Crystal Structure of the Cytochrome P450cam Mutant That Exhibits the Same Spectral Perturbations Induced by Putidaredoxin Binding*

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The cytochrome P450cam active site is known to be perturbed by binding to its redox partner, putidaredoxin (Pdx). Pdx binding also enhances the camphor monooxygenation reaction (Nagano, S., Shimada, H., Tarumi, A., Hishiki, T., Kimata-Ariga, Y., Egawa, T., Suematsu, M., Park, S.-Y., Adachi, S., Shiro, Y., and Ishimura, Y. (2003) Biochemistry 42, 14507–14514). These effects are unique to Pdx because nonphysiological electron donors are unable to support camphor monooxygenation. The accompanying 1H NMR paper (Tosha, T., Yoshioka, S., Ishimori, K., and Morishima, I. (2004) J. Biol. Chem. 279, 42836–42843) shows that the conformation of active site residues, Thr-252 and Cys-357, and the substrate in the ferrous (Fe(II)) CO complex of the L358P mutant mimics that of the wild-type enzyme complexed to Pdx. To explore how these changes are transmitted from the Pdx-binding site to the active site, we have solved the crystal structures of the ferrous and ferrous-CO complex of wild-type and the L358P mutant. Comparison of these structures shows that the L358P mutation results in the movement of Arg-112, a residue known to be important for putidaredoxin binding, toward the heme. This change could optimize the Pdx-binding site leading to a higher affinity for Pdx. The mutation also pushes the heme toward the substrate and ligand binding pocket, which relocates the substrate to a position favorable for regio-selective hydroxylation. The camphor is held more firmly in place as indicated by a lower average temperature factor. Residues involved in the catalytically important proton shuttle system in the I helix are also altered by the mutation. Such conformational alterations and the enhanced reactivity of the mutant ox complex with nonphysiological electron donors suggest that Pdx binding optimizes the distal pocket for monooxygenation of camphor.

Cytochrome P450cam (P450cam)3 (1, 2) catalyzes the regio- and stereo-specific hydroxylation of d-camphor. To activate molecular oxygen, P450cam requires two protons and two electrons. Protons are provided from bulk water through a proton shuttle system that includes Asp-251, Thr-252, and water molecules (3–6). Electrons are provided by an iron-sulfur protein, putidaredoxin (Pdx). In the electron transfer steps, P450cam forms a complex with reduced Pdx, and the P450cam active site is perturbed as indicated by various spectroscopic studies including NMR (7, 8), EPR (9, 10), resonance Raman (11–13), and infrared (14, 15). Because nonphysiological redox proteins such as ferredoxin and adenodoxin cannot support the monooxygenation reaction or induce the conformational changes (7, 16), the Pdx-induced changes in P450cam are thought to be important for the “effector” function of Pdx in addition to its reductase activity. In support of this view, a recent infrared and mutational study (15) has shown that Pdx-induced conformational changes promote the camphor monooxygenation reaction.

Recent NMR studies found that the CO complex of the L358P mutant has very similar spectroscopic property to the WT P450cam-CO in a complex with Pdx (8, 17). This suggests that the L358P mutant is a good model for Pdx-bound P450cam. However, our knowledge on the conformational change from the NMR studies is limited to only two residues, the axial cysteine ligand (Cys-357) and Thr-252 and the substrate. To overcome this limitation and to complement the NMR work, we have solved the crystal structures of the ferrous and ferrous-CO complex of the L358P mutant.

EXPERIMENTAL PROCEDURES

Protein Purification and Crystallization—WT P450cam and the L358P mutant were overexpressed in Escherichia coli and purified as described elsewhere (18). The CO-bound P450cam structure was first solved by Raag and Poulos (19) by using the initial P21 crystal form used to solve the P450cam structure. For a more accurate direct comparison between the WT and L358P structures, the ferrous and the ferrous-CO-bound structures for both WT P450cam and the mutant were determined in the P21 crystal form, thus minimizing differences because of crystal lattice and packing effects. Single crystals of WT P450cam and the mutant were obtained by the method described previously (20) for WT crystals in the same P21 space group with minor modifications. Crystals of the ferro form were grown at 15 °C. The precipitant solution was 50 mM Tris-HCl buffer, pH 7.4, containing 0.1–0.4 M KCl, 25–32% polyethylene glycol 4000, and 0.7–1.0 mM (CYP); Pdx, putidaredoxin; WT, wild type; P450cam-O2, d-camphor-bound ferrous-O2; P450cam; P450cam-CO, d-camphor-bound ferrous-carbon monoxide P450cam.

