Putidaredoxin-Cytochrome P450<sub>cam</sub> Interaction

SPIN STATE OF THE HEME IRON MODULATES PUTIDAREDOXIN STRUCTURE*

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During the monoxygenase reaction catalyzed by cytochrome P450<sub>cam</sub> (P450<sub>cam</sub>), a ternary complex of P450<sub>cam</sub>, reduced putidaredoxin, and d-camphor is formed as an obligatory reaction intermediate. When ligands such as CO, NO, and O<sub>2</sub> bind to the heme iron of P450<sub>cam</sub> in the intermediate complex, the EPR spectrum of reduced putidaredoxin with a characteristic signal at 346 milliteslas at 77 K changed into a spectrum having a new signal at 348 milliteslas. The experiment with O<sub>2</sub> was carried out by employing a mutant P450<sub>cam</sub> with Asp<sup>251</sup>→Asn or Gly where the rate of electron transfer from putidaredoxin to oxyferrous P450<sub>cam</sub> is considerably reduced. Such a ligand-induced EPR spectral change of putidaredoxin was also shown in situ in Pseudomonas putida. Mutations introduced into the neighborhood of the iron-sulfur cluster of putidaredoxin revealed that a Ser<sup>44</sup>→Gly mutation mimicked the ligand-induced spectral change of putidaredoxin. Arg<sup>109</sup> and Arg<sup>112</sup>, which are in the putative putidaredoxin binding site of P450<sub>cam</sub>, were essential for the spectral changes of putidaredoxin in the complex. These results indicate that a change in the P450<sub>cam</sub> active site that is the consequence of an altered spin state is transmitted to putidaredoxin within the ternary complex and produces a conformational change of the 2Fe–2S active center.

Cytochrome P450<sub>cam</sub> (P450<sub>cam</sub>) catalyzes the conversion of d-camphor to 5-exo-hydroxycamphor at the expense of 1 mol each of NADH and dioxygen (1). In the reaction, two reducing equivalents from NADH are sequentially transferred to P450<sub>cam</sub> via two redox-linked proteins, putidaredoxin reductase (PdR), a FAD-containing enzyme, and putidaredoxin (Pd), a 2Fe–2S protein, where Pd receives an electron from PdR and transfers it to P450<sub>cam</sub>. In the first electron transfer, reduced Pd combines with ferric d-camphor-bound P450<sub>cam</sub> and reduces it to the ferrous form. In the second electron transfer, reduced Pd forms a quarternary complex with the ferrous dioxygen complex of d-camphor-bound P450<sub>cam</sub> to decompose it into the reaction products, i.e. hydroxycamphor, water, d-camphor-free ferric cytochrome P450<sub>cam</sub>, and oxidized Pd (2, 3).

The role of Pd described above is not replaceable by the other electron donors. Low potential iron-sulfur protein such as spinach ferredoxin and bovine adrenodoxin can donate the first electron to P450<sub>cam</sub> but not the second electron, whereas reduced rubredoxin and cytochrome b<sub>5</sub>, which are incapable of giving the first electron, can provide the second electron yielding the reaction products (4). Much effort has been made therefore to understand the interaction between P450<sub>cam</sub> and Pd; results of UV-visible, electron paramagnetic resonance (EPR), and recent resonance Raman studies indicated that high spin ferric P450<sub>cam</sub> in the presence of d-camphor was partially converted to a low spin form upon the binding of oxidized Pd (5–7). Associated with this spin conversion, the heme axial ligand stretching mode ν<sub>fex</sub> of 1940 to 1932 cm<sup>−1</sup> was observed (8). With the ferrous d-camphor-bound P450<sub>cam</sub>, the binding of reduced Pd to P450<sub>cam</sub> produced a shift in ν<sub>C O</sub> of the heme-bound CO from 1940 to 1932 cm<sup>−1</sup> (9). However, the significance of such structural change evoked by the binding of Pd in the reaction catalyzed by P450<sub>cam</sub> is unknown.

