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CHAPTER 6

Structural characterization of the substrate access channel of P450cam with a paramagnetic inhibitor
Abstract

P450 enzymes bind to a variety of different compounds to catalyze monooxygenation reactions. However, the mechanisms of substrate recognition and product release of P450 enzymes are poorly understood. In the presence of substrates, the crystal structures of P450cam have been shown to adopt the closed conformation. In this state, there is no channel through which substrates can access the active site of P450cam. In this project, utilizing the paramagnetic inhibitor, N-(3-(1H-imidazole-1-yl)phenyl)-1-oxylradical-2,2,5,5-tetramethyl-8-3-pyrroline-3-carboxamide (1-PIM-3), the nature of the substrate access channel was investigated by X-ray crystallography as well as paramagnetic NMR techniques. X-ray crystallography revealed that P450cam exhibits the open conformation in the complex with 1-PIM-3. The substrate access channel was traced by paramagnetic relaxation enhancements, indicating that the A helix and the region nearby β1 sheet are part of the substrate access channel.
Introduction

Cytochrome P450cam (CYP101A1) is an archetypical bacterial P450 for which more than a hundred structures have deposited in the protein data bank. Recently, crystal structures of artificially cross-linked Pdx and P450cam were solved,[1] which raised questions about the conformational states of P450cam. In the presence of the substrate almost all of the P450cam structures show the “closed” conformation, in which there is no channel present large enough to allow substrate access to the active site. Substrate entry into P450cam had puzzled researchers for decades until Dunn et al. co-crystallized the protein with elongated substrate analogues.[2] These analogues protruded from the active site to the protein surface, forcing the substrate access channel to be opened. In 2010, Goodin and co-workers solved the crystal structure of P450cam in the absence of substrates, revealing that the opening of the substrate access channel primarily involves the movement of the F and G helices.[3] While the structure of the “open” conformation provides valuable information for understanding P450cam catalysis, it also leaves many questions to be unresolved: how fast is the exchange between the open and closed conformations? Which factors trigger the equilibrium shift? Is there a secondary substrate binding site inside the channel as suggested by a recent study?[1] Paramagnetic NMR techniques can be used to examine the substrate access channel in solution. When a substrate or inhibitor analogue with a paramagnetic center binds to the active site of P450cam, it should cause PREs mark on the residues comprising the substrate access channel. The PRE is dependent on distance between a paramagnetic center and observed residue and the sensitivity of the PRE allows the detection of minor conformational states of proteins.[4] Tracking the PRE effect on P450cam residues, the structure and dynamics of substrate access channel can be analyzed (Figure 1). In this chapter, the paramagnetic substrate analogue, 1-PIM-3-para, was used to characterize the substrate access channel of P450cam. The work demonstrates the proof-of-principle that small paramagnetic centers attached to ligands rather than protein can be helpful to characterize a complex.

Results & Discussion

1-PIM-3 binding to P450cam

The paramagnetic substrate analogue, 1-PIM-3-para, was designed on the basis of the known type I inhibitor of P450cam, 1-phenylimidazole, and a commonly used paramagnetic spin label, 1-oxyl-2,2,5,5-tetramethyl-δ-3-pyrroline-3-methyl (OTPM). The crystal structure of 1-phenylimidazole bound P450cam indicates that the closed conformation of P450cam remains intact while one nitrogen atom of the imidazole group binds the heme iron.[5] The coordination of the sixth ligand to the iron provides an important advantage to the NMR analysis since it suppresses the endogenous PRE effect caused by the high spin state of the heme iron. The diamagnetic inhibitor analogue, 1-PIM-3-dia was prepared by replacing OTPM with 1-methoxy-2,2,5,5-tetramethyl-δ-3-pyrroline-3-methyl (MTPM) group of 1-PIM-3-para.
Figure 1. Schematic representation of mapping the channel with PREs. The paramagnetic inhibitor analogue, 1-PIM-3-para reaches the active site of P450cam through the substrate access channel. The red colored regions of P450cam represent the residues that exhibit PRE.

To determine the affinity of 1-PIM-3 to P450cam, a competitive binding assay was carried out with UV/VIS spectrophotometry (Figure 2).

