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Redox Partner Recognition and Selectivity of Cytochrome P450lin (CYP111A1)

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Abstract

The strict requirement of cytochrome P450cam for its native ferredoxin redox partner, Pdx, is not exhibited by any other known P450 system and the molecular details of this selectivity are still not completely understood. We therefore examined the selectivity of a related *Pseudomonas* cytochrome P450, P450lin, by testing its activity with non-native redox partners. We found that P450lin could utilize Arx, the native redox partner of CYP101D1, to enable turnover of its substrate, linalool, while Pdx showed limited activity. Arx exhibited a higher sequence similarity to P450lin's native redox partner Ldx than Pdx, including several residues that are believed to be at the interface of the two proteins, based on the P450cam-Pdx complex structure. We therefore mutated Pdx to resemble Ldx and Arx and found that a double mutant, D38L/Δ106, displayed higher activity than Arx. In addition, we found that Pdx D38L/Δ106 does not induce a low-spin shift in linalool bound P450lin but does destabilize the P450lin-oxy complex. Together our results suggest that P450lin and its redox partners may form a similar interface to P450cam-Pdx, but with a different relative orientation.

Graphical Abstract

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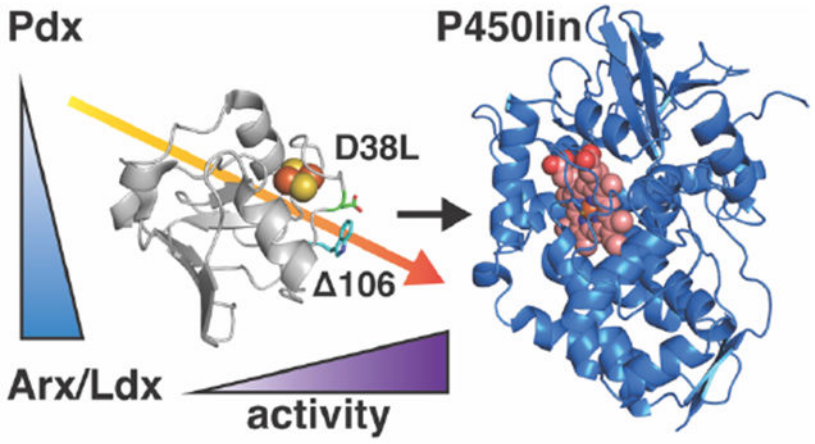

Introduction

Cytochromes P450 (CYPs or P450s) are a superfamily of heme enzymes that execute a broad scope of oxidation reactions and play essential roles in many biosynthetic and metabolic processes throughout the biosphere.[1] Due to their physiological importance and biocatalytic potential, great efforts have been devoted to defining the structure-function relationships of P450s to understand the origin of their substrate specificity, regio- and stereoselectivity, and oxidative power.[2] To achieve substrate oxidation, P450s require electrons that are typically derived from a redox partner protein. Although redox partners are usually discussed as passive components of the catalytic cycle serving as simple auxiliary electron transfer proteins, there is a growing consensus that redox partners can actively modulate P450 functionality.[3, 4]

The first evidence of redox partner induced effects came from cytochrome P450cam, a model system for P450 structure-function studies. P450cam was found to exhibit a strict requirement for its native redox partner putidaredoxin (Pdx) during the hydroxylation reaction of its substrate, camphor.[5] Over several decades, it was demonstrated that this specificity arises from a conformational change induced by the binding of Pdx necessary for initiating O_2 activation and formation of the active oxidant, Compound I.[6, 7] This ‘effector’ role of Pdx causes an opening of the active site that promotes heterolytic cleavage of the O–O bond through the formation of a proton relay network that delivers protons to the iron-linked O_2 unit. Specifically, upon Pdx binding, an active site aspartate, Asp251, is freed from ion-pairing interactions with Lys and Arg residues along the F-helix which allows the Asp to undergo a rotameric change and initiate the proton relay.[3, 8]

Thus far, the specificity of P450cam has not been found to be shared by other P450 systems as many P450s can accept both electrons from non-cognate redox partners. Moreover, it is not clear whether the lack of specificity is because the conformational change upon redox partner binding does not occur or that non-native redox partners can induce the same conformational dynamics in these systems. CYP101D1, for example, catalyzes the same reaction as P450cam, but its interaction with its redox partner is less specific as Pdx can support CYP101D1 catalysis, but the redox partner of CYP101D1, Arx, cannot support

