, R., Iizuka, T., Sakaguchi, K., and Ishimura, Y. (1982) in Oxygenases and Oxygen Metabolism (Nozaki, M., Yamamoto, S., Ishimura, Y., Goon, M. J., Ernst, L., and Estabrook, R. W., eds) pp. 467–477, Academic Press, New York.
16. Shimada, H., Makino, R., Imai, M., Horiuchi, T., and Ishimura, Y. (1991) in International Symposium on Oxygenases and Oxygen Activation (Yamamoto, S., Nozaki, M., and Ishimura, Y., eds) pp. 133–136, Yamada Science Foundation, Osaka, Japan.
17. Hinth, M. J., Mock, D. M., Peterson, L. L., Tuttle, K., and Peterson, J. A. (1982) J. Biol. Chem. 257, 14324–14332.
18. Fisher, M. T., and Sligar, S. G. (1985) J. Am. Chem. Soc. 107, 5018–5019.
19. Marcus, R. A., and Sutin, N. (1985) Biochim. Biophys. Acta 811, 265–322.
20. Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S., and Dutton, P. L. (1992) Nature 355, 796–802.
21. Rodgers, K. K., and Sligar, S. G. (1991) J. Am. Chem. Soc. 113, 9419–9421.
22. Caffrey, M. S., and Cusanovich, M. A. (1991) Arch. Biochem. Biophys. 285, 227–230.
23. Davies, M. A., Guillenette, J. G., Smith, M., Greenwood, C., Thurgood, A. G., Mauk, A. G., and Moore, G. R. (1993) Biochemistry 32, 5431–5435.
24. Stayton, P. S., and Sligar, S. G. (1988) J. Biol. Chem. 263, 13544–13548.
25. Pochapsky, T. C., Ye, X. M., Ratnaswamy, G., and Lyons, T. A. (1994) Biochemistry 33, 6424–6432.
26. Pochapsky, T. C., Ratnaswamy, G., and Patera, A. (1994) Biochemistry 33, 6433–6441.
27. Lipscomb, J. D., Sligar, S. G., Nambvedut, M. J., and Gunsalus, I. C. (1976) J. Biol. Chem. 251, 1116–1124.
28. Shimada, H., Makino, R., Unno, M., Horiuchi, T., and Ishimura, Y. (1994) in Cytochrome P450: Biochemistry, Biophysics and Molecular Biology (Lechner, M. C., ed) pp. 299–306, John Libbey Eurotext Ltd., Paris.
29. Gerber, N. C., and Sligar, S. G. (1992) J. Am. Chem. Soc. 114, 8742–8743.
30. Gerber, N. C., and Sligar, S. G. (1994) J. Biol. Chem. 269, 4260–4266.
31. Aikens, J., and Sligar, S. G. (1994) J. Am. Chem. Soc. 116, 1143–1144.