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The Conformation of P450cam in Complex with Putidaredoxin Is Dependent on Oxidation State

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S *Supporting Information*

ABSTRACT: Double electron−electron resonance (DEER) spectroscopy was used to determine the conformational state in solution for the heme monooxygenase P450cam when bound to its natural redox partner, putidaredoxin (Pdx). When oxidized Pdx was titrated into substrate-bound ferric P450cam, the enzyme shifted from the closed to the open conformation. In sharp contrast, however, the enzyme remained in the closed conformation when ferrous-CO P450cam was titrated with reduced Pdx. This result fully supports the proposal that binding of oxidized Pdx to P450cam opposes the open-to-closed transition induced by substrate binding. However, the data strongly suggest that in solution, binding of reduced Pdx to P450cam does not favor the open conformation. This supports a model in which substrate recognition is associated with the open-to-closed transition and electron transfer from Pdx occurs in the closed conformation. The opening of the enzyme in the ferric-hydroperoxo state following electron transfer from Pdx would provide for efficient O_2 bond activation, substrate oxidation, and product release.

The cytochrome P450 superfamily, which includes more than 15 000 sequences found in most organisms from bacteria to humans,[2] serves essential biological roles ranging from sterol biosynthesis to drug metabolism. These heme-containing monooxygenases catalyze the oxidation of organic substrates by molecular O_2 ($RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O$). A series of ordered steps precedes O−O bond activation and cleavage to create the catalytically competent Compound I intermediate.[3] These steps include substrate binding, one-electron reduction, O_2 binding, a second one-electron reduction, proton delivery, and O−O bond cleavage.[4] The source of electrons depends on the type of P450. While microsomal P450s utilize FAD/FMN-containing cytochrome P450 reductases, most mitochondrial and bacterial P450s use separate ferredoxins and ferredoxin reductases.[5]

Reduction and O−O bond activation are believed to be carefully controlled by the enzyme to prevent oxidative damage and unproductive side reactions. For many P450s, this control appears to be coupled to structural changes associated with substrate binding and recognition.[6−12] In the archetypical bacterial enzyme P450cam from *Pseudomonas putida*,[13] binding of the substrate camphor induces a transition from an open to a closed conformation, states which have been observed both in the crystal[12] and in solution.[14] Interestingly, it was the open conformation in which the structure near the active site showed remarkable similarities to that of the ferrous O_2 complex, including changes in the I helix bulge, a reorientation of Thr-252, and inclusion of the catalytic water above the heme,[12] each of which have been proposed to be important in O−O bond cleavage.[15,16] On this basis, it was proposed that the conformational shift between the open and closed states might be coupled to the known effector function upon binding of its reductase, putidaredoxin (Pdx).[12]

The structure of a covalently linked complex between P450cam and Pdx has recently been reported,[17] providing significant new insight into these issues. While the protein contacts in this complex are in general agreement with previous mutagenesis[18−22] and spectroscopic studies,[23−26] the structure of the P450cam−Pdx complex was observed to be in the open conformation both for crystals grown in the oxidized state and for crystals that were subsequently reduced with dithionite. Transition to the open conformation resulted in the changes noted above in the I helix, Thr-252, and the water network, allowing Tripathi et al.[17] to conclude that the effector function of Pdx binding is indeed associated with conversion of the enzyme to the open conformation. While these results provide a paradigm shift in the understanding of P450 catalysis, they also leave some important questions unresolved. It is likely that the structure of the P450cam−Pdx complex was not adversely affected by the covalent tether, because two different linkages were examined in the study. Second, it is possible that the oxidized structure was affected by at least some degree of X-ray-induced reduction. Most importantly, the reduced structure was obtained by soaking crystals of the oxidized complex with dithionite. As we have observed that crystals of substrate-free open P450cam do not convert to the closed state upon soaking with substrate,[12] it is possible that the structure of the chemically reduced P450cam−Pdx complex remained trapped in the open conformation by crystal packing effects. Therefore, it is important to characterize the conformational states of the P450cam−Pdx complex in both the oxidized and reduced states in solution.