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‡ The abbreviations used are: P450cam, cytochrome P450 (CYP101) originally isolated from Pseudomonas putida; P450, cytochrome P450.
Structure of the Leu-358 → Pro P450cam Mutant

Table I
Crystallographic and refinement statistics

| Data set          | Ferrous CO-bound L358P | Ferrous L358P | Ferrous CO-bound WT | Ferrous WT |
|-------------------|------------------------|---------------|---------------------|-----------|
| Unit cell (Å, °)  | a = 57.11              | a = 57.01     | a = 67.03           | a = 66.99 |
|                   | b = 59.30              | b = 101.17    | b = 62.23           | b = 62.39 |
|                   | c = 57.41              | c = 72.92     | c = 94.92           | c = 94.97 |
| β                 | 104.37                 | 108.30        | 90.48               | 89.57     |
| Space group       | P2₁                   | P₂₁           | P2₁                 | P₂₁       |
| Resolution range (Å) | 50–1.80               | 50–1.90       | 50–1.80             | 50–1.90   |
| Reflections (observed/unique) | 171,586/33,098        | 230,635/61,711 | 293,505/72,331 | 212,397/60,650 |
| Rmerge (%)        | 30.1 (2.6)             | 19.5 (2.4)    | 30.0 (4.4)          | 17.2 (2.2) |
| Completeness (%)  | 95.6 (82.3)            | 99.9 (100.0)  | 99.4 (98.1)         | 97.8 (95.8) |
| Rfree (%)         | 18.9/21.6              | 19.9/23.6     | 18.5/20.7           | 18.7/21.2 |
| r.m.s.d. bond length (Å) | 0.006               | 0.006         | 0.005               | 0.006     |
| r.m.s.d. bond angle (°) | 1.30                | 1.27          | 1.32                | 1.30      |
| No. water molecules | 296                  | 537           | 607                 | 510       |

Values in parentheses are for data above 2.0 Å.

Results and Discussion
L358P Mutation-induced Conformational Changes—The accompanying paper (17) shows that the CO complex of the L358P mutant has remarkably similar NMR and vibrational spectral patterns to those of the Pdx-bound WT enzyme. Furthermore, because the vibrational spectroscopic behavior of the CO complex upon Pdx binding parallels that of the ox complex, structural information gained from the CO complex can serve as a model for changes in structure upon formation of the oxy complex.

Leu-358 follows the Cys-357 heme ligand. Although the geometry between the Cys-357 sulfur and Leu-358 peptide NH is not optimal for hydrogen bonding, the 3.6-Å distance is close enough for the partial positive charge on the peptide NH to attenuate the negative charge on the thiolate sulfur. The L358P mutation eliminates such electrostatic stabilization of the Cys-357 thiolate sulfur, which should enhance electron donation from the axial Cys to the heme iron. The enhanced electron donation was confirmed by a slight lowering of 36 mV in the mutant compared with that of WT enzyme (18, 27). Vibrational spectroscopic studies on the CO, NO, and oxy complexes have shown that Pdx binding enhances electron donation from the axial Cys, thereby promoting O–O bond cleavage (11–13, 15).

Leu-358 makes a number of other nonbonded contacts with the heme pyrrole C-ring and neighboring residues such as Arg-112, Ala-115, and Asn-116 in the C helix. As depicted in Fig. 2, replacement of the Leu with Pro enables the C-terminal side of the C helix to move toward the proximal ligand. Most notably the guanidinium group of Arg-112 relocates about 1 Å closer to Pro-358 and the heme. As discussed below, this is consistent with NMR studies by Pochapsky et al. (7) and may explain the high affinity of the mutant for reduced Pdx.

