L358P Mutation on Cytochrome P450cam Simulates Structural Changes upon Putidaredoxin Binding

THE STRUCTURAL CHANGES TRIGGER ELECTRON TRANSFER TO OXY-P450CAM FROM ELECTRON DONORS*

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To investigate the functional and structural characterization of a crucial cytochrome P450cam (P450cam)-putidaredoxin (Pdx) complex, we utilized a mutant whose spectroscopic property corresponds to the properties of the wild type P450cam in the presence of Pdx. The 1H NMR spectrum of the carbonmonoxy adduct of the mutant, the Leu-358 → Pro mutant (L358P), in the absence of Pdx showed that the ring current-shifted signals arising from η-camphor were upfield-shifted and observed as resolved signals, which are typical for the wild type enzyme in the presence of Pdx. Signals from the β-proton of the axial cysteine and the γ-methyl group of Thr-252 were also shifted upfield and downfield, respectively, in the L358P mutant as observed for Pdx-bound wild type P450cam. The close similarity in the NMR spectra suggests that the heme environment of the L358P mutant mimics that of the Pdx-bound enzyme. The functional analysis of the L358P mutant has revealed that the oxygen adduct of the L358P mutant can promote the oxygenation reaction for η-camphor with nonphysiological electron donors such as dithionite and ascorbic acid, showing that oxygenated L358P is “activated” to receive electron from the donor. Based on the structural and functional characterization of the L358P mutant, we conclude that the Pdx-induced structural changes in P450cam would facilitate the electron transfer to Pdx (3). For example, the homologous iron-sulfur protein, adrenodoxin, which serves as the reductant for mitochondrial P450s, is unable to transfer an electron to give a product. Furthermore, the addition of nonphysiological small molecule reductants, including dithionite and monothiols to oxy-P450cam, produce only less than 5% of the theoretical maximum amounts of product, although they exhibit lower redox potentials than that of Pdx. These observations have led to the longstanding view first proposed by Lipscomb et al. (3) that Pdx is an effector molecule for the catalytic reaction in P450cam as well as an electron donor.

Various spectroscopic studies on the P450cam-Pdx system have demonstrated that Pdx induces structural changes in P450cam (4–12), which are generally thought to be related to the effector role of Pdx. Unno et al. (4) reported that Pdx binding increases the heme-iron axial ligand (Fe-S) stretching mode by 3 cm⁻¹, indicating the electronic donation from the axial thiolate to the heme iron. The νFe-CO and νC-O stretching modes also shifted up and down, respectively, upon Pdx binding to P450cam-CO (5), supporting enhancement of the electronic donation. Recently, we applied NMR spectroscopy to the P450cam-Pdx system to characterize further the Pdx-induced conformational changes of P450cam in detail (6). Upfield shifts of the ring current-shifted signals from the β-proton of the axial Cys and η-camphor in the P450cam-CO upon the binding of Pdx showed that the axial ligand and η-camphor move toward the heme iron by Pdx binding (6).

These structural changes in P450cam upon Pdx binding seem to be crucial for the enzymatic activity, because the enhancement of the electronic donation from the axial thiolate to the heme iron could facilitate the activation of the O–O bond and stabilize the high valent reaction intermediate of P450cam (13). Based on the radical rebound mechanism (14), the approach of η-camphor to the heme iron could also promote hydroxylation of η-camphor. In addition, a recent report by Nagano et al. (15) indicated that mutations of the amino acid residue at the putative Pdx-binding site, Arg-109 or Arg-112, inhibit the conformational changes in P450cam and lower the electron transfer rate from Pdx to oxy-P450cam (second electron transfer rate) to 39–75% of the wild type enzyme, supporting that the Pdx-induced structural changes possibly promote the second electron transfer reaction. Furthermore, Pochapsky et al. (7) examined the binding effects of adrenodoxin, which does not exhibit the effector activity, to P450cam by using multidimensional NMR spectroscopy, and they found that adrenodoxin could not perturb the structure of P450cam. This result clearly indicates that a nonphysiological electron donor such as adrenodoxin cannot induce the structural changes in P450cam, thereby giving no hydroxylation product. These
observations allow us to speculate that the Pdx-induced structural changes would be essential for the electron transfer to oxy-P450cam.

