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**Author:** Hiruma, Yoshitaka  
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CHAPTER 4

Hot spot residues in the cytochrome P450cam-putidaredoxin binding interface

Abstract

Cytochrome P450cam (P450cam) is a heme containing monooxygenase that catalyzes hydroxylation of D-camphor to produce 5-exo-hydroxycamphor. The catalytic cycle of P450cam requires two electrons, both of which are donated by a [2Fe-2S] cluster containing ferredoxin, putidaredoxin (Pdx). Recently, atomic resolution structures of the Pdx-P450cam complex have been solved by X-ray crystallography as well as paramagnetic NMR spectroscopy. The binding interface revealed the potential electron transfer pathways and interactions between Pdx Asp38 and P450cam Arg112 as well as hydrophobic contacts between Pdx Trp106 and P450cam residues. Several polar residues are found in the interface that had not been recognized to be relevant for binding before. In this study, site-directed mutagenesis, kinetic measurements and NMR studies were employed to probe the energetic importance and role of the polar residues in the Pdx-P450cam interaction. A double mutant cycle (DMC) analysis of kinetic data shows that favorable interactions exist between Pdx Tyr33 and P450cam Asp125 as well as Pdx Ser42 and P450cam His352. The results show that alanine substitutions of these residues and several others do not influence the rates of electron transfer. It is concluded that these polar interactions contribute to partner recognition rather than electronic coupling of the redox centers.
Introduction
Cytochrome P450 (P450) is a superfamily of heme-containing monooxygenases that participate in a variety of biological metabolisms. From bacteria to humans, P450 members are found in all kingdoms and at least 15,000 P450 genes have been currently reported.[1] The most common reaction that P450 enzymes catalyze is the insertion of oxygen atom into a chemically inert organic substrate (R-H + O_2 + 2H^+ + 2e \leftrightarrow R-OH + H_2O). Due to their specificities and efficiencies, P450 enzymes are widely applied in industrial fields including bioconversion, bioremediation and biosensors.[2] One of the most extensively characterized P450 family members is P450cam (CYP101A1). P450cam from *Pseudomonas putida* catalyzes the regio- and stereo-specific hydroxylation of D-camphor. The activation and cleavage of the oxygen molecule in the P450cam catalytic cycle is accompanied with two electron transfers (ETs) from the physiological ET partner protein, putidaredoxin (Pdx). Pdx shuttles electrons between the FAD-containing putidaredoxin reductase (PdR) and the monoxygenase, P450cam.[3] Interestingly, ferric P450cam can accept the first electron from diverse chemical reductants and Pdx homologues (ET1), but the second (ET2) requires Pdx as donor. The stringent requirement for Pdx in ET2 has been explained by the effector activity. Pdx not only provides the electron but also causes the start of the catalytic reaction.[4-6] However, the underlying mechanism of the effector role of Pdx remains under debate. Recently, X-ray crystallography and NMR have uncovered the three dimensional structures of Pdx-P450cam complex (see chapter 3 for the details).[7, 8] Pdx binds to the proximal surface of P450cam in which the intermolecular distance between iron sulfur cluster and heme iron is approximately 16 Å. Compared with the previously proposed model of Zhang et al.,[9] Pdx is rotated nearly 90° relative to P450cam. The crystal structures of the Pdx-P450cam complex show which residues constitute the interface.[7, 8] The functional role of Pdx Trp106 had been recognized as early as the 1970’s.[5] In the complex it is in contact with Ala113 of P450cam.[7] Another key interaction, between Pdx Asp38 and P450cam Arg112, was confirmed by the atomic resolution structures. The potential hydrogen bond between the side chains of these residues is thought to be essential for one of the electron transfer coupling pathways.[6, 10] Moreover, several other polar residues are found in the interface that had not been recognized to be relevant for binding before. For instance, in the crystal structures, Pdx Ser42 and Ser44 are positioned in the close proximity to P450cam His352 (less than 5 Å). Another potentially important residue, P450cam His361 is located at the core of the binding interface. The imidazole side chain of P450cam His361 is pointed towards the [2Fe-2S] cluster of Pdx and had previously been proposed to be the part of the ET2 pathway.[11] The flexibility of Pdx Tyr33 is vital to the interaction of PdR-Pdx complex. Upon binding to P450cam, the side chain of Pdx Tyr33 adapts a rigid configuration and forms a hydrogen bond to P450cam Asp125. In this study, site-directed mutagenesis, kinetics and NMR studies were employed
to further probe energetic importance and role of the polar residues involved in Pdx-P450cam interaction. Our experimental results indicated that turnover rates of ET remain intact upon the substitution of these residues by alanine. However, the mutations influence the dissociation rates and binding affinities of the Pdx-P450cam complex. The relevance of the work for understanding the effector activity of Pdx binding will be addressed in discussion.

