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Effect of Redox Partner Binding on CYP101D1 Conformational Dynamics

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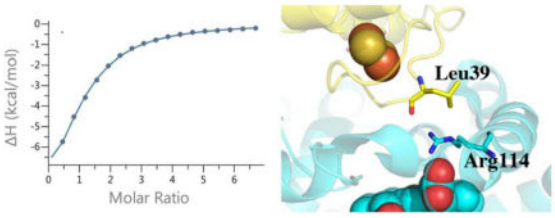
Abstract

We have compared the thermodynamics of substrate and redox partner binding of P450cam to its close homologue, CYP101D1, using isothermal titration calorimetry (ITC). CYP101D1 binds camphor about 10-fold more weakly than P450cam which is consistent with the inability of camphor to cause a complete low- to high-spin shift in CYP101D1. Even so molecular dynamics simulations show that camphor is very stable in the CYP101D1 active site similar to P450cam. ITC data on the binding of the CYP101D1 redox partner, Arx, shows that the substrate-bound closed state of CYP101D1 binds Arx more tightly than the substrate-free open form. This is just the opposite to P450cam where Pdx (redox partner of P450cam) favors binding to the P450cam open state. In addition, CYP101D1-Arx binding has a large negative ΔS while the P450cam-Pdx has a much smaller ΔS indicating that interactions at the docking interface are different. The most obvious difference is that PDX_{D38} which forms an important ion pair with $P450cam_{R112}$ at the center of the interface is Arx_{L39} in Arx. This suggests that Arx may adopt a different orientation than Pdx in order to optimize nonpolar interactions with Arx_{L39}.

Graphical Abstract

Isothermal titration calorimetry of Arx binding to CYP101D1 and a hypothetical model of the CYP101D1-Arx complex.



Keywords

Cyp101D1; P450cam; Arx; Pdx; isothermal titration calorimetry

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Introduction

All cytochrome P450 enzymes require interaction with an electron transfer redox partner. While the redox partner for a majority of well studied P450s plays a passive role and simply serves as a source of electrons without active participation in the O_2 activation process, P450cam has proven to be an exception. It has been known for some time that P450cam is highly specific for its redox partner, Pdx (putidaredoxin), for the second electron transfer step that results in O_2 activation [1]. Recent crystal structural work [2, 3] supported by a variety of spectroscopic [4–7], computational, and direct binding assays [8] shows that Pdx favors binding to the open P450cam conformer. Although it has generally been assumed that the closed form where the substrate pocket is sealed off from bulk solvent is the active conformer, the P450cam-Pdx structural work suggests that a more open form is required for O_2 activation.

One problem with this view is that looser substrate-protein interactions in the open state may not be compatible with the observed regio- and stereo-selective hydroxylation. This is important because, in the crystal structures of the P450cam-Pdx complex, P450cam is in the open conformation, yet we observe well defined electron density for the correct product, 5-*exo*-hydroxycamphor, in the active site [3]. This means that the open form is active in substrate hydroxylation and that the substrate must be held in the correct position for regio- and stereo-selective hydroxylation for x-ray driven reduction and O_2 activation. Recent computational studies have shown that Pdx stabilizes the active site and actually favors conformational states between the extremes of the open and closed forms [9]. This effector role of Pdx has been attributed to the requirement that an opening of the active site is necessary to arm the proton relay network essential for O_2 activation. In the closed state the essential Asp251[10, 11] is locked down by ion pairing with a Lys and Arg while in the more open state, these ion pairs are broken and Asp251 is free to help shuttle protons to the iron linked O_2 molecule which is necessary to promote heterolytic cleavage of the O-O bond to give Compound I.