If the Pdx-induced structural changes in P450cam trigger electron transfer to oxy-P450cam, then it might be expected that the Pdx-bound form of the enzyme would accept electrons from nonphysiological electron donors to produce the hydroxylation product. Here, in order to confirm that the Pdx-induced structural changes can be the trigger for the turnover reaction, we focussed on a P450cam mutant, Leu-358 \( \rightarrow \) Pro mutant (L358P) (16, 17), whose spectroscopic properties are similar to those of wild type P450cam in the presence of Pdx. Earlier work (16, 17) showed that mutating Leu-358 to Pro in P450cam disrupts the hydrogen bond between the amide proton of the main chain and the axial thiolate (Fig. 1), and leads to enhanced \( \pi \)-electron donation from the axial thiolate to the heme iron, which changes \( \nu_{Fe-CO} \) and \( \nu_{FeC-O} \) in the ferrous-CO form (Table I). Similar enhancement of \( \pi \)-electron donation from the axial thiolate to the heme iron is also induced by the binding of Pdx to wild type P450cam (5, 15), raising the possibility that the ferrous-CO form of L358P in the absence of Pdx is similar to wild type P450cam in complex with Pdx. This study and the accompanying paper (18) provide detailed structural and functional analyses of the L358P mutant as a model for Pdx-bound wild type P450cam in order to understand the functional significance of the Pdx-induced structural changes in P450cam.

**EXPERIMENTAL PROCEDURES**

**Expressions and Purifications of Proteins**—The constructions of the expression vectors for wild type and the L358P mutant of P450cam were reported by our group (16). Wild type and the mutant of P450cam were expressed in E. coli strain BL21 as inclusion bodies (16, 19). Heme reconstitution and purification were carried out according to the method described previously (16, 19). Purified proteins with an \( RZ \) value (\( A_{392/450} \)) greater than 1.5 were used in this study. The purified samples were dissolved in 50 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl and 1 mM \( \alpha \)-camphor and stocked at \(-70^\circ C\). The substrate-free sample was prepared by passing substrate-bound P450cam through a G-25 column before the measurements to avoid the interference from nonphysiological electron donors to produce the hydroxylated product. Here, in order to confirm that the Pdx-induced structural changes can be the trigger for the turnover reaction, we focussed on a P450cam mutant, Leu-358 \( \rightarrow \) Pro mutant (L358P) (16, 17), whose spectroscopic properties are similar to those of wild type P450cam in the presence of Pdx. Earlier work (16, 17) showed that mutating Leu-358 to Pro in P450cam disrupts the hydrogen bond between the amide proton of the main chain and the axial thiolate (Fig. 1), and leads to enhanced \( \pi \)-electron donation from the axial thiolate to the heme iron, which changes \( \nu_{Fe-CO} \) and \( \nu_{FeC-O} \) in the ferrous-CO form (Table I). Similar enhancement of \( \pi \)-electron donation from the axial thiolate to the heme iron is also induced by the binding of Pdx to wild type P450cam (5, 15), raising the possibility that the ferrous-CO form of L358P in the absence of Pdx is similar to wild type P450cam in complex with Pdx. This study and the accompanying paper (18) provide detailed structural and functional analyses of the L358P mutant as a model for Pdx-bound wild type P450cam in order to understand the functional significance of the Pdx-induced structural changes in P450cam.

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Pdx was expressed in *E. coli* strain RR1 and was purified by the method of Gunsalus and Wagner (20) with a minor modification (21). Pdx was expressed in *E. coli* strain RR1 and was purified by the method of Gunsalus and Wagner (20) with a minor modification (21). The purified Pdx had a 325 to 280 nm absorbance ratio of at least 0.6. The sample was dissolved in 50 mM potassium phosphate, pH 7.4, containing 50 mM KCl and 80 mM \( \alpha \)-mercaptomethanol and stored at \(-70^\circ C\). \( \beta \)-Mercaptomethanol was removed by passing through a G-25 column before the measurements to avoid the interference of the association with P450cam.

**NMR Measurements**—\(^1^H\) NMR measurements were carried out with BRUKER Avance DRX500 and DRX600 spectrometers at 17 and 40 °C. Peak shifts were referenced to the residual water signal that was calibrated against tetramethylsilane. A PRESAT pulse sequence was calibrated against tetramethylsilane. A PRESAT pulse sequence was performed by using the standard pulse sequence (23).