Results

Previous mutagenesis studies highlighted that the key residues in the formation of the Pdx-P450cam complex and ET include Pdx Asp38, Arg66, Trp106 and P450cam Arg109 and Arg112. The crystal structures of Pdx-P450cam complex revealed a number of other residues that exhibit close contacts (< 4Å) in the interface and participate in polar interactions. To characterize the contributions of residues, Pdx Ser42 and Ser44 and P450cam His352 and His361 were substituted to alanine. In addition, Pdx Y33A and P450cam D125A constructs were prepared. These residues are not found in the core but on the rim of the binding interface, yet were predicted to be important in partner recognition. Figure 1 illustrates the mutation sites used in this study.

To investigate the effects of the mutations on the ET1 reaction, pre-steady state kinetics were studied by stopped-flow spectrophotometry. As described in the early studies, the rate of ET1 can be derived from the rate of formation of carbon

![Figure 1](image_url)

Figure 1. The crystal structure of the Pdx-P450cam complex (PDB entry, 3W9C) is shown in a ribbon representation. The residues substituted by alanine and the heme are labeled and shown in sticks. The [2Fe-2S] cluster is represented in spacefill.
monoxide (CO) bound ferrous P450cam (P450cam\textsuperscript{red-CO}). Equation 1 shows the model of the formation of P450cam\textsuperscript{red-CO} species.

\[
Pdx^{\text{red}} + P450\text{cam}^{\alpha} \xrightleftharpoons[k_{\text{off}}^{\text{ET1}}]{k_{\text{on}}^{\text{ET1}}} Pdx^{\text{ox}} - P450\text{cam}^{\alpha} + P450\text{cam}^{\text{red}}\]

To investigate the effects of the mutations on the ET\textsuperscript{1} reaction, pre-steady state kinetics were studied by stopped-flow spectrophotometry. As described in the early studies, the reaction can be described by a rapidly established binding/dissociation equilibrium and a slow ET reaction. Thus, ten milliseconds after mixing, a steady state condition is valid for Pdx\textsuperscript{red-P450camox}, and its concentration can be taken to be constant. The data are then described by the rate constant \(k_{\text{obs}}\)\textsuperscript{[15]}

\[
k_{\text{obs}} = \frac{k_{\text{ET1}} \cdot k_{\text{on}} \cdot \langle \text{Pdx} \rangle}{k_{\text{off}} + k_{\text{ET1}} + k_{\text{on}} \cdot \langle \text{Pdx} \rangle}
\]

Because \(k_{\text{ET1}} \ll k_{\text{off}} \cdot k_{\text{on}} \cdot \langle \text{Pdx} \rangle\), equation (2) can be written as:

\[
k_{\text{obs}} = k_{\text{ET1}} \cdot \frac{\langle \text{Pdx} \rangle}{K_D + \langle \text{Pdx} \rangle}
\]

By varying the \(\langle \text{Pdx} \rangle\), \(K_D\) and \(k_{\text{ET1}}\) can be fitted. The first points of the stopped-flow curves, before the steady state of the intermediate is established, contain information about the rate of formation of the Pdx\textsuperscript{red-P450camox} complex. Therefore the reaction curves were simulated numerically to extract the values of \(k_{\text{ET1}}, K_D\) and \(k_{\text{off}}\) (Figure 2).