An important question is whether or not redox partners play a similar effector role in other P450s. In addressing this question CYP101D1 [12] has been particularly useful. This P450 catalyzes exactly the same reaction as P450cam with the same coupling efficiency and product. In addition, the structure of CYP101D1 and its redox partner called Arx are very similar to P450cam and Pdx, respectively, with rms deviations of backbone atoms less than 1Å [13] with 44% and 29% sequence identity between CYP101D1 vs. P450cam and Arx vs Pdx, respectively [13]. Even so the two P450s behave very differently. For example, CYP101D1 does not strictly require its own redox partner for catalysis[14] [15] while P450cam has a strict requirement for Pdx since Pdx can support CYP101D1 catalysis but Arx cannot support P450cam catalysis. Furthermore, we found that Arx shifts the spin state equilibrium toward high-spin [15] which is consistent with tightening the P450 structure while Pdx shifts P450cam more toward the low-spin and more open state [7].

Isothermal titration calorimetry (ITC) has proven to be a sensitive tool for comparing the open and closed states of P450cam [8, 16] since ITC studies show that Pdx binds more

tightly to the open substrate-free P450cam. Here we present an ITC study of the CYP101D1-Arx interaction.

MATERIALS AND METHODS

Protein Expression and Purification

E. coli codon-optimized genes coding for full-length CYP101D1 and Arx were subcloned into vector pET28a (Novagen Inc.) and a N terminal 6X-His tag was incorporated for both proteins (between sites *NdeI* and *BamHI*). Expression for both was done in the BL21(DE3) strain of *E. coli* grown at 37°C in 1 liter flasks of Luria-Bertani broth (LB) media with the kanamycin as the selection antibiotic. After growing at 37°C for six hours, protein expression was induced by addition of 1 mM isopropyl 1-thio-$_D$-galactopyranoside and set to shake overnight at 25°C. Cells were then harvested by centrifugation, resuspended in 50 mM potassium phosphate (pH 7.4) and 150 mM NaCl, and lysed by sonication at 4°C. This crude extract was then centrifuged at 15,000 rpm for 1 hour and 10 minutes and the supernatant was loaded onto an Ni-IMAC column (Bio-Rad). The column was then washed with lysis buffer containing 8 mM imidazole followed by elution of the protein using 50 mM potassium phosphate with 200 mM imidazole. The eluted fractions were pooled, concentrated, buffer exchanged into 50 mM potassium phosphate, and left to incubate for 5 hours with approximately 50 units/mg of high-purity bovine thrombin to cleave the 6-His tag. After thrombin digestion, the sample was loaded once more onto the Ni-IMAC column and the flow through was collected and concentrated. Finally, gel filtration chromatography on a Superdex 75 (GE Healthcare) size-exclusion column was used to obtain fractions of pure CYP101D1 in 50 mM potassium phosphate (pH 7.4) buffer, which were then pooled and concentrated. Arx was also purified using similar protocol and same buffer composition as described for CYP101D1.

Spectroscopy

UV–visible spectroscopy was conducted using a Cary 3 spectrophotometer. The CYP101D1 concentrations were measured using an extinction coefficient of 107 mM^{-1} cm^{-1} at 418 nm [17]. The Arx concentrations were measured using an extinction coefficient of 9.3 mM^{-1} cm^{-1} at 414 nm [17].

Isothermal Titration Calorimetry

All experiments were carried out using the MicroCal PEAQ-ITC instrument from Malvern. All samples used in the experiments were in 50 mM potassium phosphate with all potential reducing agents removed. For experiments that required the substrate, D-camphor, a final concentration of 1mM camphor was used in solution. All experiments were run in a reaction cell at 25°C containing 300 μL of 50 μM CYP101D1 with 2 mM Arx in the injection syringe. Each titration experiment involved 18 injections of 2 μL with a 4 second duration and a 150 second pause between injections using a reference power of 5, high feedback mode, and a 750-rpm stirring speed. Data were analyzed using the MicroCal PEAQ-ITC analysis software and all enthalpy values were corrected for the enthalpy of dilution with values obtained using titrations in identical conditions with buffer alone in the sample cell. For ITC runs measuring camphor binding to CYP101D1, 600 μM to 1mM D-camphor

solution was titrated into 40–50 μM CYP101D1 with same titration setting as described above. Each run was repeated 3–5 times.

Molecular Dynamics

The methods used for molecular dynamics simulations were the same as previously published for P450cam [9]. The starting structure was the ferric camphor bound structure (4C9K). The one difference here is simulations with compound I. A ferryl O atom was placed 1.6Å from the iron and the forcefield for the P450 compound I heme was used [18].