Because of steric crowding, the rigid Pro-358 side chain also forces a 0.4-Å movement of the heme pyrrole ring C away from the Pro-358 side chain as well as movement of the 357–359 backbone away from the heme (Fig. 2). Heme rings A and B move toward the proximal side by 0.3–0.4 Å. These changes slightly increase heme ruffling. Similar ruffling has been observed for the mutant P450cam where Arg-112 was substituted with Lys (15). Most interestingly, a Pro at the position corresponding to 358 in P450cam has been found in a number of P450 enzymes such as CYP121 (28) and CYP128 (29) from Mycobacterium tuberculosis, CYP7A1 from rabbit (30), and CYP104 from Agrobacterium tumefaciens (31). The crystal structure of CYP121 also shows a ruffled heme conformation because of steric crowding by the Pro ring (28). Because the plausible Pdx-binding site includes Arg-112, a critical residue...
for the electron transfer reaction and conformational change, it is reasonable to propose that Pdx binding to WT P450cam also pushes the C-ring up as in both the L358P and R112K mutants as well as CYP121. Although the relevance of such ruffling to P450 function remains unclear at present, a ruffled heme conformation could be important in the oxy-P450cam-Pdx complex because spectroscopic studies on metalloporphyrins have shown that ruffling decreases porphyrin π-metal dπ interactions and provides smaller inner sphere reorganization energies and thus enhances electron transfer rates (32).

To clarify the effect of CO binding on the conformational change, we also determined structures in the ferrous state (structure not shown). The differences between the WT and mutant structures in the proximal pocket of the ferrous proteins are the same as found in the ferrous-CO structure. Therefore, proximal side changes are due to the mutation itself and not a combination of the mutation with CO binding. In sharp contrast, there is no significant difference in the distal pocket between WT and the mutant when the ferrous structures are compared. Therefore, differences in the distal pocket discussed in the next section are due to CO binding in combination with the mutation and not simply the mutation.

In combination with the steric effect of CO binding, movement of the heme away from Pro-358 and toward the distal pocket where substrate binds leads to small but significant changes in the distal pocket (Fig. 2). Most notable is a repositioning of the substrate relative to the heme which is best described as a slight rotation about the C-1 atom, which results in a maximum movement of about 0.6 Å in the C-3, C-4, and C-5 atoms. The neighboring groups that contact the substrate like Leu-244 shift away from the distal pocket to allow the reorientation of the substrate.

The conformation of the CO ligand in WT P450cam is very similar to that found in the CO-bound form structure reported previously (19). In this earlier work and the current study, the ligand in WT P450cam lies off the heme normal, bent away from the bound substrate, and directed toward Gly-248 in the I helix. The tilt angle between the heme normal and Fe–C bond and bend angle for the Fe–C–O bond are 7° and 151°, respectively, in the WT structure. The CO ligand of L358P also is positioned off of the heme normal and points away from the substrate with an Fe–C–O bond of 7° and 156°. However, because of the repositioning of the substrate in the L358P mutant compared with WT, the terminal O atom of the ligand moves closer to Thr-252 by 0.7 Å, which is similar to the WT O2 or CN− complexes (33).

In the mutant CO complex, $F_o - F_c$ electron density maps clearly indicate that Asp-251 and Thr-252 have two alternate conformations that have been designated conformers A and B (Fig. 3). In conformer A, the peptide carbonyl of the Asp-251 peptide points toward the substrate, whereas Thr-252 is slightly further away from the substrate. Associated with conformer A are two new water molecules. However, we do not observe multiple conformations nor are the two new water molecules present in the WT-CO complex. This indicates that the two new waters are associated with conformer A of Asp-251 to Thr-252 because of the mutation and not CO binding.

Conformer A of Asp-251 to Thr-252 was also observed in the O2 or CN−-bound forms, and most interestingly, both ligands have very similar bent conformations even though CN− favors
a linear Fe-C-N angle (33). Electrostatic interactions between the distal ligand and Thr-252 could be a key factor in the observed alternate conformations of Thr-252 and Asp-251. A CN⁻ ligand has a negative charge formally localized at the N atom. Although an O₂ molecule is neutral, the oxy complex is usually considered to be in equilibrium between the Fe(II)-O-O and Fe(III)-O-O resonance forms, or at the very least, the significant negative charge is delocalized on the distal O atom of O₂ (34). Electrostatic interaction between the negatively charged distal ligand and O-γH of Thr-252 could be responsible for the conformation A observed in the O₂ and CN⁻ complexes (20, 26). Because a neutral CO ligand does not have such an electrostatic interaction, the conversion from conformer B to A does not occur in the WT-CO complex. However, the A and B conformations are observed in the L358P-CO complex. In this case, the camphor has moved in the mutant closer to the CO compared with WT that forces the oxygen atom in CO to orient toward Thr-252. Therefore, Thr-252 must adopt the A conformation to avoid steric clashes with the ligand and to make electrostatic interaction between the O-γ atom and the ligand.