Figure 2. Competitive binding assay of 1-PIM-3 and camphor to P450cam. (A) Overlaid traces of absorption spectra of P450cam. Upon the titration of 1-PIM-3, the maximum absorption peak at 391 nm shifts to the 417 nm. (B) The plot of $\Delta A_{417}$ against the concentration of 1-PIM-3. The blue points and red line represent the experimentally derived $\Delta A_{417}$ values and the simulated curve, respectively.
In the presence of the physiological substrate, camphor, the changes of the Soret maximum peak at 417 nm wavelength of P450cam were monitored with increasing concentration of 1-PIM-3. The dissociation constant ($K_D$) of 1-PIM-3 was estimated to be $7.5 \pm 0.5 \, \mu M$ by simulating the curve with the binding affinity for 1-PIM-3 as well as camphor and the maximal change of the absorbance at 417 nm ($\Delta A_{417}$) as variable (Figure 2B). The $K_D$ of camphor was found to be $7 \pm 1 \, \mu M$, which is in line to the previous study of $K_D = 10.2 \pm 0.6 \, \mu M$.[6] The details of the simulation are described in the Materials and Methods. The obtained $K_D$ value of 1-PIM-3 is compared with those of camphor and 1-phenylimidazole in Table 1. The binding affinity of 1-PIM-3 is comparable to that of camphor. The difference of $K_D$ by 70–80 folds with 1-phenylimidazole is attributed to the presence of OTPM function group.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$K_D ($\mu M$)</th>
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<tbody>
<tr>
<td>Camphor</td>
<td>$7 \pm 1$</td>
</tr>
<tr>
<td>1-phenylimidazole</td>
<td>0.1$^a$</td>
</tr>
<tr>
<td>1-PIM-3</td>
<td>$7.5 \pm 0.5$</td>
</tr>
</tbody>
</table>

(a) previously determined by Poulos, T.L. and Howard, A.J.[5]

**Crystal structure of P450cam bound to 1-PIM-3**

Having established the affinity of 1-PIM-3 for P450cam, we obtained the structural information of P450cam bound to 1-PIM-3 by X-ray crystallography. The formation of crystal was observed under the same conditions as described by Lee, Y.T. et al.[7] The crystal structures of both 1-PIM-3-para and -dia bound P450cam were solved with maximum resolution of 2.2 Å and 2.0 Å, respectively. Data processing and refinement were carried out by Dr. Igor Nederlof (Biophysical Structural Chemistry group at Leiden University).

In line with the findings for 1-phenylimidazole,[5] the crystal structure of 1-PIM-3 shows that the nitrogen atom of imidazole group coordinates the heme iron (Figure 3A). While the electron density of the 1-phenylimidazole group as well as the amide linker is well-defined, that of the OTPM functional group was less clear. This observation points toward mobility of the OTPM group, which has also been reported for MTSL labeled samples in NMR studies.[8-9]

To further characterize the 1-PIM-3 binding, a comparison was made with the crystal structure of P450cam bound to 1-phenylimidazole.[5] The position of the axial nitrogen atom of imidazole group is very similar (Figure 3B), indicating that the nature of type I inhibition of 1-phenylimidazole is conserved in 1-PIM-3. However, the orientation of the 1-PIM-3 imidazole ring was found to be rotated by 55 degrees relative to that...
of 1-phenylimidazole. It is also worth mentioning that the position of OTPM group coincides to the previously observed second substrate binding site.\[^{[1]}\] The potential link between the occupancy of the second binding site and conformation state of P450cam will be studied in future.

Interestingly, 1-PIM-3 bound P450cam is present with substrate access channel in the open conformation, which distinctively differs from the closed conformation of P450cam bound to 1-phenylimidazole (Figure 4). The conformations of substrate access channel can be classified by the structural features of antiparallel F and G helices and intervening F–G loop.\[^{[7]}\] The F–G loop region exhibits the largest movement upon the conformational change in P450cam.

The superimposed structures of the F–G loop illustrate that 1-PIM-3 bound P450cam adopts the structural features of the open conformation (Figure 4). In fact, the degree opening appears to be even slightly larger than in the substrate-free structure. It is plausible that the presence of bulky OTPM functional group forces the protein into this conformation. Other residues that are known to correlate with the conformation change are shown in Figure 4B and C. The formation of the unusual hydrogen bond between hydroxyl group of Thr252 and carboxyl backbone of Gly248 is a unique feature of the closed conformation. It has been reported to regulate the position of catalytic water in the active site.\[^{[10]}\] Another characteristic aspect of P450cam conformational states is the arrangement of the B’ helix. In the intermediate and open states, the B’ region exhibit large mobility and does not form the defined secondary structure. However, the binding of a potassium ion enhances the stability

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**Figure 3.** Crystal structure of P450cam bound to 1-PIM-3-dia. (A) Details of the electron density map with a maximum resolution of 2.0 Å. The structure was solved by molecular replacement using P450cam in the open conformation (PDB entry 3L61\[^{[10]}\]). (B) Positions and orientations of ligands are superimposed. Gray, pink and yellow sticks represent 1-PIM-3-dia, 1-phenylimidazole and camphor, respectively.
of B’ helix. It increases the binding affinity of camphor by ten-folds, which leads to the closed conformation.\cite{10} Taken together, we concluded that 1-PIM-3 binding forces the substrate access channel of P450cam into the open state.