RESULTS

ITC of Camphor Binding

The ITC runs for the binding of D-camphor to CYP101D1 and binding of Arx to CYP101D1 in presence and absence of the D-camphor were measured multiple times. The range of K_D for binding of D-camphor to CYP101D1 was between 12 μM to 15 μM (Figure 1). This is in good agreement to the spectroscopic dissociation constant reported by Yang et al. [13]. The binding of camphor to CYP101D1 was about 10 fold weaker compared to P450cam (K_D ~1.2 μM). Table 1 provides a comparison with the ITC results obtained with P450cam [9]. In both cases ΔH is positive so the binding of camphor is entropically driven. The weaker binding of camphor to CYP101D1 also is reflected in the inability of camphor to induce a complete low- to high-spin shift [12, 15]. One potentially important structural difference contributing to substrate affinity is the K^+ binding site in P450cam which is missing in CYP101D1. K^+ helps to stabilize the region around Tyr96 which donates an H-bond to the camphor carbonyl oxygen and is generally thought to help stabilize the active site pocket.

Molecular Dynamics

We carried out several 100ns MD simulations to determine the stability of camphor in the CYP101D1 binding pocket. The analysis is similar to a recent study carried out with P450cam [9]. The percent of 5000 snapshots over a 100ns simulation where the C5-Fe distance is ≤ 4.5Å (Figure 3) relative to other camphor carbon atoms is presented in Table 2. In addition the % of frames where the Tyr98-camphor carbonyl O atom is ≤ 3.5Å (Fig. 3) is presented in Table 2. Finally, we carried out two 100ns simulations with compound I. Here the % of snapshots where the H15-ferryl O atom (Fig. 3) distance is ≤3.0Å and the Fe-O-H15 angle is between 120°–140° was followed. These restrictions are consistent with H15 abstraction by the ferryl O atom. These simulations indicate that camphor is every bit as stable in the CYP101D1 pocket as in P450cam.

ITC of Redox Partner Interaction

Next we measured the binding of the redox partner Arx to CYP101D1 in the absence of camphor (Figure 2). The accuracy of using ITC to measure redox partner complex formation can be assessed by comparing results from two different labs for the P450cam-Pdx complex shown in Table 1. The results from our experiments gave a measured K_D value ranging between 94.8 μM to 97.7 μM in substrate-free CYP101D1. In the presence of camphor, when the CYP101D1 is in the closed substrate-bound, state, the ITC data yielded a K_D

between 41.1 μM to 43.3 μM. These experiments are summarized in Figure 3 and compared to P450cam in Table 1. These results suggest that, opposite to the dynamics of the P450cam-Pdx complex, Arx forms a more stable complex when binding to the closed substrate bound form of CYP101D1. The thermodynamics of redox partner binding also are different. In P450cam the P450cam-Pdx interaction is driven by ΔH. With CYP101D1, ΔH is significantly larger but binding is opposed by a large negative TΔS. This suggests that the binding interface may be different in the CYP101D1-Arx complex. Figure 4 is a hypothetical model of the CYP101D1-Arx complex based on the known structure of the P450cam-Pdx complex with the assumption that Arx adopts the same orientation as Pdx. There are two major differences. First, P450cam forms a critically important ion pair between Pdx_{D38} and $P450cam_{R112}$. In Arx, Asp38 is Leu39 while Arg112 (Arg114 in CYP101D1) is conserved. Second, Arx lacks the critically important C-terminal Trp106 found in Pdx. These differences, especially the replacement of PDX_{D38} with Arx_{L39}, indicates that the orientation of Arx may be quite different in the CYP101D1-Arx complex. As shown in Fig. 4 if Arx adopts the same orientation as Pdx then Arx_{Leu39} would not form any favorable nonpolar interactions with CYP101D1 suggesting that Arx may adopt a different orientation is order to accommodate Leu39. This could also contribute to the differences in the thermodynamics of complex formation. Another important difference is that in the P450cam-Pdx the C-terminal Trp106 plays a key role in binding and is known to be essential for catalysis [19, 20]. Even if Arx adopts a much different orientation than Pdx, it is clear that the C-terminal Glu105 is too far from the P450 to play any significant role in binding. The crystal structure of the complex formed between the steroidogenic CYP11A1 and adrenodoxin [21] shows that the redox partner indeed can adopt a different orientation relative to the P450cam-Pdx complex. As shown in Fig 4C, adrenodoxin binds to the same proximal surface on the P450 but Adx rotates approximately 90° relative to Pdx. In addition, the homolog to $Pdx_{Asp\ 38}$ and Adx_{Leu39} is Adx_{Ala51}. A recent spin labeling study on another bacterial P450 system, CYP199A2 [22], shows that its Fe_2S_2 redox partners once again binds to the same proximal P450 surface but in a different orientation.