In summary, the mutation causes Arg-112 to move closer to the heme and the Pro-358 pushes the heme C-ring toward the distal site, resulting in reorientation of the substrate and ligand. This reorientation further changes the conformation of the proton supply system and hydrogen bonding network around the ligand (Fig. 4a). We speculate that a relocation of Arg-112 also results from Pdx binding (Fig. 4b), which optimizes the site for interactions with Pdx. This could explain why the L358P mutant binds Pdx more tightly (17). This relocation would push the bulky side chain Leu-358 toward the C-ring, resulting in similar reorientation of the heme, ligand, and substrate. Thus, it is possible that the proton supply system is also changed by Pdx binding. The significance of these changes is discussed below.

Comparison with Pdx-induced Structural Changes Inferred from NMR—As we expected from the similar ring current-shifted NMR patterns for the CO-L358P and CO-WT-Pdx complex, the changes due to the L358P mutation we observed in the crystal structure closely parallel the changes in the NMR spectrum when Pdx binds to the CO-WT complex (8). The NMR work shows that the resonances from the 5-exo and 9-methyl protons of p-camphor and one of the β-protons of Cys-357 shift upfield by 1.28, 0.20, and 0.12 ppm, respectively, upon Pdx binding. On the basis of these spectral shifts and the Johnson-Bovey model for ring current shift (35, 36), such high field shifts indicate that these protons move closer to the heme plane and/or the heme normal passing through the iron atom (z axis) by 0.5, 0.15, and 0.1 Å, respectively. This is qualitatively consistent with the CO-L358P crystal structure because both the C-5 and C-9 atoms of the substrate are closer to the z axis compared with that of the CO-WT complex (Table II and Figs. 3 and 5) by 0.1 and 0.3 Å, respectively. NMR also indicates that C-γ of Thr-252 moves further away from the heme group when Pdx binds. As shown in Fig. 5 and Table II, conformer A of Thr-252 C-γ is about 0.6 Å further from the z axis. Finally, the NMR data also indicate that one of the β-protons of the proximal ligand move toward the heme iron by 0.1 Å upon Pdx binding. Although the position of C-β relative to the heme is not changed by the mutation, the NMR result is consistent with the L358P crystal structure because, to avoid a steric clash with the mutant Pro-358 side chain, the torsion angle between the C-α-C-β and Fe–S-γ bonds increases from 88 to 101° (Fig. 5B), which moves the β-protons closer to the heme plane.

Functional Relevance of Substrate Movement—As already noted, there is a significant parallel between conformational changes found in the crystal structures and those predicted from NMR data. Moreover, Pdx binding effects on the vibrational spectroscopic properties of the CO complex (14, 15) are very similar to that of the oxy complex (12). Therefore, it is reasonable to expect that the conformational changes induced by the L358P mutation also occur upon Pdx binding to the O₂-WT complex. Such changes involve both the protein and substrate. We first consider changes in the substrate.

Abstraction of the H atom from C-5 of p-camphor is optimized by a co-linear arrangement of the C-5 H atom with the Fe-O intermediate generally considered to be the species re-
sponsible for hydroxylation (37, 38). The mutant-induced move-
movement of the substrate and changes in the heme places C-5 in
a more optimal position for H atom abstraction. The angle be-
tween the camphor C-5 and 5-\textsuperscript{exo} H atoms and C atom of the
CO ligand (equivalent position of the O atom of the Fe-O
intermediate) increased from 152° in the WT structure to 176°
in the L358P mutant. In addition, the mutant holds the cam-
phor more firmly in place as indicated by a decrease in the
average \textit{B} factor (Fig. 6). As in the earlier structure of the
CO-WT complex (19), the average temperature factor for the
D-camphor atoms notably increases in the CO complex but not
for protein and heme atoms. A similar increase was also found
upon O\textsubscript{2} binding (20), indicating that the binding of CO or O\textsubscript{2}
results in greater substrate mobility. For the L358P mutant,
however, very similar average temperature factors for the sub-
strate are observed for both the CO-bound and -unbound forms,
suggesting that the substrate is held more firmly in place in the
active site even after CO and, probably, O\textsubscript{2} binding. Because
the L358P mutant closely mimics the Pdx\textsubscript{-}H\textsubscript{18528}
WT complex, it could be that Pdx binding induces changes in the active site, which
optimizes the stereochemistry for camphor hydroxylation by
slightly reorienting and holding the substrate firmly in place
for proper catalysis.