PRE analysis of P450cam bound to 1-PIM-3
To investigate the dynamics and structural components involved in the substrate access channel, a paramagnetic NMR experiment was carried out. In the current study, due to the limited availability of [\( ^{2}\text{H}, \ ^{15}\text{N} \)] labeled proteins, \([^{15}\text{N}] \) isotopically enriched P450cam C334A/H352A mutant was used to optimize the experimental conditions. The catalytic activity of this mutant was shown to be intact in a previous study (Chapter 4). The two dimensional \([^{15}\text{N}]^{-1}\text{H} \) HSQC spectra were acquired for the P450cam samples in the presence of 1-PIM-3-para/-dia. In addition to the amide-proton resonances of P450cam, large peaks caused by dimethylformamide (DMF) were observed across the indirect dimension at 7.8 ppm of the \( ^{1}\text{H} \) chemical shift (Figure 5A). The 3% of DMF used to solubilize 1-PIM-3 in the sample is the cause of this signal. The contribution of DMF peaks can be minimized by utilizing deuterated DMF in the future. The NMR spectra were overlaid with the closed conformation of P450cam in high spin and low spin states (kindly provided by Simon Skinner at Leiden University). Most of the assigned peaks in closed state were either shifted or disappeared, presumably due
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to the P450cam being in the open conformation and the chemical shift perturbations caused by 1-PIM-3 binding. Out of 414 residues, 116 residues were assigned on the basis of the assignments of closed conformation. As a future perspective, the number of unambiguous assignments can be increased by acquiring NMR spectra in a titration of camphor. In addition, the exchange regime (on the NMR time-scale) of the association and dissociation of 1-PIM-3-dia binding needs to be established. From the ratios of the peak intensities of 1-PIM-3-para/-dia ($I_{\text{para}}/I_{\text{dia}}$), the PREs were calculated. The observed PREs are a weighted average of 1-PIM-3 bound form and substrate-free P450cam. Given the $K_D = 7.5 \, \mu\text{M}$, ca. 90% of P450cam was bound to 1-PIM-3 in this experiment. Therefore, the observed PREs were extrapolated to the 100% bound

![PRE analysis of P450cam bound to 1-PIM-3. (A) Overlaid HSQC spectra of P450cam. Blue and red peaks represent 1-PIM-3-dia and -para samples, respectively. (B) $I_{\text{para}}/I_{\text{dia}}$ ratios (B) and PREs (C) are plotted against P450cam residue number. (D) Ribbon representation of the crystal structure of P450cam bound to 1-PIM-3. P450cam residues were color coded according to PRE with $\gtrsim 30 \, \text{s}^{-1}$, red; 20–30 s$^{-1}$, orange; 15–20 s$^{-1}$, yellow; $< 15 \, \text{s}^{-1}$, blue. Unassigned residues are shown in gray. (E) Zoom-in of the region affected by PRE. Sticks and sphere represent 1-PIM-3-dia and heme.](image)
state by dividing the PREs by 0.9. The calculated PREs were color coded on P450cam as shown in Figure 5.

It has been reported that the secondary structural elements helices, F, G and I as well as the F–G loop exhibit distinct movements in the transition between the conformational states.\[7\] Due to the significant chemical shift changes, it was impossible to assign the resonances of residues in those elements. The B’ helix, comprising residues 90–95, is also known to be important for the conformation shift of P450cam. Significant PREs (>25 s⁻¹) indicate that those residues are in close proximity of the paramagnetic center of 1-PIM-3. In addition to the previously described structural motifs, other regions, such as the A helix and the nearby β1 sheet also display strong PREs (Figure 5E). Most of these residues are located within 22 Å from the paramagnetic center, which agrees well with the distance range in which a nitoxide spin label is expected to cause PREs (14–22 Å). Further NMR experiments are required to characterize the dynamics and nature of the substrate access channel in the solution state.

Conclusions
The current study demonstrated binding of a paramagnetic inhibitor analogs to P450cam. The binding affinity of 1-PIM-3 was found to be $K_D = 7.5 \mu$M, which is comparable to the physiological substrate, camphor. The crystal structure of P450cam bound to 1-PIM-3 exhibits a more open conformation than that of substrate-free form. The preliminary results of the PRE analysis indicate that paramagnetic ligand analogues can be used to characterize protein-ligand complexes.