DISCUSSION

Of the many well characterized bacterial P450s, CYP101D1 is the most similar to P450cam. CYP101D1 catalyzes exactly the same reaction with the same rate and coupling efficiency and the structures of both the P450s and redox partners are very similar [13]. CYP101D1 thus provides an opportunity to dissect out subtle structural features that contribute to functional differences. One of the most important differences is the effect of redox partner binding. P450cam is highly selective for its own redox partner, Pdx, while CYP101D1 is more promiscuous. ITC shows that Pdx favors binding to the open form of P450cam which now is thought to contribute to the effector role of Pdx. This more open form of P450cam is required in order for the proton relay network required for O_2 activation to be established. Arx, however, has just the opposite effect on CYP101D1 and favors the closed form. These results indicate that Arx does not play a major effector role in CYP101D1 catalysis. This implies that if all P450s must establish a proton relay network for proper O_2 activation, then formation of this network does not require redox partner induced structural changes in CYP101D1. Evidence that this may be true is that substrate hydroxylation in crystals of

ferric substrate-bound CYP101D1 can be driven by x-ray reduction but not in P450cam. CYP101D1 has the same critical Asp residue as Asp251 in P450cam but in CYP101D1 this Asp is tied up with only one ion pair to an Arg residue while in P450cam, Asp251 is tied down buy ion pairing with both an Arg an Lys. Thus, the essential catalytic Asp in CYP101D1 is more exposed to external solvent thereby enabling the proper proton relay network to more readily form. This enables x-ray induced reducing equivalents to drive substrate hydroxylation in crystals. Further evidence along these lines is that when the Asp-Arg ion pair is disrupted in P450cam, the enzyme retains substantial activity and product now forms in the x-ray beam similar to CYP101D1 [9]. These comparative studies between P450cam and CYP101D1 have provided important insights on how redox partner binding is coupled to the O_2 activation machinery enabling a fairly detailed mechanistic picture to emerge. Even so, the question of why P450cam exhibits such selectivity remains open. One would expect that there should be some biological advantage to having this level of control. Given the enormous number of P450s in Nature, it might be expected that other P450s would behave similar to P450cam in the requirement for specific redox partner induced structural changes. Perhaps future studies will discover such P450s which then should enable a better understanding on whether or not there is some common biological advantage for a P450 to evolve a strict requirement for redox partner binding induced structural changes essential for catalysis.

Acknowledgments

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Highlights

- Isothermal titration calorimetry (ITC) shows important differences between the interaction of the redox partner with P450cam and its close homologue, CYP101D1.
- While the P450cam redox partner, Pdx, shifts P450cam to the more open conformation, the CYP1011D1 redox partner, Arx has the opposite effect and favors binding to the closed state.
- A comparison between the P450-redox partner interface shows that where Pdx has a critical Asp that forms an ion pair with an Arg in P450cam, Arx has a Leu.
- The Asp/Leu difference indicates that the orientation of Arx is quite different than in the P450cam-Pdx complex.
- Camphor binds more weakly to CYP101D1 than to P450cam although molecular dynamics simulations show that camphor is quite stable in the CYP101D1 active site.