Double electron−electron resonance (DEER) was used to determine the solution conformational states of P450cam in complex with Pdx. Two spin labels were placed on P450cam, and the distance between the spins could be determined from their dipolar coupling by DEER.[27] Samples of the P450cam-(4S,2C) mutant containing two surface cysteines at positions 48 and 190 were labeled with 1-oxyl-2,2,5,5-tetramethyl-Δ3-

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pyrroline-3-methyl methanethiosulfonate (MTSL).[14] Figure 1 shows background-subtracted X-band DEER traces and their corresponding distance distributions using Tikhonov regularization.[28] We have previously shown that measured spin−spin distances of 4.8 and 5.6 nm from such DEER data represent the closed substrate-bound and open substrate-free states of P450cam, respectively.[14] As shown in Figure 1, in the absence of Pdx and the presence of 1 mM camphor, the major peak in the distance distribution appeared at 4.8 nm for both ferric and CO-bound ferrous P450cam, indicating that both forms are in the closed conformation (Figure 1a). A small peak in the distance distribution at 4.3 nm was also observed for both samples and most likely resulted from a minor alternate MTSL rotamer.

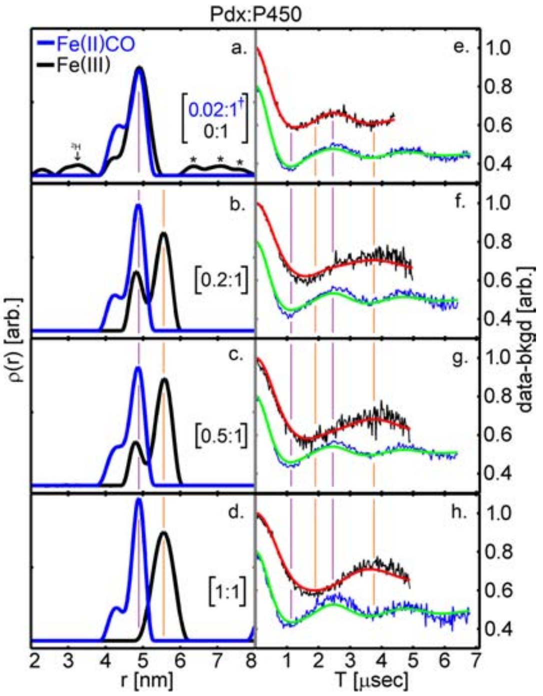

Figure 1. X-band DEER at 30K for the titration of Pdx with aerobic camphor-bound P450cam (black) and with anaerobic camphor-bound P450cam under reducing conditions (blue). †In panel a., Pdx:P450 was 0.02:1 for the Fe(II)CO species, whereas it was 0:1 for the Fe(III) species. Deuterium modulations (^{2}H) and background subtraction errors(*) in panel a. are as demarcated. The closed and open states are marked with the purple and orange lines, respectively.

When ferric spin-labeled P450cam-(4S,2C) was titrated with 0.2, 0.5, and 1.0 molar equiv of oxidized Pdx, a clear and progressive change in the DEER echo modulation frequency was observed (Figure 1e−h). This resulted in a shift in the peak of the distance distribution from 4.8 to 5.6 nm (Figure 1a−d), exactly corresponding to that for conversion from the closed to the open conformation.[14] Quantitation of the relative contributions of the two conformations was challenging because of the different effects of low- and high-spin heme on MTSL spin relaxation, but the qualitative effect clearly showed that addition of oxidized Pdx to the closed substrate-bound enzyme causes its conversion to the open state. Moreover, electron paramagnetic resonance (EPR) measurements on these samples showed a decrease in the amount of high-spin heme as Pdx was titrated into P450cam (Figure S1 in the Supporting Information). It has been known for many years that binding of Pdx to P450cam induces an increase in the low-spin species,[29,30] and in hindsight these results are consistent with the conversion from the closed to the open state.

In stark contrast to the above results, Figure 1 shows that samples of ferrous-CO P450cam displayed very well defined DEER modulations with no significant changes upon addition of reduced Pdx. Instead, the spin−spin distance remained at 4.8 nm, exactly that characteristic of the closed conformation. UV/vis spectra of these samples showed the characteristic Soret peak at 446 nm (Figure S2). As many previous observations have shown specific interactions between ferrous-CO P450cam and reduced Pdx,[25,26,31,32] we consider it unlikely that the ferrous-CO P450cam does not bind to reduced Pdx in our samples. Thus, the DEER measurements show that in solution, addition of reduced Pdx does not result in conversion of substrate-bound ferrous-CO P450cam from the closed to the open state.