Materials and Methods
Chemicals
1-PIM-3-para/-dia were kindly provided by Wei-Min Liu (Leiden University).

Protein production
P450cam C334A as well as [¹⁵N] isotopically enriched P450cam C334A/H352A were produced and purified as described in Chapters 3 and 4. Camphor was removed by Superose 12 gel filtration column (120 mL) pre-equilibrated with 20 mM Hepes, pH 7.4. The fractions containing P450cam with absorbance ratio of $A_{417}/A_{280} > 1.55$ were pooled.

Competitive binding assay
The reaction mixtures (2 mL) contained 5 μM P450cam C334A in 20 mM Hepes, pH 7.4 and 100 mM KCl. First, P450cam was saturated with three molar equivalents of camphor. 1-PIM-3 was titrated into camphor bound P450cam and the change of the Soret maximum peak at 417 nm ($\Delta A_{417}$) was monitored at 293K.
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The following model was used to describe the binding of camphor and 1-PIM-3.

\[
A + B \rightleftharpoons AB + C \\
\text{AC}
\]

(1)

where A, B, C, AB and AC represent P450cam, camphor, 1-PIM-3, camphor bound P450cam and 1-PIM-3 bound P450cam, respectively. The system is described by two \(K_D\) values and the mass laws (eq. 2-7)

\[
K_{D}^{AB} = \frac{A \cdot B}{AB}
\]

(2)

\[
K_{D}^{AC} = \frac{A \cdot C}{AC}
\]

(3)

\[
B = \frac{B \cdot K_{D}^{AB}}{K_{D}^{AB} + A}
\]

(4)

\[
B_i = B + AB
\]

(5)

\[
C = \frac{C \cdot K_{D}^{AC}}{K_{D}^{AC} + A}
\]

(6)

\[
C_i = C + AC
\]

(7)

The experimental absorbance change at 417 nm was simulated with eq. 8 for each point \(i\) in the titration, by varying \(K_{D}^{AB}, K_{D}^{AC}\) and \(\Delta A_{417}^{\text{max}}\), the maximal change in absorbance (for 100% P450cam-1-PIM-3 complex).

\[
\Delta A_{417}(i) = \frac{AC_i}{A_i} \cdot \Delta A_{417}^{\text{max}}
\]

(8)

The error associated with the dissociation constant was estimated by measuring the agreement between experimental and simulated data.

**Crystallization**

The stock solution contained 200 μM P450cam C334A in 20 mM Hepes, pH 7.4 and 2 mM 1-PIM-3-para/-dia (10% DMF). Crystals of P450cam bound to 1-PIM-3 were grown by the sitting-drop vapor diffusion method at room temperature from 12–22% PEG 8000, 100 mM sodium cacodylate, pH 5.5–7.0 and 100–200 mM KCl. The crystals were mounted on nylon loops containing glycerol as a cryoprotectant and flash-frozen in liquid nitrogen.
X-ray diffraction data were collected at European synchrotron radiation facility in Grenoble. Data collection and crystal structure determination were performed by Dr. Igor Nederlof (Biophysical Structural Chemistry group at Leiden University).

**NMR samples and experiments**

The fractions from Superose 12 column were pooled and buffer exchanged to 100 mM cacodylic acid, pH 6.5, 200 mM KCl, 12.5% D₂O by ultrafiltration using a centicon (5K). The NMR samples contained 250 μM [¹⁵N] P450cam C334A/H352A and 300 μM 1-PIM-3-para/-dia in 80 mM cacodylic acid, 160 mM KCl, pH 6.5, 5 mM HEPES, 10% D₂O and 3% DMF. Two-dimensional ¹⁵N–¹H HSQC spectra were recorded at 298 K on a Bruker Avance III HD 850-MHz spectrometer equipped with a TCI-Z-GRAD cryoprobe. NMR data were processed in NMRPipe[13] and analyzed in CCPNMR.[14]

**NMR assignment**

The amide resonances of P450cam C334A/H352A bound to 1-PIM-3-dia/-para were assigned on the basis of cyanide and camphor-bound oxidized P450cam C334A (provided by Simon Skinner, Leiden University).

**Restraint analysis**

The ratios of \( I_{\text{para}} / I_{\text{dia}} \) were normalized by dividing them by the averaged values of the 85 largest \( I_{\text{para}} / I_{\text{dia}} \) ratios (0.96). PREs were determined as described in the Chapter 3 and 5.

**References**