On the other hand, only a few reports have been found in the literature on the structural changes of Pd upon binding to P450<sub>cam</sub>. Sligar and Gunalsus (10) reported an elevation of redox potential upon binding to P450<sub>cam</sub> by 10 mV, whereas Pochapsky et al. (11) noted two-dimensional NMR signal changes of some amino acid residues in Pd upon P450<sub>cam</sub> binding. In this study, we examined the effects of P450<sub>cam</sub> binding to the structure of Pd with the aid of EPR spectroscopy. Results revealed that the binding of reduced Pd with ferrous d-camphor-bound P450<sub>cam</sub> induced an EPR-detectable conformational change in the iron-sulfur cluster of reduced Pd. Furthermore, a change in the spin state of heme iron by ligand binding to P450<sub>cam</sub> in the same complex caused a distinct and even larger conformational change in Pd than those that occurred in the complex formation. Thus, the structural change in the
active site of P450<sub>cam</sub> occurred as the consequence of the altered spin state is transmitted to the redox center of another component protein in the intermediate complex presumably through pathway(s) including particular surface amino acid residues.

**EXPERIMENTAL PROCEDURES**

**Growth of Bacteria—**Pseudomonas putida cells (strain ATCC17453) were grown on d-camphor as a sole carbon source as described elsewhere (12). The cells harvested by centrifugation were washed twice with 50 mM potassium phosphate, pH 7.4, containing 50 mM KCl and 1 mM d-camphor (buffer A), suspended in the same buffer, and stored at −20 °C until use. For the expression of P450<sub>cam</sub>, Pd, and PdR, Escherichia coli strain JM109 was grown in LB medium supplemented with ampicillin and isopropylthio-β-D-galactoside as described previously (13).

**Enzyme Preparations and Mutagenesis—**The P450<sub>cam</sub> and Pd genes were mutated by employing an oligonucleotide-directed mutagenesis system according to the manufacturer’s protocol (Takara Biomedical, Kyoto, Japan). The wild-type P450<sub>cam</sub> and its mutants expressed in E. coli were purified with the procedures described previously (13). Puriﬁed preparations with an RZ value (A<sub>392</sub>/A<sub>280</sub>) greater than 1.5 were employed in this study. Pd, its mutant protein, and PdR were purified from E. coli to a homogeneity on SDS-polyacrylamide gel electrophoresis according to the methods of Gunsalus and Wagner (14). Chemicals used were of analytical reagent grade and were used without further purification.

**EPR Spectroscopy**—A 200-μl solution containing either Pd or Pd plus P450<sub>cam</sub> in buffer A was transferred into a screw-topped EPR tube. After screwing a cap with rubber septum into the tube, the protein was degassed by repeating three cycles of evacuation and subsequent flushing with oxygen-free N<sub>2</sub> gas. Then proteins were reduced with a trace amount of solid sodium dithionite, which had been placed on the inside of the tube, and then allowed to stand on ice for 5 min to complete the reduction. The mixture was frozen by immersing the tube into liquid nitrogen, which usually took 15–30 s. When necessary, CO or NO gas was anaerobically introduced to the tube containing reduced Pd and ferrous P450<sub>cam</sub>. When O<sub>2</sub> was introduced in place of CO and NO, the EPR tube was rapidly immersed into liquid nitrogen to freeze the protein as quickly as possible after the mixing of the protein and O<sub>2</sub>; the procedure was completed within 3 s.

EPR measurements were carried out on a Varian E-12 EPR spectrometer (San Fernando, CA) at 9.363 GHz of X-band microwave frequency under the following instrumental parameters: microwave power, 5 milliwatts; modulation frequency, 100 kHz; and modulation amplitude, 0.5 mT. An immersion Dewar flask was used for the measurements at liquid nitrogen temperature (77 K). The microwave frequency and magnetic field of the instruments were calibrated by a microwave frequency counter (Takeda Riken, model TR5212) using an Mn<sup>2+</sup> signal in MgO as a standard. Accuracy of the g values was approximately ±0.001. Other details were described under appropriate figure legends.