The circles in Figure 2A represent the experimental datasets of P450cam H352A for a range of concentrations of Pdx\textsuperscript{red}. The curves were simulated globally to extract the kinetic parameters. Figure 2B shows the simulated concentrations for all species as function of reaction time for \(\langle \text{Pdx} \rangle = 2.5 \mu\text{M}\), as an example. Note the subtle biphasic behavior representing the pre-steady state and steady state phases of the reaction. The \(k_{\text{off}}\) values are derived from the first few time points, limiting their precision, whereas those of \(k_{\text{ET1}}\) and \(K_D\) are derived from the steady state phase and are better defined. The \(k_{\text{on}}\) was calculated from the \(K_D\) and \(k_{\text{off}}\) values (\(K_D = k_{\text{off}} / k_{\text{on}}\)). Table 1 summarizes \(k_{\text{ET1}}, K_D, k_{\text{on}}\) and \(k_{\text{off}}\) for the WT and mutant complexes.

Figure 2C shows the simulated \(k_{\text{obs}}\) values, obtained from an exponential fit of the simulated [P450cam\textsuperscript{red-CO}] as a function of time to present the results in a familiar way. An advantage of the numerical simulation is that no steady-state assumption is

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made. With this assumption the binding and dissociation are taken to be so fast that a steady state is reached for the Pdxred-P450camox species within the dead time of the stopped-flow apparatus. In that case, the absorption changes for the formation of the P450camox-CO species obey simple exponential behavior, yielding the classical \( k_{obs} \). For low Pdx concentrations, the simulated \( k_{obs} \) values deviate from this \( k_{obs} \). Also no pseudo first-order assumption ([Pdx] \( \gg \) [P450cam]) needs to be made. The simulations show that for low [Pdx] this condition is not met, which slightly influences the rates obtained.

Figure 2. Analysis of ET1 reaction by stopped-flow spectrophotometry. A) \( A_{447} \) is plotted as a function of the reaction time for several Pdxred concentrations. The \( k_{ET1} \) curves were simulated numerically to obtain \( k_{off} \) and \( K_D \). B) The simulated concentrations of all species as function of time for the curve of [Pdx] = 2.5 \( \mu \)M are plotted. C) \( k_{obs}^{sim} \) was plotted against concentration of Pdxred for the WT and mutant proteins. The labels indicate the Pdx and P450cam WT or variants, respectively. The simulated \( k_{obs}^{sim} \) values were obtained by an exponential fit of the simulated curves for the [P450camox-CO], as shown in panel B. The lines connect the positions to guide the eye. The stopped flow measurement was carried out by 1:1 mixing of 2 \( \mu \)M P450camox and 5–150 \( \mu \)M Pdxred at 22°C in CO saturated 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl, 1 mM camphor, 1 mM glucose and an oxygen-scrubbing system.
The $k_{ET1}$ value of wild type (WT) complex was determined to be $42 \pm 1$ s$^{-1}$, which corresponds to the previously reported value of $41 \pm 1$ s$^{-1}$.[11] Compared to WT complex, $k_{ET1}$ values of Pdx and P450cam variants remain intact. In contrast, $K_D$ values for Pdx Y33A and S42A mutants were 20–30-fold higher than for WT (see Table 1). The $k_{off}$ values of these variants were one order of magnitude higher, indicating that the substitution of Pdx Tyr33 and Ser42 alter the binding affinity rather than ET1. The mutations of P450cam Asp125 and His352, which form hydrogen bonds to Pdx Tyr33 and Ser42, respectively, result in modest changes of $K_D$ with four to eight fold increase.

To investigate the extent of the interaction of Tyr33(Pdx)–Asp125(P450cam) and Ser42(Pdx)–His352(P450cam), double-mutant cycle (DMC) analysis was performed. The free energy of interaction, $\Delta \Delta G_{int}$ (see Experimental Procedures, eq. 6),[16] between the residue pairs was calculated to be $-4.2$ kJ/mol and $-3.7$ kJ/mol for Tyr33(Pdx)–Asp125(P450cam) and Ser42(Pdx)–His352(P450cam), respectively. Since the experimental error of $\Delta \Delta G_{int}$ for this system was calculated to be ±1.0–1.7 kJ/mol, both cases indicate significant favorable interactions.