P450cam. Despite this lack of selectivity, CYP101D1 remains highly coupled, meaning nearly 100% of the electrons derived from NADH are utilized to form hydroxylated product.[9] X-ray crystal structures along with spectroscopic and thermodynamic data indicate that formation of the proton relay in CYP101D1 may not require the conformational change that is necessary in P450cam.[9–11] Given this discrepancy between two closely related P450s, the origin of the P450-redox partner specificity remains to be clarified. This is increasingly important as redox partner interactions have been shown not only to induce conformational changes, but even change the regioselectivity or type of chemical reaction (e.g. epoxidation vs hydroxylation) as well.[12–15]

As such, our lab has focused on understanding the molecular origins of this P450cam-Pdx interaction as well as examining other similar P450-redox partner interactions for insights. Like P450cam, P450lin (CYP111A1) is a plasmid-borne Class 1 P450 derived from *Pseudomonas incognita* and was the second bacterial P450 to be expressed and characterized. P450lin catalyzes the conversion of linalool to 8-hydroxylinolool (Scheme 1) and is expressed along with its redox partners, Linredoxin Reductase (LdR) and Linredoxin (Ldx).[16–18] Here, we explore the interaction of P450lin with several ferredoxin-type redox partners and characterize redox-partner induced effects on the stability of the oxycomplex (Fe^{II}–O_2).

Materials and Methods

Protein Expression and Purification

P450lin was encoded on a pET28a+ vector with N-terminal His6-tag (Genscript). Cells were grown in Luria Broth (LB) overnight culture of 100 ml with 50 mg/L kanamycin. 10 mL of overnight culture were inoculated into 7 flasks each containing 1 L of Terrific Broth with 50 mg/L kanamycin. Cells were grown at 37 °C and 220 RPM until OD_{600} = 0.8 – 1. Expression was induced by addition of 1 isopropyl β-d-1-thiogalactopyranoside (IPTG) and supplemented with 0.4 mM 5-aminolevulinic acid (D-ALA) The shaker conditions were turned down to 25°C and 100 RPM. Protein was expressed over 48 hours, and then the cells were harvested. Cells were resuspended in lysis buffer (50 mM KPi pH 7.4, 250 mM NaCl, 2 mM BME) and lysed by two passes through a microfluidizer. The cell lysate was centrifuged at 15K RPM for 1 hour at 4°C (Beckman Coulter Avanti JA-17). The supernatant was loaded onto a NiNTA column (ThermoFisher) pre-equilibrated with lysis buffer. After washing with 3-5 column volumes (CVs) of lysis buffer, the protein was eluted with 1 CV of lysis buffer containing 250 mM imidazole. Fractions containing the red-colored protein were pooled. A few granules of thrombin (ThermoFisher) were added into the pooled solution. The protein solution was then dialyzed against lysis buffer overnight at 4°C. The dialyzed protein was loaded and passed through a second NiNTA column pre-equilibrated with lysis buffer to remove any residual protein retaining a His-tag. Protein was buffer exchanged into wash buffer (50 mM KPi pH 7.4, 2 mM BME) by several rounds of centrifugation in 30 kDa centrifugal filters (Millipore Amicon), then loaded onto a pre-equilibrated Q Sepharose column (Cytiva). The column was washed with 5 CVs of wash buffer followed by a gradient elution of the protein with 0 – 600 mM NaCl in wash buffer. Collected fractions with an optical purity ratio (Reinheitzahl, R/Z, A_{417}/A_{280}) > 1.4

were pooled and concentrated to less than 2 mL. The protein was then loaded onto a size exclusion column (SEC) (Cytiva Sephacryl S-200 HR) equilibrated with SEC buffer (50 mM KPi pH 7.4, 5 mM DTT). Fractions with R/Z > 1.6 were collected, concentrated, and flash frozen for storage at −80°C.