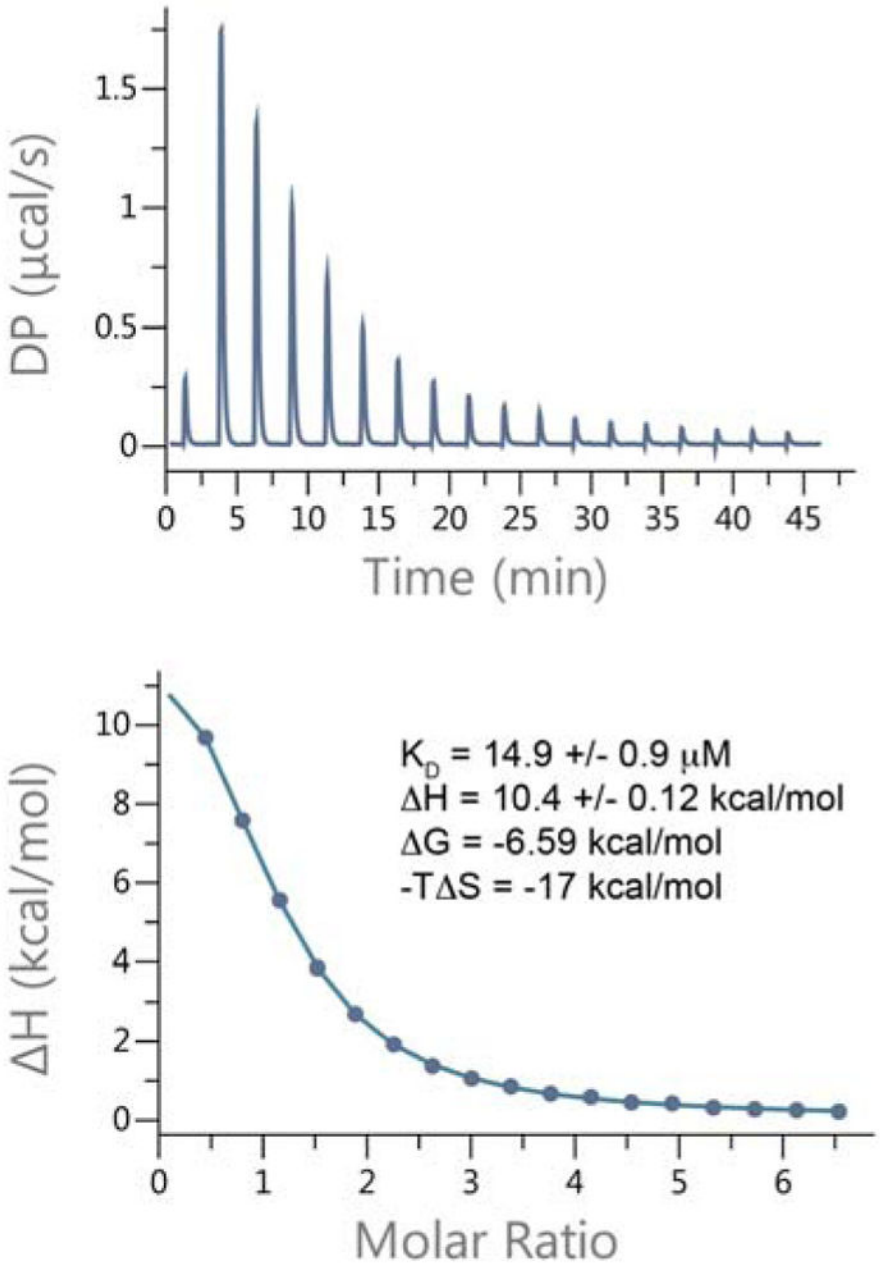

Figure 1.
ITC runs for binding of CYP101D1 to D camphor. Experimental details are provided in methods.