Having established the effect of mutations on the ET1 process, we examined the steady state kinetics of NADH oxidation. Camphor turnover activity of P450cam is known to be tightly coupled with two ET events (ET1 and ET2). Since the ET steps are rate limiting in the P450cam reaction cycle,[14] the catalytic turnover was measured by the NADH consumption rate in the PdR-Pdx-P450cam reconstituted system as previously described.[17] In this assay, Pdx is kept in the reduced state by ensuring [PdR] >> [P450cam]. The spectral changes at 340 nm, indicative of NADH oxidation, were used to determine the steady state P450cam turnover rate. To minimize the contributions of the binding affinities to the ET rates, the concentration of Pdx was 100 μM, which is above the $K_D$ values determined in the stopped-flow experiments. As shown in Table 3, the catalytic turnover of WT(Pdx)-WT(P450cam) complex was found

<table>
<thead>
<tr>
<th>Pdx</th>
<th>P450cam</th>
<th>$k_{ET1}$ (s$^{-1}$)</th>
<th>$K_D$ (μM)</th>
<th>$k_{on}$ (10$^7$ M$^{-1}$s$^{-1}$)</th>
<th>$k_{off}$ (10$^2$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>42 ± 1</td>
<td>2.2 ± 0.2</td>
<td>3.6–11.4</td>
<td>0.8–2.5</td>
</tr>
<tr>
<td>Y33A</td>
<td>WT</td>
<td>40 ± 1</td>
<td>50 ± 5</td>
<td>6–8</td>
<td>30–40</td>
</tr>
<tr>
<td>S42A</td>
<td>WT</td>
<td>42 ± 2</td>
<td>55 ± 8</td>
<td>5.5–7.5</td>
<td>30–41</td>
</tr>
<tr>
<td>S44A</td>
<td>WT</td>
<td>42 ± 1</td>
<td>6 ± 0.5</td>
<td>6.7–11.7</td>
<td>4–7</td>
</tr>
<tr>
<td>WT</td>
<td>D125A</td>
<td>36 ± 2</td>
<td>9 ± 0.5</td>
<td>5.6–11.1</td>
<td>5–10</td>
</tr>
<tr>
<td>WT</td>
<td>H352A</td>
<td>42 ± 2</td>
<td>16 ± 2</td>
<td>5.6–10.6</td>
<td>10–19</td>
</tr>
<tr>
<td>WT</td>
<td>H361A</td>
<td>35 ± 3</td>
<td>11 ± 2</td>
<td>7–13</td>
<td>7–12</td>
</tr>
<tr>
<td>Y33A</td>
<td>D125A</td>
<td>42 ± 1</td>
<td>38 ± 2</td>
<td>2.6–5.3</td>
<td>10–20</td>
</tr>
<tr>
<td>S42A</td>
<td>H352A</td>
<td>42 ± 2</td>
<td>90 ± 10</td>
<td>2.2–5.6</td>
<td>20–50</td>
</tr>
</tbody>
</table>

Table 1. Kinetic parameters of ET1. The reaction conditions were the same as given in the caption of Figure 2.
to be $38.2 \pm 0.2 \text{ s}^{-1}$, which is close to the reported value of $35.9 \pm 1.1 \text{ s}^{-1}$. Similar values were measured for all mutant complexes (Table 2). On the basis of equation (3), the maximum turnover at $[\text{Pdx}] = 100 \mu\text{M}$ ($k_{\text{max}}$) can be predicted assuming ET1 is the rate limiting step. The results indicate that the effect of the mutations is solely on binding affinity and that the ET steps are not affected significantly.

**Table 2.** The steady state turnover activities of P450cam catalytic cycle. NADH consumption rates were monitored at 340 nm at 22°C. The reaction mixture contained 0.05 μM P450cam, 100 μM Pdx, 1.0 μM PdR and 400 μM NADH in 50 mM Tris-HCl buffer, pH 7.4, consisting of 100 mM KCl and 1 mM camphor.