NMR samples were prepared by the method described previously (6). Substrate-bound P450cam was dissolved in 10% D\(_2\)O containing 50 mM potassium phosphate, 50 mM KCl, and 1 mM \( \alpha \)-camphor, pH 7.4. Substrate-free sample was dissolved in 10% D\(_2\)O containing 50 mM potassium phosphate, 50 mM KCl, pH 7.4. The concentrations of P450cam in the NMR samples were 0.2 and 0.5–1.0 mM for the one-dimensional NMR and two-dimensional nuclear Overhauser enhancement spectroscopy measurements, respectively. Five hundred \( \mu \)L of sample was transferred into the NMR tubes, which were capped with rubber septa. After flushing the samples with argon gas, samples were reduced by the addition of a small aliquot of the degassed sodium dithionite solution. CO gas was anaerobically introduced into the NMR tube to prepare the ferrous-CO form of P450cam. The formation of carbonmonoxy P450cam was confirmed by the electronic absorption spectrum.

**Determination of the Dissociation Constant of Pdx to P450cam**—Following the previous report (6), we determined the dissociation constant (\( K_c \)) of Pdx to P450cam. We measured one-dimensional NMR spectra for carbonmonoxy L358P in the presence of various concentrations of reduced Pdx (Pdx\(^{-}\)) at 17 and 40 °C. The concentration of Pdx\(^{-}\) was from 50 to 800 \( \mu \)M. The \( K_c \) value of Pdx\(^{-}\) to carbonmonoxy P450cam was estimated by the shifts of the NMR signals upon the complexation with Pdx\(^{-}\). We confirmed that the equilibrium between Pdx-bound P450cam, and Pdx-free P450cam was the “fast exchange process” on the NMR time scale at 40 °C (see “Results”), the nuclei in two states gave a single resonance at the population weighted average chemical shift of the nuclei during the titration, and \( K_c \) can be determined by the following Equation 1 (24).

\[
\delta_{obs} = \delta_0 + \frac{\delta_{max} - \delta_0}{[P450]_{tot} + [Pdx]_{int}} \cdot \frac{[P450]_{tot}}{[Pdx]_{int}} - \sqrt{\frac{[K_c] + [P450]_{tot} + [Pdx]_{int}}{2} - 4 \cdot [P450]_{tot} \cdot [Pdx]_{int}}
\]

(Eq. 1)

where \( \delta_{obs} \) denotes the observed chemical shift of P450cam in the titration experiments. \( \delta_{max} \) and \( \delta_0 \) represent the chemical shifts of Pdx-bound P450cam and Pdx-free P450cam, respectively. [P450]\(_{tot}\) and [Pdx]\(_{int}\) represent the total concentrations of P450cam and Pdx, respectively. [Pdx]\(_{int}\) denotes the concentration of Pdx below which the Pdx-P450cam complex can be considered to be in the slow exchange limit on the NMR time scale.
A mutant of P450cam mimicking the putidaredoxin-bound form

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**RESULTS AND DISCUSSION**

**Heme Environment of the Ferrous-CO Form of L358P**—Upshift and downshift of ν_{Fe-CO} and ν_{C=O}, respectively, in the L358P mutant indicate that the electronic structure of L358P-CO likely corresponds to that of the Pdx-bound wild type enzyme. To confirm the structural similarity between the L358P mutant and the Pdx-bound wild type enzyme, we investigated the heme environment of the ferrous-CO form of the L358P mutant by using NMR spectroscopy. Fig. 2 displays the NMR spectra for carbonmonoxy wild type P450cam (trace A) in the absence and presence of Pdx^{red} (trace B), and carbonmonoxy L358P in the absence of Pdx^{red} (trace C) in the upfield region at 40 °C. As seen in Fig. 2, the signals x (9-methyl group of n-campher) and y (5-exo proton of n-campher), both of which are only detected as resolved peaks in the presence of Pdx^{red} for the wild type enzyme (trace B), were observed as resolved peaks for the L358P mutant in the absence of Pdx^{red} (trace C). Furthermore, the signals a (β-proton of the axial Cys) and d (γ-methy1 group of Thr-252) showed upfield and downfield shifts, respectively, in the L358P mutant, which were also observed in the wild type enzyme upon Pdx binding. Thus, the NMR spectral pattern for carbonmonoxy L358P in the absence of Pdx^{red} (Fig. 2, trace C) is similar to that of the wild type enzyme in the presence of Pdx^{red} (trace B), indicating that the heme environmental structure of the mutant would be similar to that of the Pdx-bound wild type enzyme.