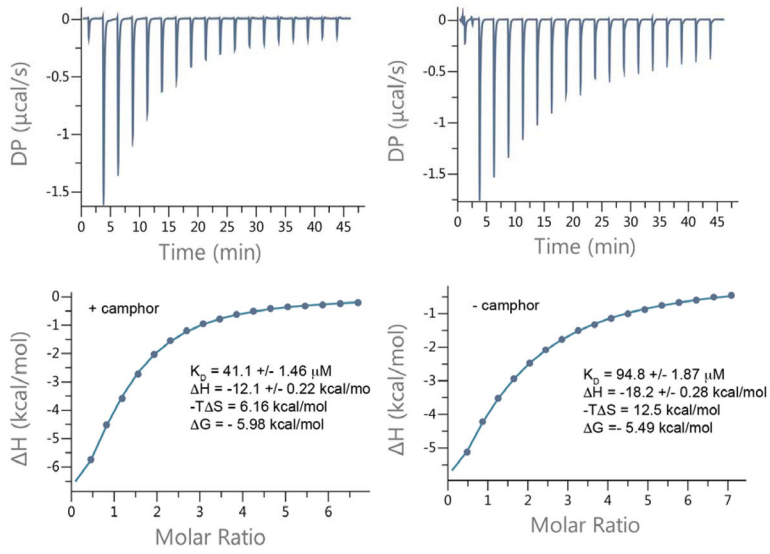

Figure 2.

ITC runs for binding of CYP101D1 to redox partner Arx in the presence and absence of substrate, D-camphor. Experimental details are provided in methods.