<table>
<thead>
<tr>
<th>Pdx</th>
<th>P450cam</th>
<th>$k_{\text{max}}$</th>
<th>NADH consumption (s$^{-1}$)</th>
<th>relative to WT-WT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>41.1</td>
<td>$38.2 \pm 0.2$</td>
<td>100</td>
</tr>
<tr>
<td>Y33A</td>
<td>WT</td>
<td>26.7</td>
<td>$34.9 \pm 2.5$</td>
<td>91</td>
</tr>
<tr>
<td>S42A</td>
<td>WT</td>
<td>27.1</td>
<td>$34.1 \pm 0.7$</td>
<td>89</td>
</tr>
<tr>
<td>S44A</td>
<td>WT</td>
<td>39.6</td>
<td>$37.4 \pm 0.6$</td>
<td>98</td>
</tr>
<tr>
<td>WT</td>
<td>D125A</td>
<td>33.0</td>
<td>$40.3 \pm 0.7$</td>
<td>106</td>
</tr>
<tr>
<td>WT</td>
<td>H352A</td>
<td>36.2</td>
<td>$36.3 \pm 0.6$</td>
<td>95</td>
</tr>
<tr>
<td>WT</td>
<td>H361A</td>
<td>31.5</td>
<td>$34.1 \pm 0.4$</td>
<td>89</td>
</tr>
<tr>
<td>Y33A</td>
<td>D125A</td>
<td>30.4</td>
<td>$26.3 \pm 0.3$</td>
<td>69</td>
</tr>
<tr>
<td>S42A</td>
<td>H352A</td>
<td>22.1</td>
<td>$17.9 \pm 0.6$</td>
<td>47</td>
</tr>
</tbody>
</table>

The mutations Y33A and S42A resulted in a large decrease of the affinity of Pdx for P450cam. To address the question of why these mutants significantly perturb the binding mode, NMR $^{15}$N HSQC spectra (Figure 3A) were recorded of all the Pdx mutants and compared to the Pdx WT to see if the mutation caused substantial structural changes. The differences of the chemical shifts between Pdx mutants and WT (in absence of P450cam) were evaluated using the average of chemical shift differences ($\Delta\delta_{\text{avg}}$) (Figure 3C–E).

The NMR spectra of both Pdx S42A and S44A variants overlapped well with Pdx WT without significant $\Delta\delta_{\text{avg}}$ (Figure 3A, D, E), indicating that they are not structurally different. It is noted that NMR signals of the amide of residues 36-50 are broadened beyond detection because they are close to the paramagnetic [2Fe-2S] cluster in Pdx (Figure 3C–E). In the NMR spectrum of Pdx Y33A several peaks were shifted when compared to the spectrum of Pdx WT (Figure 3A). The $\Delta\delta_{\text{avg}}$ values shown in Figure 3C were mapped on the crystal structure of Pdx (PDB: 1XLP) [18], Figure 3B. The large values of $\Delta\delta_{\text{avg}}$ were observed not only the residues directly adjacent to the mutation site but also extended to spatially neighboring residues. Pdx residue Asp103, in particular, was not found in the spectrum, possibly due to its large chemical shift change or its
extensive line broadening. Taken together, we conclude that the structure of Pdx Y33A mutant is likely to be altered by mutation, which may contribute to the reduction of the affinity for P450cam.