**RESULTS**

**Effects of Carbon Monoxide on the EPR Spectrum of the P450<sub>cam</sub>-Putidaredoxin Complex**—EPR spectrum of reduced Pd was measured in the presence and absence of camphor-bound ferrous P450<sub>cam</sub> and CO at 77 K (Fig. 1). The spectrum of reduced Pd in its free state has a signal at 2.02 (331 mT) and g<sub>z</sub> signal at 1.94 (345 mT) (Fig. 1, A) as originally reported by Tsihrib et al. (15). Upon the addition of camphor-bound ferrous P450<sub>cam</sub> Pd changed its spectrum in the g<sub>z</sub> region (Fig. 1, B). The trough of the derivative type signal was broadened with an appearance of a shoulder around 348 mT. The spectral changes
were more evident in a difference spectrum presented in Fig. 2, a (spectrum B minus A of Fig. 1). Titration of reduced Pd with ferrous P450\textsubscript{cam} in the presence of \textit{d}-camphor indicated that changes in the spectrum of Pd were caused by the formation of a 1:1 complex of the two proteins (data not shown).

The addition of CO to the 1:1 complex resulted in further spectral changes, particularly in the \( g \) region as seen in Fig. 1, C; the position of the trough at 346 mT moved to 348 mT with a shoulder at 346 mT. The CO-induced spectral changes were also demonstrated in a difference spectrum (spectrum C minus B of Fig. 1) in Fig. 2, b, showing clearly that the CO addition caused larger spectral changes in Pd than those that occurred upon binding to P450\textsubscript{cam}. The two difference spectra in Fig. 2 also indicated that spectral changes of Pd induced by cytochrome P450 binding to Pd and those of CO binding are different from each other in that the positions of the major trough and peak were clearly distinct. As seen in Fig. 1, C, a minor change was also observed in the \( g \) region by the addition of CO; a small increase in the \( g \) value was observed. In the absence of P450\textsubscript{cam}, exposure of reduced Pd to CO did not alter the spectrum (data not shown).

In Fig. 3, Pd was titrated with an increasing amount of ferrous P450\textsubscript{cam} in the presence of excess CO. With an increment of P450\textsubscript{cam}, the intensity of difference spectra recorded against reduced Pd increased following a set of isosbestic points. Further spectral change was not observed upon addition of an equimolar amount of P450\textsubscript{cam}, indicating that the observed spectral changes are because of the formation of a 1:1 complex with CO-ferrous P450\textsubscript{cam}. The CO-induced spectral changes of reduced Pd were found to be reversible; the original spectrum was restored after an extensive evacuation of CO under an illumination of light (data not shown). Thus, the binding of CO to the heme in P450\textsubscript{cam} altered the EPR spectrum of reduced Pd in its binary complex with camphor-bound ferrous P450\textsubscript{cam} suggesting that Pd senses the spin state changes of P450\textsubscript{cam} in the complex.

The effect of CO on the EPR spectrum was also observed in situ in \textit{P. putida} cells where PdR, Pd, and P450\textsubscript{cam} were contained in a 1:8:8 ratio (16). The cells, which were reduced with sodium dithionite, showed an EPR spectrum of reduced Pd complexed with ferrous P450\textsubscript{cam}, together with an unidentified signal around \( g \) (333 mT) (Fig. 4, A). The \( g \) signal arising from Pd changed on exposure of the cells to CO to an almost identical one that is shown in Fig. 1, C (Fig. 4, B).

**Effects of Nitric Oxide on the EPR Spectrum of the P450\textsubscript{cam}**

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Next we examined the effects of NO binding to the heme of P450\textsubscript{cam} in the complex of Pd with camphor-bound ferrous P450\textsubscript{cam}. In Fig. 5, EPR spectra of reduced Pd, NO-ferrous P450\textsubscript{cam}, and reduced Pd in the presence of NO-ferrous P450\textsubscript{cam} are compared. The \( g \) signal of Pd in the complex with NO-ferrous P450\textsubscript{cam} (Fig. 5, C) was indistinguishable from that of the CO-induced signal in Fig. 1, C. Other signals, except for the \( g \) signal of reduced Pd, were derived from NO-ferrous P450\textsubscript{cam}, whose spectrum is presented in Fig. 5, B for comparison. Inspection of the spectra of NO-ferrous P450\textsubscript{cam} and its complex with reduced Pd also leads to the finding that the \( g = 2.075 \) signal of NO-ferrous P450\textsubscript{cam} shifted to \( g = 2.070 \) upon complex formation with Pd. Such a spectral change suggests an alteration of the NO-heme geometry as had been observed for the structure of heme-bound CO in CO-ferrous P450\textsubscript{cam} (9). NO is known to react with an iron-sulfur protein yielding an EPR-active nitrosyl Fe\(^{3+}\) (17), but it was not the case here; reduced Pd did not exhibit any spectral change upon exposure to NO, although an addition of NO to oxidized Pd, which is EPR silent, was found to produce EPR signals indicative of a nitrosyl Fe\(^{3+}\) formation (data not shown). Thus, the aforementioned effects of NO on Pd associ-
ated with P450<sub>cam</sub> suggested that NO binding to the heme iron of the cytochrome was sensed by Pd as was found in CO binding. A similar spectral change of Pd was obtained by in situ EPR measurements of <i>P. putida</i> cells with NO.