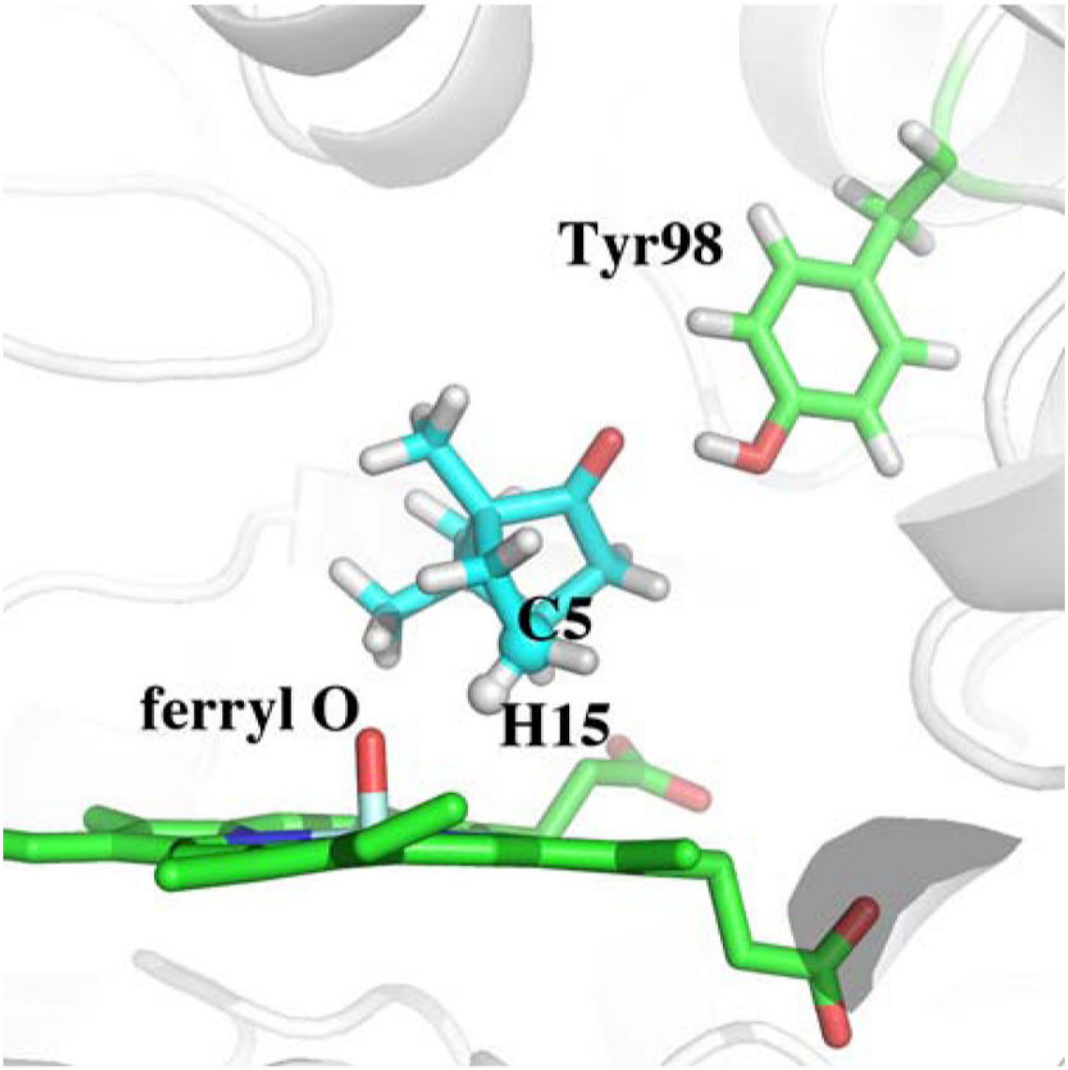

Figure 3.
Active site of CYP101D1 compound 1.

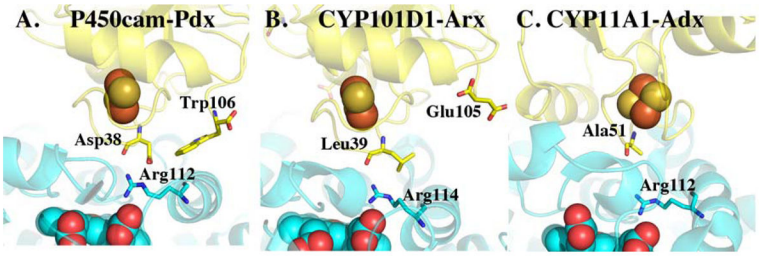

Figure 4.

The P450cam-Pdx complex crystal structure and a hypothetical model of the CYP10D1-Arx complex. The CYP101D1-Arx model was obtained by superimposing CYP101D1 on to P450cam in the P450cam-Pdx. Arx then was superimposed on Pdx in the P450cam-pdx complex. Also shown is the crystal structure of CYP11A1-Adx complex (3N9Y). In all three diagrams the P450 is in cyan (bottom structure) and the redox partner is yellow (top structure).

Table 1

Summary of ITC results. Data for P450cam are taken from our previous work [9] and those published by Liou et al. [15].

Camphor					
	K_D (μM)	**ΔH (kcal/mol)**	**-TΔS (kcal/mol)**	**ΔG (kcal/mol)**	**reference**
P450cam	1.2 +/− 0.4	2.13 +/− 0.09	−10.2	−8.09	[9]
CYP101D1	14.9 +/−0.9	10.40 +/− 0.2	−17.0	−6.59	This work
Redox partner-campbor					
P450cam	19.4 +/− 1.23	−6.97 +/− 0.13	0.54	−6.43	[9]
P450cam	15.2	−9.3	2.7	−6.6	[16]
CYP101D1	94.8 +/− 1.87	−18.2 +/− 0.28	12.5	−5.5	This work
Redox partner +camphor					
P450cam	44.6 +/− 2.97	−7.93 +/− 0.23	1.99	−5.94	[9]
P450cam	29.0	−6.5	0.33	−6.19	[16]
CYP101D1	41.1 +/− 1.46	−12.1 +/− 0.22	6.2	−6.0	This work

Table 2

Results from several 100 ns molecular dynamics simulations. The total % of 5000 frames where the C5-Fe distance ≤ 4.5Å was followed. Also followed is the H-bond between Tyr96 and the camphor carbonyl O atoms. Finally, the % of frames where the camphor H15 atom is in position for H atom abstraction by the ferryl O atom relative to other camphor carbon atoms is presented.

	Run 1	Run 2	Run 3	Run 4	Run 5
% C5-Fe ≤4.5Å	99.9	97.9	99.6	99.4	99.5
% Y98-camphor≤3.5Å	97.5	96.8	98.4	98.5	99.2
% H15-ferryl O ≤3.0ÅFe-O-H15 120°–140°	68.0	76.5			