To provide additional support that oxidized Pdx causes a shift in the enzyme conformation, the rate of camphor dissociation from P450cam was measured as a function of [Pdx] and [K$^+$] by stopped-flow mixing with the strong-binding competitive inhibitor metyrapone. Figure 2 shows that the rate of camphor dissociation further increased in the presence of Pdx at low [K$^+$]. The camphor dissociation rate was not influenced by oxidized Pdx at high salt concentrations, possibly because of disruption of the salt bridge between P450cam-Arg112 and Pdx-Asp38. These results are consistent with the observation that binding of oxidized Pdx to P450cam results in conversion to the open conformation.

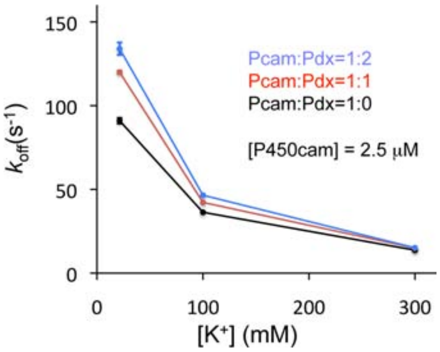

Figure 2. Effects of oxidized Pdx on camphor dissociation from ferric P450cam. The rate of camphor dissociation was calculated using the spectral change from 391 nm (camphor-bound) to 421 nm (metyrapone-bound).[1]

This study supports recent X-ray crystallography results[17] suggesting that the P450cam−Pdx complex favors the open conformation in the oxidized state. Importantly, our results show that this effect is observed in the solution state and that addition of oxidized Pdx to the substrate-bound closed conformation causes its conversion to the open conformation. This removes any possibility that crystal contacts or X-ray-induced reduction is the source of the effect. In hindsight, a significant body of previous work also supports this observation. For example, resonance Raman[30] and EPR[29] studies have shown that oxidized Pdx shifts the spin state of P450cam from high- to low-spin even in the presence of camphor. In addition, Glascock et al.[33] observed that binding of oxidized Pdx to oxyferrous P450cam resulted in a 100-fold increase in the rate of auto-oxidation.

Our results also show that in solution, the ferrous-CO state of the enzyme remains in the closed substrate-bound conformation upon addition of reduced Pdx. In support of this, while small changes have been noted by NMR spectroscopy for both proximal and distal residues upon binding of reduced Pdx to ferrous-CO P450cam, a large change in structure was not indicated.[34] However, these conclusions appear to contrast with the results of Tripathi et al.,[17] in which dithionite-reduced crystals did not cause a shift from the open conformation seen in the oxidized complex. We propose three possible explanations for this difference. First, the effects of Pdx binding on the ferrous and ferrous-CO states may be distinct. The former may represent the state of the complex prior to O_2 binding, while the latter may more accurately reflect the state before the second electron transfer (ET) step. Second, it is possible that the interactions between Pdx and the enzyme are different for the ferrous-CO complex and the ferrous-O_2 complex. DEER studies of the ferrous and ferrous-O_2 complexes with Pdx are under active investigation to provide insight into this important issue. Third, to account for the observation of the open conformation in the dithionite-reduced crystals, we propose that the complex becomes trapped in the open conformation during crystallization of the oxidized forms and resists conformational change upon subsequent reduction. This is supported by our observation that crystals of the open conformation did not convert to the closed conformation upon soaking in substrate.[12]

As our DEER results suggest that reduced Pdx favors the closed state of substrate-bound P450cam, we propose that ET may also occur in this state. In this view, if the effector function of Pdx results from the redox-state-dependent triggering of the closed-to-open transition, this would occur naturally as a slow conformational transition following the second ET from Pdx. We propose that a redox-dependent conformational change induced by binding of Pdx to P450cam permits such a dual role. Whether that also occurs during the first reduction remains uncertain. It should also be noted that the effector function of Pdx may not require a transition of P450cam from the closed to the completely open state, as we have previously observed an intermediate conformation for some tethered substrates that also results in occupation of the catalytic water.[35,36]

In summary, our observations in solution are consistent with the recently proposed model for Pdx effector function,[17] but with one important difference. In this view, substrate recognition by P450cam is associated with conversion from the open to the closed conformation. The same open and closed states have been observed by X-ray crystallography[12] and DEER spectroscopy[14] and for a large number of tethered substrate analogues.[36] However, our results suggest that binding of reduced Pdx to the substrate-bound closed conformation does not cause conversion to the open conformation but instead allows ET to occur in the closed state if substrate is bound. As soon as the second electron is transferred, the system will exist as the ferric-hydroperoxo complex bound to oxidized Pdx. It is this state that we propose to be converted to the open conformation. Further clarification of these possibilities is under active investigation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental methods and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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