Because the contribution of the ring current shifts mainly depend on the position of the proton relative to the heme plane (29, 30), the ring current-shifted signals for the L358P mutant provide more detailed information on the local heme environment of the L358P mutant. By using the Johnson Bovey model (29), a useful tool for calculating the ring current contribution, the chemical shifts in the mutant indicate that the β-proton of the axial Cys and the 9-methyl group and 5-exo proton of n-campher approach closer to the heme iron by −0.15, 0.1, and 0.2–0.3 Å, respectively, upon the mutation. On the other hand, the γ-methyl group of Thr-252 moves away from the heme iron by −0.05 Å in the L358P mutant. In our previous work we found that Pdx binding to P450cam results in the movement of the axial Cys, 9-methyl group, and 5-exo proton of n-campher closer to the heme iron by −0.05–0.15, −0.15, and 0.5–0.7 Å, respectively, whereas the γ-methyl group of Thr-252 moves by −0.25 Å away from the heme iron (6). Although the magnitude of the structural changes on the Leu-358 to Pro mutation is smaller than from Pdx binding, the same trend in the structural changes in the heme environment indicates that the heme environment of L358P-CO has similar property to the Pdx-bound wild type enzyme.

**Effects of Pdx Binding on Ferrous-CO L358P**—If the L358P mutant in the absence of Pdx corresponds to the Pdx-bound wild type enzyme, it is expected that the heme environment in the mutant might not be perturbed by Pdx binding. We measured the NMR spectra for L358P-CO in the presence of Pdx^{red} to examine the effects of Pdx binding on the heme environmental structure of L358P-CO. Fig. 3 illustrates the NMR spectra for the ferrous-CO form of the L358P mutant in the presence of 0.5–4 eq of Pdx^{red} at 40 °C. By increasing the concentration of Pdx^{red}, the ring current-shifted signals showed upfield (signals a, x, and y) and downfield shifts (signal d) as observed for the wild type enzyme (6). These NMR spectral changes indicate that the binding of Pdx further induces structural changes around the heme in the L358P mutant.

To compare the magnitude of the Pdx-induced structural changes in the L358P mutant with those of the wild type enzyme, the limiting shifts of the ring current-shifted signals in the L358P mutant were estimated by NMR titration experiments. No significant line broadening of the NMR signals, except for signal y, in the L358P mutant upon the binding of Pdx implies that the exchange rate between free and bound proteins is fast enough on the NMR time scale in the L358P-Pdx system, which is the same as that in the wild type enzyme (6). In the fast exchange process, we can use Equation 1 for determining the limiting shifts (24). Fig. 4 shows a plot of the shifts for the signal x in the L358P mutant and the wild type enzyme as a function of the ratio of Pdx^{red} to P450cam. The experimental data points for the L358P mutant were fitted by
In addition to the limiting shift, the $K_d$ value estimated from the titration experiment also supported the view that the L358P mutant could correspond to the Pdx-bound wild type enzyme. The $K_d$ value of Pdx to the L358P mutant (4.8 µM) is 1/30th that of wild type P450cam (149 µM) (6), suggesting that the L358P mutant binds more tightly to Pdx due to the structural changes in the Pdx-binding site by the mutation. NMR titration studies at 17 °C further confirm tighter binding to Pdx in the L358P mutant than that in the wild type enzyme. Fig. 5 represents the NMR spectra for the ferrous-CO form of wild type P450cam (left panel) and the L358P mutant (right panel) in the presence of 0–3 eq of Pdxred at 17 °C. For the wild type enzyme, the signal $x$ at $-1.29$ ppm, which is derived from the 9-methyl group of $\beta$-camphor, was shifted to the upfield region with increasing concentrations of Pdxred (Fig. 5, left panel), as observed at 40 °C (6). In sharp contrast to the wild type enzyme, a new signal at $-1.45$ ppm appeared and increased its intensity with a decrease in the intensity of the signal $x$ at $-1.35$ ppm upon the addition of Pdxred to the L358P mutant (Fig. 5, right panel). The separated signals were observed for the Pdx-free and Pdx-bound L358P unlike the titration of Pdx to wild type P450cam, showing that the bound and free proteins are in slow exchange on the NMR time scale (24). It is likely that tighter binding to Pdx in the L358P mutant might be caused by the structural rearrangements at the Pdx-binding site, which optimize the interaction between the L358P mutant and Pdx. Because previous reports (6, 15, 31) indicated that Pdx induces the structural changes in P450cam through the hydrogen bond between one of the heme propionates and Arg-112 at the putative Pdx-binding site, the structural changes around the heme could also evoke the structural changes in the Pdx-binding site. Therefore, we suggest that the Pdx-binding site of the L358P mutant as well as the heme environment correspond to the Pdx-bound wild type enzyme.