**Discussion**

The structure of the Pdx-P450cam complex provided molecular insight into the ET pathway and effector role of Pdx (see chapter 3 for the details). In this work, mutagenesis was employed to examine the importance of several polar interface residues highlighted by the structure of the complex. Most of these had not been considered to be important before the structure was available.
Stopped-flow studies indicated that most of the mutations do not influence the rate of ET1 reaction. This is in line with the suggestions in the previous study (chapter 3), where the ET pathways were predicted by the program HARLEM. None of the residues studied in this report are directly involved in the suggested ET1 pathways. However, Pdx Y33A and S42A substitutions lower the binding affinity by 20–30 fold. Pdx Tyr33 is located at the beginning of the loop region that surrounds the [2Fe-2S] cluster (Figure 3B). An NMR study demonstrated that Tyr33 is mobile on the Pdx surface and occupies several conformational states on the μs–ms timescale. The result of our NMR data indicated that the substitution of Tyr33 with alanine changes the structure of the loop region, which transmits to distant residues of Pdx, including Asp103. The functional role of Tyr33 has been recognized in the binding of Pdx both to PdR and P450cam. The DMC analysis indicated that Pdx Tyr33 has a favorable interaction with P450cam Asp125. As illustrated in the crystal structures of the complex, Pdx Tyr33 adapts a rigid configuration upon P450cam binding and forms a hydrogen bonding to P450cam Asp125. Pdx Ser42 and Ser44 are positioned adjacent to the [2Fe-2S] cluster (Figure 1) and are part of the polar interactions within the interface. In the DMC analysis, Pdx Ser42 was shown to have a favorable interaction with P450cam His352. The chemical shift perturbation analysis showed that the structure of Pdx S42A mutant is similar to Pdx WT, suggesting that the side chain of Ser42 make a major contribution to the interaction. P450cam His361 is located at the proximal site of heme-binding loop and is in close vicinity to Cys357, the axial ligand of the heme iron. A previous mutagenesis study demonstrated that substitution of the adjacent residue Gln360 with glutamate causes the $K_D$ to increase with 25-fold and the $k_{ET1}$ to decrease with 5-fold. Moreover, a computational study predicted that along with Gln360, His361 is a part of the ET2 pathway. The stopped flow measurements on P450cam H361A resulted in the small reduction of the binding affinity and ET1 rate, which suggests that P450cam His361 plays a minor role in ET.

To investigate if the mutations influence the overall ET (ET1+ET2), the catalytic turnover activity of P450cam was monitored by measuring the NADH consumption rate. In accord with the results of the ET1 reaction, all Pdx and P450cam mutants exhibit a turnover rate close to that of WT (considering the higher $K_D$ in some cases). It is concluded that the polar interactions in the Pdx-P450cam interface studied here contribute to the complex formation rather than ET reactions.

In this work, the polar residues that were targeted for the substitution were established on the basis of the crystal structures of Pdx-P450cam complex. In the structures, P450cam is found in open and semi-open conformation (see chapter 3 for the details). The authors of reference interpreted this finding to implicate that Pdx forces P450cam into the open state upon binding and related this to the effector activity. The authors of reference suggested the open state may be a crystallization effect. A very recent study employing double electron-electron resonance (DEER)
spectroscopy provide evidence showing that P450cam adapts a closed conformation in the ET events in solution.\cite{21} To evaluate the changes in the binding interface with the conformational state of P450cam, the crystal structures of Pdx-P450cam complex (PDB entries, 3W9C, 4JX1 and 4JWS)\cite{7, 8} were superimposed with the closed state of free P450cam (PDB entry, 1DZ4),\cite{22} as shown in Figure 4.

The crystal structures of both the oxidized (3W9C\cite{8} and 4JWS\cite{7}) and dithionite reduced, CO bound (4JX1\cite{7}) Pdx-P450cam complex have been reported. However, the actual redox state of the metals is difficult to establish in the crystal because the metals are prone to reduction by X-ray radiation.\cite{18}

The comparison reveals that majority of the prominent interactions in the binding interface changes only very subtly between the different P450cam conformations (open, semi-open and closed). The hydrogen bond between Asp38$_{\text{(Pdx)}}$–Arg112$_{\text{(P450cam)}}$
important for ET, remains intact. The minimal distance between Pdx Trp106 and P450cam A113 is only slightly larger in the model of the complex of Pdx with the closed state of P450cam than in the ox-ox complex (4.4 Å vs. 3.8 Å in 3W9C). The H-bond between the Pdx Tyr33 and P450cam Asp125 is also present in both these complexes. Only the P450cam His352 is significantly further from Pdx Ser42 (5.5 Å vs. 4.2 Å). Given that the complex of the closed state with Pdx bound is not a crystal structure but a superposition, these differences can be considered small and it is reasonable to assume that Pdx is capable to bind both closed and open conformational states of P450cam. Thus, we believe it is plausible that the open/closed state of the complex is influenced by the crystal packing effects, as we suggested before (see chapter 3)[8] and was also concluded by Myers et al.[21] This implies that the question of what represents the effector activity remains open. Various suggestions have been made. The classic model of a “push” effect suggests that Pdx binding is physically transmitted to the heme-binding loop of P450cam and accelerates ET by influencing the axial thiolate ligand of heme iron.[23] Another mechanism suggested by Asciutto et al. involves cis/trans isomerization of the Ile88–Pro89 peptide bond of P450cam.[24] Upon the complex formation, the structural perturbation in the Pdx binding site mechanically conveys to remote regions of P450cam, which leads to the closure of the substrate access channel. The substrate is thereby trapped in the active site of P450cam, which minimizes the solvation of the substrate and the diffusion of physiologically harmful peroxide intermediates.[24] Structural studies of the complex in solution using NMR spectroscopy can complement the frozen solution (EPR) and crystal studies (X-ray diffraction) and may be able to shed more light on the issue.