P450cam, Pdx, PdR, Arx, ArR, were purified according to previously published procedures.[9, 19, 20] Several unsuccessful attempts were made to express and purify the natural P450lin redox partner , Ldx. We therefore generated mutants of Pdx that converted the expected binding interface to that of Ldx. The two most important differences at the binding interface are Asp38 in Pdx which is a Leu in Ldx and Trp106 of Pdx is missing in Ldx. The D38L mutation was made using the primers in Table S1 by standard polymerase chain reaction protocols (Takara PrimeSTAR Max). The vector was transformed into *E. coli* C41(DE3) stock and plated onto ampilcillin-supplemented Luria Broth (LB) plates. Successful mutation was verified by miniprep (Macherey-Nagel) and sequencing (Genewiz) using standard protocols. The gene sequence for Ldx is known[17]; however, expression of the ferredoxin has proven elusive despite many attempts in different vectors, cell lines, and expression protocols.

Spectroscopy

The NADH consumption assays were run using a Cary Series UV-Vis Spectrophotometer by monitoring the absorbance at 340 nm. The reaction mixtures contained 0.5 μM P450, 0.5 μM reductase, 5 μM ferredoxin in 1 ml of 50 mM KPi pH 7.4. PdR was used with all Pdx proteins, and ArR was used with Arx. The reaction was blanked, and then 2 μl of 185 mM NADH was added to collect the background oxidation rate. Substrate solution (20 μl was then added to initiate the reaction. Reactions with P450cam and P450lin used 8 mM camphor and 10 mM linalool, respectively. Rates were calculated using a least-squares regression method. Traces of representative experiments can be found in Figure S1.

Stopped-flow Kinetics

Stopped-flow experiments were performed using an Applied Photophysics SX18 stopped-flow spectrophotometer. Spectra were acquired using photodiode array (PDA) detection at room temperature. The P450 concentration was 15 μM, and the redoxin concentration was 30 μM, both in 50 mM KPi pH 7.4 with 1 mM linalool. The P450 was reduced with dithionite in degassed buffer. The experiments were performed in a single mix injection, where reduced P450 was mixed with the redoxin in oxygenated buffer in equal volumes. The data were fit to exponential models (eq S1 and S2) using the ProData SX software. In some cases, the initial data points were removed to allow for convergence of the fit as they contributed too much to noise. Additional spectra can be found in Figure S3.

Results

The specificity of P450lin for non-native redox partners was determined by NADH consumption assays. The NADH consumption assay is a simple technique for testing redox partner selectivity. Since electrons must flow from NADH to the reductase, to the redoxin, and finally to the P450 for catalysis and the first electron transfer from the ferredoxin to the

P450 is the rate limiting step, the consumption of NADH acts as an indirect reporter of the turnover rate.[21] Therefore, if the ferredoxin is not compatible with the P450, there will be negligible NADH consumption upon addition of substrate.

It is well established that P450cam is selective for its native redox partner, Pdx. P450lin, on the other hand, was capable of turnover with non-native redox partners Pdx and Arx (Table 1). However, neither of these rates are as fast as the previously published rate using the native LdR and Ldx from the host *Pseudomonas incognita*.[16] Amongst the non-native ferredoxins, Arx displayed the highest turnover rate. Examination of the sequence similarity between Ldx, Arx, and Pdx revealed that a critical residue for the P450cam-Pdx interaction, Asp38[22], is a leucine in both Ldx and Arx. Furthermore, the C-terminal residue of Pdx, Trp106, which also plays an important role in P450cam-Pdx binding and electron transfer[22, 23], is not present in either of the other redoxins. We therefore mutated Pdx to resemble Ldx and Arx by changing Asp38 to a Leu in both WT and a tryptophan knockout variant, Δ106. While both the D38L and Δ106 variants increased NADH consumption slightly over WT Pdx, the D38L/Δ106 double mutant enabled the highest rate of turnover of the ferredoxins with P450lin. Additionally, as expected, the single and double mutants of Pdx did not exhibit turnover with P450cam.