**Reactivity of Oxy-P450cam with Electron Donors**—Besides the enhanced push effect in the L358P mutant (16, 17), our current NMR data show that both $\beta$-camphor and the $\beta$-proton of the axial Cys approach closer to the heme-iron as observed in the Pdx-bound wild type enzyme. Therefore, we expected that the mutant should exhibit functional properties characteristic of Pdx-bound P450cam. If the L358P mutant functionally mimics the Pdx-bound wild type enzyme, oxy-L358P might be able to accept an electron from electron donors that have lower redox potentials than Pdx but cannot donate electrons to wild type oxy-P450cam to produce product. In this study, we used dithionite as an electron donor, which has a lower redox potential than that of Pdx, but gives less than 5% of the theoretical maximum amounts of product upon mixing with wild type oxy-P450cam (3).

The reactivity of oxy-P450cam with the electron donors including Pdx and dithionite was evaluated by measuring the amounts of product, 5-exo-hydroxyacamphor, after mixing oxy-P450cam and the electron donors in the presence of the substrate, $\beta$-camphor. Quantitation of 5-exo-hydroxyacamphor was performed by gas chromatography. Fig. 6 shows the gas chromatogram of the reaction mixture after mixing oxy-P450cam and the electron donors. As can be seen in Fig. 6 (traces A and B), mixing of oxy-P450cam and Pdxred yields product whose retention time was 13.7 min under the conditions we used here. On the basis of the peak areas for 5-exo-hydroxyacamphor, the amount of product was estimated to be 4.3 and 4.8 µM for wild type P450cam and the L358P mutant, respectively, which correspond to 86 and 96% of the maximal yield that was estimated from the initial concentration of oxy-P450cam. The addition of Pdxred to oxy-L358P yields almost the same amount of product as that obtained by the addition of Pdxred to wild type P450cam.

The addition of dithionite to wild type oxy-P450cam also...
absence and presence of Pdx red at 17°C. The buffer used for the measurements was 50 mM potassium phosphate, pH 7.4, containing 10% D2O, 50 mM KCl, and 1 mM D-camphor. 

When L358P was mixed with oxy-L358P, the yield of the product was 3.5-fold higher than that of oxy-L9262 (0.21% of the maximal yield). On the other hand, when dithionite was added to ferric P450cam, however, the L358P gave increased amounts of product (26). Upon the addition of dithionite to oxy-L358P, the yield was only 7% of the maximal yield. The enhanced reactivity of oxy-L358P is because of the promotion of the substrate binding, the reduction of ferric P450cam or the oxygen binding, and not because of the acceleration of the electron transfer. Therefore, we suggest that the Pdx-induced structural changes would facilitate the electron transfer from donor to oxy-P450cam. As we expected from the spectral features of the L358P mutant, these results show that nonphysiological reductants can reduce oxy-L358P to yield the product. 

TABLE II

Specific assignments for the ring current-shifted signals in the 1H NMR spectra of the ferrous-CO forms of the enzymes in the absence and presence of Pdx

| δ (ppm from tetramethylsilane) | Pdx-free | Pdx-bound | Δδ |
|-------------------------------|----------|-----------|----|
| β-Proton of the axial Cys      | Wild type | −2.76     | −2.88 | −0.12 |
|                               | L258P    | −2.95     | −2.02 | 0.87  |
| 9-Methyl group of d-camphor    | Wild type | −1.25     | −1.45 | −0.20 |
|                               | L358P    | −1.37     | −1.45 | −0.08 |
| 5-exo-Proton of d-camphor      | Wild type | −1.18     | −2.46 | −1.28 |
|                               | L358P    | −2.02     | −2.63 | 0.61  |
| γ-Methyl group of Thr-252      | Wild type | −0.88     | −0.69 | 0.19  |
|                               | L358P    | −0.80     | −0.67 | 0.13  |

*Δδ = δPdx-bound − δPdx-free.