In conclusion, mutagenesis studies presented in this report show the importance of the interactions of the polar residues in the Pdx-P450cam interface. These polar interactions were found to be important in the partner recognition, rather than in ET.

**Experimental procedures**

**Cloning**

The plasmid harboring the cDNA of PdR was purchased from GeneArt®, Life technologies (Invitrogen). The gene was designed to be subcloned into the pET28a expression vector by introducing NcoI and XhoI restriction sites. A His tag sequence was not included. The pET28a vectors containing cDNA of Pdx and P450cam were prepared as described in chapter 3.[8] Pdx Y33A, Pdx S42A, Pdx S44A, P450cam D125A/C334A, P450cam H352A/C334A and P450cam H361A/C334A constructs were prepared by following the Quikchange® site-directed mutagenesis protocol (Stratagene, La Jolla, CA). The cysteine of P450cam at position 334 was substituted with alanine. The C334A mutation has been reported to prevent the dimerization of the protein and retain the catalytic turnover activity.[17] P450cam C334A was treated as WT in this report.
Protein production

All Pdx and P450cam variants were produced as described in chapter 3. The gene expression and purification of PdR was performed by following the previous report with several modifications. Briefly, pET28a plasmid harboring PdR gene was transformed into the BL21 Star™ (DE3) chemically competent E. coli strain (Life technologies, Invitrogen). Single colonies were picked and inoculated into 5 mL LB medium with 50 μg/mL kanamycin at 37°C for 4 hours. Two mL of pre-culture was transferred into 500 mL LB medium with 50 μg/mL kanamycin and incubated at 37°C until $A_{600\text{nm}} = 0.6–0.7$. Gene expression was induced by adding 0.5 mM IPTG. The cultures were incubated at 22°C for 40–50 hours. Cells were resuspended in a KP buffer (20 mM KP, pH 8.0, 150 mM KCl, 1 mM DTT, 1 mM PMSF and 1 mM DNase). Samples were flash frozen in N₂ (l) and stored at −80°C.

The resuspended cells were defrosted rapidly at room temperature. A few mg of crystals of lysozyme were added and the sample was stirred for 30 minutes at 22°C. The lysate was further disrupted by French-press and sonication and spun down by centrifugation at 35000 r.p.m. for 30 minutes at 4°C. The pH of supernatant was adjusted to ~7.5 by addition of a few drops of KOH. Ammonium sulfate powder was added slowly to the sample to 30% (w/v). The sample was then stirred in cold room for 30 minutes. Following centrifugation at 35000 r.p.m. for 30 minutes at 4°C, the supernatant was dialyzed overnight against 4 L of KP buffer A (20 mM KP, pH 7.5, 1 mM DTT). The dialysate was loaded on a HiTrap DEAE FF anion-exchange column (60 mL). The protein was eluted with a linear gradient of 0.1-0.3 M KCl in KP buffer A and the yellow fractions were collected. The fractions were pooled and concentrated by ultrafiltration using a Centricon 3K (Millipore) and injected into a Superose 12 size exclusion column pre-equilibrated with KP buffer B (20 mM KP, pH 7.4, 150 mM KCl, 1 mM DTT). Ammonium sulfate crystals were added (30% w/v) to the pooled fractions and the sample was stirred for 20 minutes at 4°C. Subsequently, the sample was centrifuged for 20 minutes to remove precipitation and the supernatant was applied to a HiTrap Phenyl FF hydrophobic column (10 mL). The yellow fractions containing PdR were collected after elution with a linear gradient 20–0% ammonium sulfate in KP buffer A. The fractions with the $A_{280\text{nm}}/A_{455\text{nm}}$ value below 7.0 were collected. Then the fractions were pooled and dialyzed overnight against 4 L of KP buffer B. The concentration of PdR was calculated using extinction coefficient of 11.0 mM⁻¹cm⁻¹ at 455 nm wavelength. Approximately 30 mg of PdR was obtained from 1 L of culture.