**Effects of KCl and Pudaredoxin Reductase on the Ligand-induced Spectral Change**—The present findings suggested that reduced Pd sensed the binding of ligands such as CO and NO to the cytochrome P450 heme when Pd forms a 1:1 complex with d-camphor-bound ferrous P450<sub>cam</sub>. The requirement for the complex formation in this phenomenon was tested by the addition of reagents that promote the dissociation of a protein-protein complex. The Pd-P450<sub>cam</sub> complex is known to be stabilized by ion pairs between acidic and basic residues of Pd and P450<sub>cam</sub>, respectively (11, 18–20). When KCl concentration in the buffer (50 mM potassium phosphate, pH 7.4, supplemented with 50 mM KCl and 1 mM d-camphor) was raised to 1 M, the ligand-induced spectral change was found to decrease to approximately 40% of that in the original buffer (Fig. 6, B). In this experiment, the titration data shown in Fig. 3 were used as the calibration curve.

It has been suggested by Holden et al. (19) that the binding site of P450<sub>cam</sub> in Pd is overlapped with that of PdR based on the observation that mutations of some amino acid residues in Pd altered both interactions of Pd with P450<sub>cam</sub> and PdR. Thus, the Pd-P450<sub>cam</sub> complex is expected to dissociate by an excess amount of PdR. Addition of a 5 M excess of PdR over the complex made the CO-induced spectral change decrease by about 60% (Fig. 6, C), giving additional evidence for a competitive binding of PdR to Pd with P450<sub>cam</sub> and PdR. Thus, the Pd-P450<sub>cam</sub> complex was frozen immediately after exposure to dioxygen.

**Effects of Oxygen Binding on the P450<sub>cam</sub>-Pudaredoxin Complex**—A complex of reduced Pd with oxyferrous P450<sub>cam</sub> is not stable and hence does not allow its EPR measurements under ordinary conditions; oxygen is a substrate for the P450<sub>cam</sub>-catalyzed reaction. One way to see the effects of dioxygen coordination to ferrous P450<sub>cam</sub> on the spectra of reduced Pd is to employ a mutant P450<sub>cam</sub>, which forms a stable complex. A mutant of P450<sub>cam</sub> with Asn, Ala, or Gly substituted for Asp<sup>251</sup> may be such a mutant; the oxyferrous form of these mutants readily forms the complex with reduced Pd, but its decay into reaction products is 500–1000-fold smaller than that of the wild-type P450<sub>cam</sub> (21, 22). Then the effects of dioxygen on Pd were tested in the complex of Pd and the mutant P450<sub>cam</sub>. Fig. 7 shows the EPR spectra of reduced Pd in its complex with the two mutants of P450<sub>cam</sub> before and after oxygenation. The complex was frozen immediately after expos...
Lys112 did not show spectral changes observed in the complex of P450 cam. The effects of mutations of Arg79, Arg109, and Arg112 as well as spectrum of reduced Pd in the g_1 field (Fig. 7, A and B). These spectral characteristics were very similar to the spectral changes of Pd on exposing the Pd-P450cam complex to CO or NO. As will be described below, the mutation at Asp251 (per se) did not affect the CO- and NO-induced EPR spectral changes of Pd (data not shown).