The redox partner selectivity of P450cam is also associated with a spectral shift towards a low spin state upon the binding of Pdx in the presence of high concentrations of camphor (Figure 2).[25–28] We therefore examined whether P450lin undergoes similar changes in its UV-vis spectral profile upon association with the Pdx D38L/Δ106 variant that supports catalysis. However, in contrast to P450cam, we did not observe a shift towards a low-spin population of P450lin in the presence of linalool and Pdx D38L/Δ106 despite its appreciable turnover rate. Due to challenges in the recombinant expression of Ldx, we were unable to test whether the native redox partner of P450lin shifts the spectrum accordingly. As such, Ldx might still induce the low-spin spectral shift. Regardless, these results indicate that the residues that allow Ldx, Arx, and Pdx D38L/Δ106 to support P450lin turnover are distinct from those that may induce a spin-state change in P450lin's active site.

We now know that the connection between a P450's spin-state and its global conformation is much more complicated than previously thought.[25, 29, 30] This observation means that while there is no apparent spin shift upon addition of Pdx D38L/Δ106 to substrate bound P450lin, a change in conformation cannot be ruled out. Since the selectivity of P450cam for Pdx is, in part, due to a conformational change driven by Pdx binding, we sought to determine whether alternative redox partners could induce a similar structural change in P450lin. In the absence of direct structural information, the stability of the reduced oxygen bound P450 or "oxycomplex" has been used to provide insight into redox partner induced conformational changes.[31, 32] As Pdx binding pushes P450cam towards a more open conformation, it was reasoned that redox partner induced structural changes would destabilize the oxycomplex resulting in a faster decay compared to the rate of autooxidation. Accordingly, the addition of Pdx increases the rate of oxycomplex decay by ~150 fold over the autoxidation rate.[33]

In P450lin, the decay of the oxycomplex was monitored with stopped-flow kinetics by the decrease in absorption at 431.5 nm and the growth of the ferric substrate-bound Soret at 388.8 nm. The rate of autooxidation of the P450lin oxycomplex in the absence of redox partner was fit to a monoexponential with both wavelengths exhibiting similar rates of decay and growth (Table 2 and Figure 1). In the presence of redox partner, the rates for P450lin were best fit to a biexponential model where the first component is attributed to the decay of the oxycomplex or growth of the oxidized ferric species. (Table 2 and Figure 1). Consistent with the NADH consumption assays, Pdx D38L/Δ106 displayed the highest oxycomplex decay rate, at ~150x, remarkably similar to P450cam with Pdx. Other redox partners had less of a pronounced effect on the oxycomplex but are also consistent with the trend in NADH consumption rates with Arx increasing the decay rate more than Pdx.

Discussion

The ability of redox partners to modulate the structure and activity of P450s is an active area of research and there is very limited structural information on how these proteins associate and induce such pronounced effects.[14, 15] As such, co-crystallized complexes like those of P450cam-Pdx have allowed for a deeper understanding of redox partner selectivity and origin of induced conformational changes.[7, 8] However, conservation of the selectivity of P450cam and the redox partner induced transitions across other P450s remains unclear. Based on the similarities between P450cam and P450lin, we hypothesized that these two systems would behave quite similarly. When P450lin was first expressed and purified from the host *Pseudomonas incognita*, the selectivity was tested with Pdx and PdR.[16] Our results with P450lin with Pdx and PdR showed the same level of selectivity, albeit at slightly different rates of turnover. As we were interested in the origin of selectivity and efficient electron transfer, we wanted to expand the scope of redox partners tested for activity. Arx, the native redox partner of CYP101D1, is more promiscuous than Pdx as it services many P450s in its native organism.[34, 35] NADH consumption assays revealed that Arx enables turnover in P450lin significantly higher than those found with Pdx.

A sequence alignment of Pdx, Arx, and Ldx revealed high degrees of similarity between Ldx and Arx (54.90% identity) and moderate conservation between Ldx and Pdx (33.98% identity) (Figure 3). We noted several important differences between the three ferredoxins including at a critical aspartate of Pdx, Asp38, that forms an interfacial ion pair with Arg112 of P450cam.[7] Mutation of D38 in Pdx kills P450cam turnover activity.[22] In the analogous position of Arx and Ldx, there is a leucine residue. We therefore mutated Asp38 of Pdx to Leu and found a slight increase in NADH consumption activity. Additionally, both Arx and Ldx lack a terminal tryptophan that is key for Pdx to bind and transfer electrons to P450cam (Figure 3). Using a knockout variant of Pdx, Δ106, we created a double mutant including the D38L substitution. While Δ106 itself allowed for only slight improvement in turnover compared to Pdx (8.67 min^{-1}), the D38L/Δ106 variant displayed a substantial increase in activity (198 min^{-1}) over both WT ferredoxins and the single mutant. The ~5 and ~20-fold increase in activity of D38L/Δ106 compared to the single mutant D38L and knockout, respectively, highlights how sensitive the interfacial dynamics are between P450s and their redox partners. Based on these mutations, it appears that P450lin and Ldx likely share a similar interface to those found in other Class 1 systems like P450cam. However,