Stopped flow spectrophotometry assay

Stopped flow experiments were performed on SX-18MV Microvolume Stopped-Flow Spectrofluorometer (Applied Photophysics). D-glucose (1 mM) and 1 unit/mL (final concentration) of oxygen scrubbing proteins, glucose oxidase and catalase were added to Tris buffer C (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM camphor, 1% MeOH) and made anaerobic by flushing argon and subsequently carbon monoxide (CO) gases for
20 minutes each. Immediately prior to experiment, NADH was added to Pdx reaction mixtures and incubated for a few minutes at room temperature. Final reaction mixtures contained 5–150 μM Pdxred, 0.1 μM PdRred, 400 μM NADH and saturated level of CO, whereas the P450cam reaction mixture contained 2 μM P450camox and CO. The absorption at 447 nm wavelength was monitored for one second at 22°C.

DMC analysis
The dissociation constant ($K_D$) relates to the difference in free energy between bound and unbound states, equation (4)

$$\Delta G_B = RT \ln K_D$$  \hspace{1cm} (4)

The difference in $\Delta G_B$ caused by a substitution of a residue by alanine ($\Delta \Delta G_B$) is calculated by equation (5)

$$\Delta \Delta G_B = RT \ln \left( \frac{K_D^{\text{mut}}}{K_D^{\text{WT}}} \right)$$ \hspace{1cm} (5)

where $K_D^{\text{mut}}$ and $K_D^{\text{WT}}$ are the dissociation constants of the Pdx and/or P450cam mutants and WT complex, respectively. The free energy of interaction ($\Delta \Delta G_{\text{int}}$) between two residues equals to:

$$\Delta \Delta G_{\text{int}} = \Delta \Delta G_B^{\text{mut,mut}}_{(\text{Pdx, P450cam})} - \Delta \Delta G_B^{\text{mut}}_{(\text{Pdx})} - \Delta \Delta G_B^{\text{mut}}_{(\text{P450cam})}$$ \hspace{1cm} (6)

where $\Delta \Delta G_B^{\text{mut,mut}}_{(\text{Pdx, P450cam})}$ represents $\Delta \Delta G_B$ of P450cam and Pdx variants derived from equation (5). A negative value of $\Delta \Delta G_{\text{int}}$ indicates that the interaction between the mutated residues is attractive whereas a positive value of $\Delta \Delta G_{\text{int}}$ suggests a repulsive interaction.\[16\]

NADH consumption rates
All of the P450cam WT and variants were exchanged into Tris buffer C by Superprose 12 size exclusion column immediately before each experiment. The NADH consumption rates were measured on Perkin Elmer Lambda 25 spectrophotometer as described by Nickerson and Wong.\[17\] Briefly, 400 μM of NADH (final concentration) was added to 0.2 mL reaction mixture containing 0.05 μM P450cam, 100 μM Pdx, 1.0 μM PdR to initiate the reaction. The absorption at 340 nm was monitored for 180 s at 22°C. The catalytic turnover of NADH oxidation was determined from the slope of a linear region in the plot of $A_{340}$ vs. time. The concentration of NADH was estimated using extinction coefficient of 6.22 mM⁻¹cm⁻¹ at 340 nm.

NMR
Approximately 1 mM of $^{15}$N Pdx WT and variants were prepared in Tris buffer C with 6% D₂O for lock. All NMR samples were measured on a Bruker Avance III 600-MHz spectrometer equipped with a TCI-Z-GRAD cryoprobe at 298 K. $^{15}$N HSQC spectra were processed with NMRPipe\[27\] and analyzed by CCPNMR.\[28\] The NMR assignment
of Pdx amide resonances were based on previous work.\textsuperscript{[29]} Chemical shift perturbations (CSP average) were analyzed as previously described.\textsuperscript{[30]}

References