Essential Amino Acid Residues for the Ligand-induced Spectral Changes—To elucidate the mechanism(s) by which CO, NO, and O_2 bind to the heme in P450cam and to induce the structural changes of Pd in the P450cam-Pd complex, we tested the effects of mutations of Arg^{109}, Arg^{109}, and Arg^{112} as well as those of Asp^{251} and Thr^{252} in P450cam on the EPR spectral changes. The former 3 amino acid residues are in the putative binding site of P450cam for Pd (11), whereas the latter two locate in the distal region of the active center of P450cam (23) and are essential for the monoxygenation reaction (13, 21, 24).

A mutation of Ala^{252}, Asn^{251}, or Gln^{245} gave similar results with those obtained with the wild-type P450cam (data not shown), whereas the mutation at positions 109 and 112 did not. Reduced Pd complexed with the mutant with Gln^{109}, Lys^{109}, Met^{112}, or Lys^{112} did not show spectral changes observed in the complex formation with the wild-type P450cam (Fig. 8, dotted line in spectrum A, B, C, or D, respectively). Furthermore, upon the addition of CO to the complex, a spectral change, which occurred with the wild-type P450cam, was not observed except in the Lys mutants; a subtle change (indicated by arrows) was observed. In the measurements with the mutants at positions 109 and 112, the concentrations of P450cam were raised from 200 µM to 2 mM against 200 µM Pd. We did so because a mutation at a surface amino acid residue such as Arg^{112} → Lys has been known to lower the binding affinity of P450cam to reduced Pd (20). The subtle changes observed were not intensified by a further addition of the Lys^{109} and Lys^{112} mutant P450cam. The significance of the subtle changes that occurred with the Lys mutants is unknown at present, but these changes proved that a complex of P450cam and Pd was in fact formed under the experimental conditions; otherwise no effect of CO exposure on Pd spectrum could be observed. Thus the lack of change in the EPR spectrum of Pd with the 112 and 109 mutants is not because of their incapability to form the Pd-P450cam complex but is because of the uncoupling of ligand binding to the structural change in Pd.

Substitution of Amino Acid Residues Around the 2Fe-2S Cluster of Putidaredoxin—In the next series of experiments, the structure of Pd was perturbed by changing the amino acid residues that surrounded the 2Fe-2S cluster using site-directed mutagenesis; Asp^{38}, Ser^{44}, Thr^{47}, and Cys^{86} were replaced by Gly, Ala, or Val. These amino acids sit next to Cys^{39}, Cys^{45}, Cys^{48}, and Cys^{86}, which coordinate directly to Fe^{3+} atoms in the iron-sulfur center of Pd. As seen in Fig. 9, every Pd mutant with a substitution at Ser^{44} (spectra E, F, and G) showed a different EPR spectrum from that of the wild type except for the Ala^{44} mutant (spectrum B), the latter of which was indistinguishable from that of the wild type (spectra D and H). Among them, the EPR spectrum that mimicked well the spectrum of reduced Pd upon the ligand binding to the heme of P450cam in the P450cam-Pd complex (Fig. 1, C) was that of the Gly^{44} mutant; upon CO binding, the trough in the g_1 signal shifted to a higher magnetic field, although essentially no change was observed in the g_1 signal region. Replacement of Thr^{47} with Val also produced a similar spectrum to that obtained upon the ligand binding to the heme in the P450cam-Pd complex in the g_1 region, although the g_1 signal moved to a higher frequency by 1 mT. On the other hand, mutations at Asp^{38} or Cys^{86} showed no change in the EPR spectrum (data not shown). It is of note that the trough of the g_1 signal was sensitive to the size of the amino acid residues.
incorporated into the position; the trough shifted from a higher to a lower magnetic field as the volume of side chain increased, Gly < Ala ~ Ser < Val (Fig. 9, A–C). The EPR spectrum of reduced Pd was also sensitive to the mutation at Thr47, but the changes were apparently random as seen in Fig. 9, E–G.