the nature of the interactions between the two proteins is what dictates their orientation and selectivity.

As there is no crystal structure of P450lin, we utilized the Alphafold predicted structure for P450lin to analyze the potential interfacial interactions with Ldx (Figure 4).[36, 37] The predicted structure exhibits the expected conserved P450 triangular fold. The areas of lowest confidence are also coincidentally the areas that vary the most from P450cam and likely participate in substrate binding. The Alphafold structure appears in an open conformation and while we do not know whether P450lin exhibits an open-to-close transition upon substrate binding, the P450cam-Pdx complexes are found in a slightly open conformations even in the presence of substrate, allowing for a reasonable comparison.

Many of the crucial P450cam residues that interact with Pdx originate from the C helix.[7, 38, 39] Overlaying the C helix from the P450cam-Pdx complex structure (PDB: 4JWU) with the Alphafold structure of P450lin (Figure 5) reveals that their structural alignment correlates well with the sequence alignment for this structural element. In P450cam, there are several residues that point towards Pdx, namely Arg109, Arg112, Ala113, and Asn116. The corresponding residues of P450lin are Thr100, Arg104, Lys105, and Met107. Only one of these residues is conserved, Arg104, which in P450cam is Arg112 that is essential for electron transfer and forms a hydrogen bond with one of the heme propionates.[39] The retention of this residue and its role in electron transfer makes the results of our selectivity experiments quite surprising as D38 in Pdx interacts with Arg112 and its mutation to a Leu in Ldx and Arx would likely weaken the strength of this contact, but instead enhances the rate of NADH consumption. This result may point to the fact that while the interfaces between P450lin and its redox partner are conserved, the orientation of the two proteins is not. Additionally, the increased activity of the double mutant supports this hypothesis as the removal of the C-terminal tryptophan may allow Pdx to adopt a new preferred orientation with P450lin that allows for more efficient electron transfer.

Beyond its electron transfer role, Pdx induces structural changes that allow for the formation of a proton relay network in P450cam. Without crystallographic or NMR structural information, it is difficult to assess whether similar changes occur in P450lin upon redox partner binding. P450lin has a conserved Thr at position 248 that, in other P450s, provides a hydrogen bond to stabilize the oxycomplex and promotes the heterolytic cleavage of the iron-linked oxygen molecule.[40] It also displays an adjacent Glu at position 247 where P450cam employs a similar Asp251. This distinction is subtle but important because when D251 is changed to a Glu in P450cam, activity is completely lost and Pdx loses its ability to destabilize the oxycomplex.[11] CYP101D1, however, also has the corresponding Asp but it is tied up in less salt bridges than P450cam and the D259E variant retains partial activity and displays a large kinetic isotope effect (KSIE).[11] The two residues that form the ion pair with D251 in P450cam are Arg186 which is conserved in CYP101D1 and Lys176 which is a glycine. This lack of a second ion-pair in CYP101D1 is what allows for the retention of activity in the D259E variant. The D259E P450cam mutant is inactive as the more flexible Glu cannot be freed from the strong local H-bonding interactions upon Pdx binding, while in D251E CYP101D1 is able to break the single R186 contact. In P450lin, the alignments suggest no analogous ion-pairing for E247. Although the Alphafold structure indicates this

area of the F-helix is of moderate confidence, the corresponding residues of P450lin are Y168 and G176. These differences suggest one reason why P450lin exhibits less selectivity for its redox partner.