Fig. 5. 1H NMR spectra of the ferrous-CO forms of wild type P450cam (left panel) and the L358P mutant (right panel) in the absence and presence of Pdxred at 17°C. The traces represent the NMR spectra of the ferrous-CO forms of the enzymes in the absence (A) and presence of 0.25 (B), 0.5 (C), 0.75 (D), 1 (E), 2 (F), and 3 eq (G) of Pdxred. The buffer used for the measurements was 50 mM potassium phosphate, pH 7.4, containing 10% D2O, 50 mM KCl, and 1 mM d-camphor. The concentration of P450cam was 200 μM. TMS, tetramethylsilane.

produces product (Fig. 6, trace C), although the yield was only 7% of the maximal yield. On the other hand, when dithionite was mixed with oxy-L358P, the yield of the product was 3.5-fold larger than that of the wild type enzyme (Fig. 6, trace D). The yields of product are summarized in Table III. The enhanced reactivity in the mutant suggests that the mutation accelerates the electron transfer from dithionite to oxy-P450cam, because the electron transfer is the rate-determining step in the hydroxylation reaction (26). Upon the addition of dithionite to ferric P450cam, however, the L358P gave increased amounts of product (0.81 μM), compared with that in the wild type enzyme (0.21 μM) (data not shown), showing that the addition of an excess amount of dithionite to oxy-P450cam could result in the multiple turnover. We cannot exclude the possibility that the increased yield of product in oxy-L358P is because of the promotion of the substrate binding, the reduction of ferric P450cam or the oxygen binding, and not because of the acceleration of the electron transfer.

In order to delineate the mutational effects on the electron transfer, we examined the reactivity of oxy-P450cam with an electron donor that is unable to lead to multiple turnover, i.e.

We confirmed no production of 5-exo-hydroxycamphor after mixing ferric enzyme with ascorbic acid in the presence of d-camphor by using gas chromatography.
decomposition of oxy-P450cam is suppressed in the L358P mutant, we examined the life span of oxy-P450cam. Fig. 7 shows time courses of absorbance at 418 nm, which is the Soret maximum of oxy-P450cam, after exposure of oxy-P450cam to the aerobic condition in the absence of the electron donor at 4 °C. In both wild type P450cam and the L358P mutant, decay of the oxygen adduct was observed, which is indicative of the autooxidation reaction. The isosbestic point at 409 nm is clear evidence that the autooxidation reaction is followed by the two-state behavior (inset of Fig. 7). By fitting a single exponential function, the rate constants for the autooxidation reactions (k_ox) were estimated to be $4.7 \times 10^{-10}$ s$^{-1}$ and $1.7 \times 10^{-13}$ s$^{-1}$ for wild type P450cam and the L358P mutant, respectively. Whereas Pochapsky et al. (7) suggested that Pdx binding could decrease autooxidation reaction, it is more plausible that Pdx binding would accelerate the autooxidation reaction.

In addition to the autooxidation reaction, the dissociation rate of oxygen from oxy-P450cam would be one of the determinants for the life span of oxy-P450cam. In other words, the suppressed dissociation of oxygen from ferrous P450cam in the L358P mutant might result in longer life span of oxy-L358P than that of wild type oxy-P450cam, and an increase in the product formation upon mixing oxy-P450cam with reductants. Based on the negative correlation between the autooxidation reaction rate and the affinity for the oxygen complex of myoglobin (32), however, it is likely that the increased autooxidation rate in the L358P mutant corresponds to the lower affinity of ferrous L358P to oxygen than that of wild type P450cam.