DISCUSSION

It has been known that a ternary complex of ferrous P450<sub>cam</sub> with <i>d</i>-camphor and reduced Pd is formed as an obligatory intermediate of the reaction during the catalysis of <i>d</i>-camphor monoxygenase (2, 3). With the aid of EPR spectroscopy, we have shown in this study that a change in the spectrum of reduced Pd occurs upon its binding to ferrous P450<sub>cam</sub> in the presence of <i>d</i>-camphor, indicating that the formation of the intermediate complex accompanies a structural change in the iron-sulfur center of Pd. More interestingly, a further structural change was observed in the reduced Pd upon binding of O<sub>2</sub>, CO, or NO to the ferrous heme of P450<sub>cam</sub> in the intermediate complex, it was distinct from that induced by the binding to P450<sub>cam</sub>. When O<sub>2</sub> is the ligand, the resulting complex of ferrous P450<sub>cam</sub> with <i>d</i>-camphor, O<sub>2</sub>, and reduced Pd is another obligatory intermediate of the reaction, the degradation of which leads to the formation of the final reaction products, 5-exo-hydroxycamphor, H<sub>2</sub>O, ferric P450<sub>cam</sub>, and oxidized Pd. Such a structural change in Pd induced by the heme ligand is unlikely to be an artificial observation in vitro because we observed a similar spectral change upon the binding of CO and NO to P450<sub>cam</sub> in situ in <i>P. putida</i> (Fig. 4).

The structural changes in Pd induced by the binding of O<sub>2</sub>, CO, and NO were indistinguishable from each other as judged from their EPR spectra, and all these ligands are known to convert the spin state of the heme iron from a high to a low spin state. Accordingly, the change in the spin state of heme iron in the intermediate complex appears to trigger a series of struc-
Arg112 residue is involved in the sensing of a structural change conceivably accompanies structural alteration of P450cam through a trough at g = 1.94 well separated from the g_y signal at g = 1.88. A separation of the two signals in 2Fe–2S proteins becomes wider as the rhombic character at ferrous iron is intensified, and Pd is reported to be less rhomic than spinach and parsley ferredoxins (26). If the ligand-induced EPR spectral change of reduced Pd in the Pd-P450cam complex resulted from a splitting of the g_y signal from the composite, the structural change of Pd described here is interpretable as an intensification of the distortion at the ferrous iron. However, it is also of note that, by an increase in line width of the g_x signal, intensification of the distortion at the ferrous iron. Thus, it must be considered that the substitution for Ser with smaller Gly increases solvent access to the cluster, because they are located at a position that can affect the solvent access to the iron-sulfur cluster. Consideration of the effects of the mutation on the EPR spectrum of reduced Pd leads to a hypothesis that an association of reduced Pd with liganded camphor-bound ferrous P450cam induces the structural alteration of Pd in a way to increase the solvent accessibility of the iron-sulfur cluster. Moreover, because Ser^{44} is in the vicinity of the ferrous iron of reduced Pd (28), a change occurred at Ser^{44}, which altered the access of solvent to the iron-sulfur cluster, induces distortion around the ferrous iron simultaneously.

The side chain of Arg^{112} of P450cam at the putative binding site for Pd is oriented toward the inside of the protein and forms a hydrogen bond with the propionyl side chain at the heme periphery. The same propionyl is also hydrogen-bonded to His^{355}, the second amino acid residue from the endogenous heme ligand Cys^{357}. Accordingly, it is not surprising that the Arg^{112} residue is involved in the sensing of a structural change occurring at the heme and its vicinity. As stated above, ligand binding to the ferrous camphor-bound P450cam alters the spin state of a heme iron from high to low spin. This spin change conceivably accompanies structural alteration of P450cam, at least in its active site, involving the movement of ferrous iron from the out of plane position to the in plane position as observed in myoglobin and hemoglobin (29, 30). Therefore we propose that a structural change occurred at the heme and its vicinity in P450cam, which upon liganding is transmitted to reduced Pd via Arg^{112} as illustrated schematically in Fig. 10. On the other hand, Arg^{209}, which was also essential to the ligand-induced structural change of Pd, has no direct bonding interaction with the heme and hence may not have a primary role for the sensing. Rather, this residue would be essential for a proper docking of Pd, thus assisting a signal transduction via Arg^{112}. It is possible that the signaling pathway in the binary complex of Pd and P450cam proposed here is also responsible for the electron transfer from the iron-sulfur cluster of Pd to the heme iron of P450cam. In any event, modulation of the Pd structure by the conformational change in the P450cam active site associated with the altered spin state of heme iron could be an important factor in the electron and proton transfers in the reaction intermediate and hence in the d-camphor monooxygenation catalyzed by P450cam.

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