While the selectivity of P450lin is decreased compared to P450cam, this difference does not preclude a conformational change upon redox partner binding. While Pdx pushes P450cam towards a low spin state, the Pdx D38L/Δ106 mutant that allows for the highest rate of NADH consumption does not induce a similar shift in the UV-vis spectrum of linalool-bound P450lin. There is, however, one feature shared by several bacterial P450s including P450lin.[31, 33, 41] The productive binding of a redox partner results in destabilization of the P450 oxycomplex. This could be due to back electron transfer from the ferrous oxycomplex to iron-sulfur cluster of the redox partner. Alternatively, there is the functionally much more interesting possibility that redox partner binding in all these P450s results in similar protein conformational changes or more subtle changes in the heme and ligands required for activation of the oxy-complex. If so, then redox partner binding serving an effector role in the activation of the P450 oxy-complex may be a general feature in P450 catalysis.

In conclusion, P450lin is less selective for its redox partner than P450cam despite originating from a highly homologous system. Even so, the binding interface between the redox partner and the P450 in both P450cam and P450lin are the same although the specific types of interactions at the interface and possibly orientation differ. Like other P450s, the interaction of P450lin with its redox partner does result in a change upon redox partner binding that leads to a destabilization of the iron-linked O_2 unit. Although the selectivity of P450cam is so far unique, how redox partner binding influences stability of the oxycomplex, which possibly plays an important role in activation of the oxycomplex, may be conserved in many P450s.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The redox partner selectivity of P450cam and P450lin were tested against two wild-type ferredoxins, Pdx and Arx and three Pdx variants. Pdx was mutated to resemble Arx and the native Ldx at two key residues, D38L and Δ106.
- P450lin is not as selective for its redox partner and exhibits turnover with WT Arx and the Pdx variants.
- P450cam retains its selectivity with only wild-type Pdx.
- Addition of redox partners increases the decay rate of the P450lin-oxy complex.
- The double mutant, D38L/Δ106 of Pdx, leads to the highest turnover rate in P450lin and the fastest decay rate of the P450lin-oxy complex.
- P450lin-redox partner interface might be the same, but orientation might be different.