\textbf{L358P as a Mimic for the Effector Role of Pdx—}\n
The main question regarding the effector role of Pdx is why reduction of
the P450cam oxy complex is so specific for Pdx (16). The most
plausible explanation is that Pdx binding induces structural
changes that optimize the system for electron transfer. As
shown in Fig. 4, the distal side changes involve the proton
shuttle machinery, Asp-251, Thr-252, and the ordered water
molecule, required for oxygen activation. If similar changes

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
 & Distance from the heme plane\textsuperscript{a}/Å & Distance from \textit{z} axis\textsuperscript{a}/Å & Pdx-induced difference\textsuperscript{b} & \\
 & WT & L358P & Difference & WT & L358P & Difference \\
\hline
Camphor C-5 & 3.7 & 3.7 & 0.0 & 2.5 & 2.4 & -0.1 \\
Camphor C-9 & 3.9 & 4.0 & +0.1 & 2.9 & 2.6 & -0.3 \\
Thr-252 C-\textgamma & 3.8 & 4.2 & +0.4 & 3.3 & 3.0 & -0.3 \\
 & 3.8\textsuperscript{c} & 0.0\textsuperscript{d} & & 3.6\textsuperscript{c} & +0.6\textsuperscript{d} & \\
Cys-357 C-\textbeta & 3.2 & 3.2 & 0.0 & 1.5 & 1.5 & 0.0 \\
\hline
\end{tabular}
\caption{Geometric parameters for the substrate, Thr-252, and Cys-357 relative to the heme.}
\end{table}

\textsuperscript{a} Plane fitting to the heme (except for methyl, vinyl, and propionate groups) was done by the molecular geometry calculation program GEOMCALC (41). Distances from the average heme plane and \textit{z} axis (the heme normal passing through the heme iron) were also calculated by using GEOMCALC (41).

\textsuperscript{b} The differences between Pdx-bound and -unbound P450cam predicted from the NMR study. The distances are from the heme plane or \textit{z} axis.

\textsuperscript{c} Conformer B.

\textsuperscript{d} Conformer A.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{Changes of the substrate, Thr-252, and Cys-357. L358P and WT are shown as yellow and cyan stick models, respectively. The C-\textgamma atom of Thr-252, C-5 and C-9 atoms of \textit{d}-camphor, and C-\textbeta atom of Cys-357 are shown in gray, orange, dark pink, and purple, respectively. The torsion angle between Fe–C-\textalpha and C-\textalpha–C-\textbeta was increased from 88 to 101 by the L358P mutation. The structures are aligned with the heme C-1–C-8 and \textit{meso}-C atoms by using LSQMAN (39).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{Average temperature factors for the peptide C-\textalpha atoms, heme, and \textit{d}-camphor of the mutant and WT P450cam in ferrous-
or ferrous-CO-bound forms. Dark hatched bars, ferrous WT; solid bars, ferrous-CO-bound WT; light hatched bars, ferrous L358P; shaded bars, ferrous-CO-bound L358P.}
\end{figure}
accompanying paper (17), the L358P mutant-oxy complex exhibits enhanced hydroxylated product formation by using nonphysiological electron donors compared with the WT enzyme. Therefore, we propose that the L358P mutation mimics the Pdx effector activity by optimizing the proton shuttle machinery required for oxygen activation and lowering the reorganization energy required for electron transfer.

Conclusions—The vibrational and NMR spectral properties of the L358P P450cam mutant in a complex with CO mimic the spectral changes observed when Pdx binds to the P450cam-CO complex. Structural changes induced by the L358P mutation may thus mimic changes that occur when Pdx binds to WT P450cam. The L358P mutant also binds Pdx about 30-fold more tightly than WT P450cam, indicating that the L358P mutation optimizes the Pdx-binding site because of the observed changes in and around Arg-112, a residue known to be important for Pdx binding. The L358P mutant also pushes the heme pyrrole C-ring toward the substrate-binding pocket, which leads to changes in the I helix and a slight reorientation of the substrate that favors proton transfer to oxygen ligand and H-atom abstraction from the C-5 atom of camphor. Because the proposed Pdx-binding site includes Arg-112, Pdx binding also could “push” Arg-112 and Leu-358 toward the heme resulting in the heme ruffling, a repositioning of the substrate for the optimal H atom abstraction, and optimization of the proton shuttle machinery required for reduction and activation of the oxy complex.

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