In order to estimate the affinity of ferrous P450cam to oxygen, we measured the kinetics of $O_2$ displacement by CO. The apparent rate constants were estimated to be 2.6 and 0.47 s$^{-1}$ for wild type P450cam and the L358P mutant, respectively, indicating that the dissociation of oxygen from oxy-P450cam would be retarded in the L358P mutant. On the other hand, we cannot determine the association rate of oxygen to ferrous P450cam by the laser flash photolysis, because of the instability of oxy-P450cam. Therefore, we were unable to estimate the affinity of ferrous P450cam to oxygen.

and the life span of oxy-L358P is shorter than that of wild type oxy-P450cam. This result allows us to suggest that the larger yield of product given by the mixing of oxy-L358P with reductants than that of wild type P450cam could be ascribed to the enhanced reactivity of oxy-P450cam with reductants and not to the longer life span.

Functional Roles of Pdx-induced Structural Changes in the Catalytic Reaction—Because the electron transfer step is rate-limiting in the reaction pathway for the monooxygenation reaction (26), it is likely that the Pdx-induced structural changes could promote the electron transfer reaction. Although it is often difficult to apply the electron transfer theory to electron transfer reactions coupled with the non-electron transfer event.

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### TABLE III

| Protein     | Pdxred | Dithionite | Ascorbic acid |
|-------------|--------|------------|---------------|
| Wild type   | 4.3 ± 0.073 (86%)$^a$ | 0.34 ± 0.023 (7%)$^a$ | < 0.05$^a$ |
| L358P       | 4.8 ± 0.082 (96%)$^a$ | 1.2 ± 0.013 (24%)$^a$ | 0.21 ± 0.010 (4%)$^a$ |

$^a$ The values in parentheses are (hydroxylation product)/(oxy-P450cam (5 μM)).

$^b$ The hydroxylation product was less than the limit of detection, which corresponds to 0.05 μM under the condition used here.
such as conformational changes, it is still worth using the electron transfer theory to discuss how the acceleration of the electron transfer rate would occur (33). To analyze the electron transfer event between oxy-P450cam and reductants, we utilized the Marcus theory (34). Equation 2 (Marcus equation) is given as follows:

\[ k_{ET} = \frac{4\pi^2H_{AB}^2}{h(4\pi R_T)^2} \exp \left[ -\frac{(\Delta G^0 + \lambda^2)}{4RT} \right] \]  

(Eq. 2)

where \( h \) is Planck’s constant; \( R \) is gas constant; \( T \) is temperature; \( \lambda \) is the reorganization energy; \( \Delta G^0 \) is the redox potential difference between donor and acceptor, and \( H_{AB} \) is the electronic coupling term, which describes the degree of wave function overlap between the donor and acceptor sites. In Marcus theory, \( k_{ET} \) is determined from \( \Delta G^0, \lambda, \) and \( H_{AB} \). Although the redox potential of the oxy-P450cam/reduced oxy-P450cam couple has not been reported, our mutational study on the electron transfer reaction between P450cam and Pdx exhibited a significant correlation with the redox potential for the Fe\(^{3+}/Fe^{2+} \) couple and the electron transfer rate from Pdx to oxy-P450cam (35). Based on the Fe\(^{3+}/Fe^{2+} \) couple (\( E_m = -135 \) and \(-170 \) mV versus NHE for wild type and the L358P mutant, respectively), we can therefore assume that the redox potential of the oxy-P450cam/reduced oxy-P450cam couple of the L358P mutant is lower than that of the wild type enzyme (16, 17). Furthermore, up- and downshifts of the Fe-CO and C-O stretching modes in the L358P mutant show the enhanced electronic donation from the thiolate ligand to the heme iron in L358P-CO (16, 17), which also supports the lowered redox potential in oxy-L358P. Because the possible negative shift in the redox potential should decrease the electron transfer rate, the decrease in \( \lambda \) and/or increase in \( H_{AB} \) would be required to facilitate the electron transfer reaction between oxy-L358P and the non-physiological reductants.

One of the factors to alter \( \lambda \) is the structural perturbation in the heme distal site in the L358P mutant. The NMR spectrum of the Leu-358 mutant indicates the positional change of Thr-113 in the heme distal site of the L358P mutant (18) provides further information on the Pdx-induced structural changes in P450cam and the effector function of Pdx. The following paper on the x-ray structural analysis of the L358P mutant (18) provides further information on the Pdx-induced structural changes in P450cam and the effector function of Pdx.

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