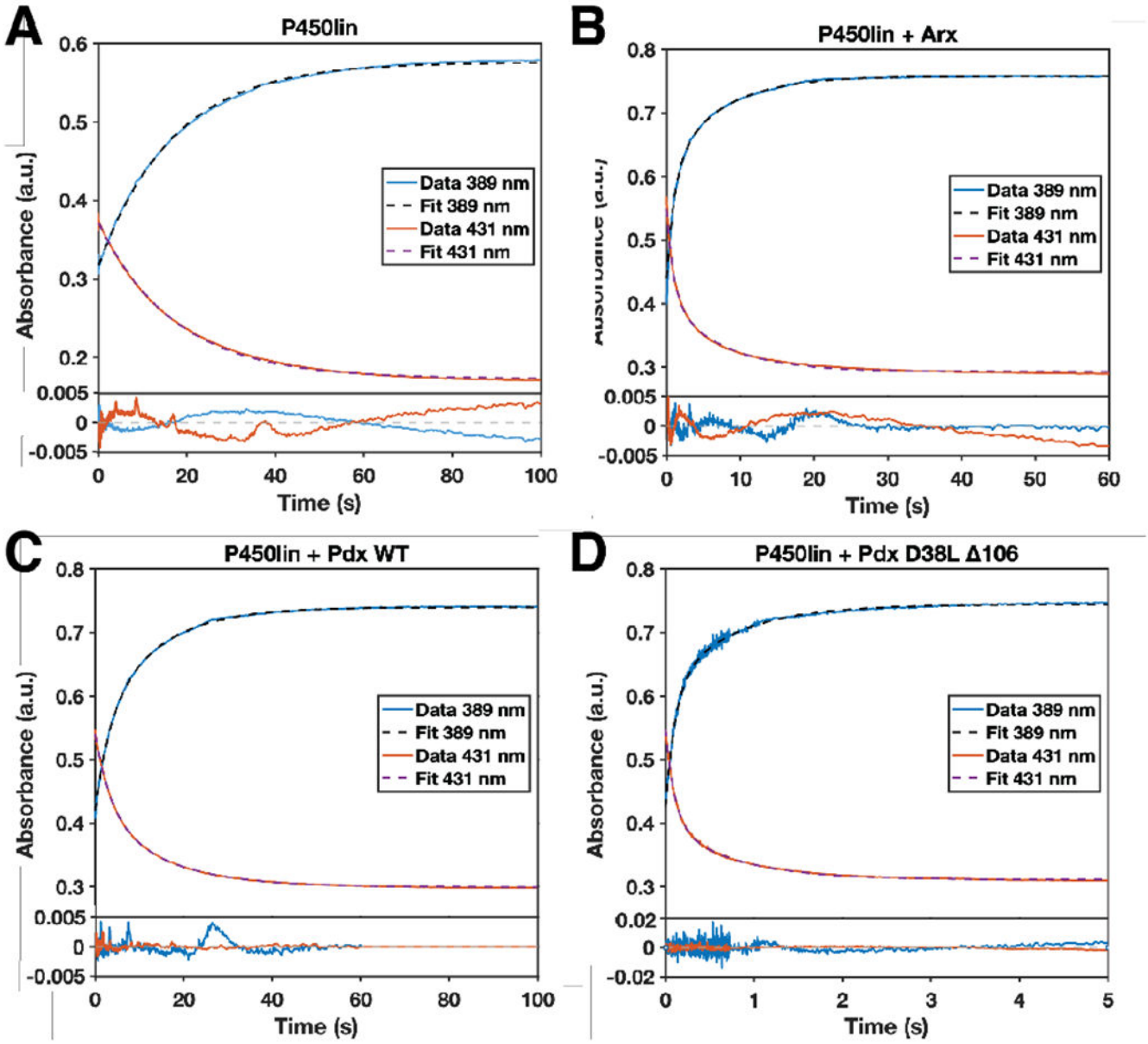


Figure 1.

Stopped-flow kinetics of P450lin oxycomplex decay. (A) P450lin oxycomplex decay monitored at 432 nm and return to ferric at 389 nm in the (A) absence of redox partner and in the presence of (B) Arx at 2-fold concentration, or (C) Pdx at 2-fold concentration, or (D) Pdx D38L/Δ106 at 2-fold concentration.

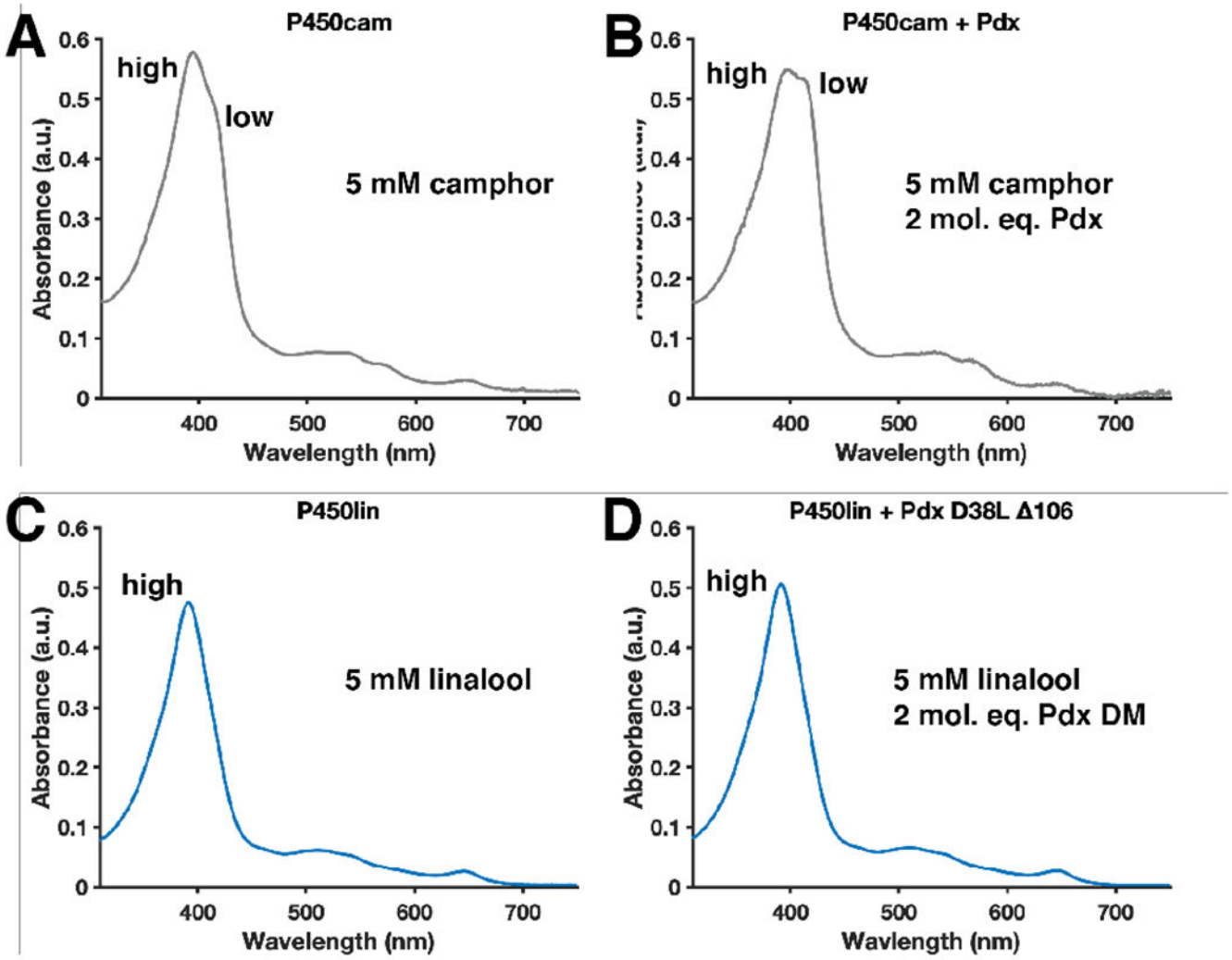


Figure 2.

UV-vis spectra of the P450 spin-shift in presence of redox partner. P450cam camphor in 10 mM KPi pH 7.4 containing 5 mM camphor in the (A) absence and (B) presence of 2 molecular equivalent of Pdx. P450lin in 10 mM KPi pH 7.4 with 5 mM linalool in the (A) absence and (B) presence of 2 molar equivalents of Pdx double mutant (DM, D38L/Δ106).

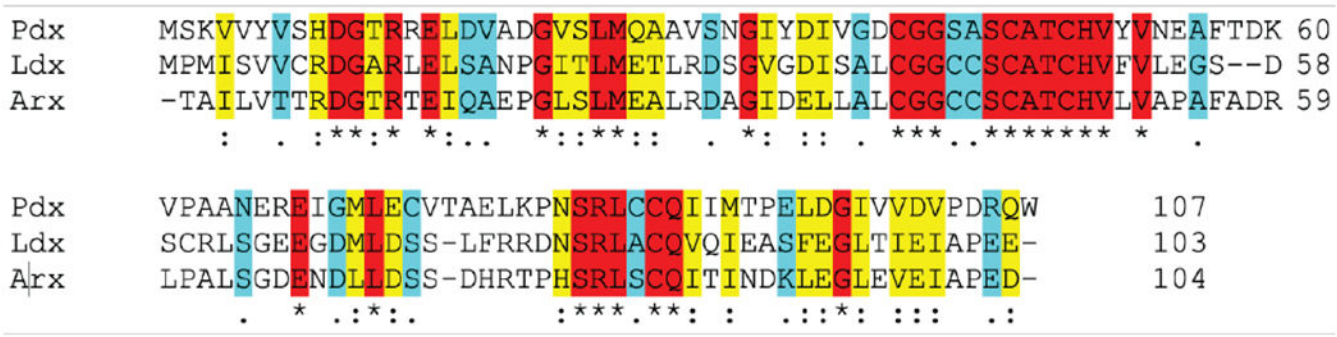


Figure 3.

Ferredoxin sequence alignment. Conserved residues are highlighted in red and are marked with an asterisk. Highly similar residues are highlighted in yellow and are marked with two dots. Similar residues are highlighted in cyan and are marked with one dot. Ldx and Pdx have 33.98% identity. Ldx and Arx have 54.90% identity.

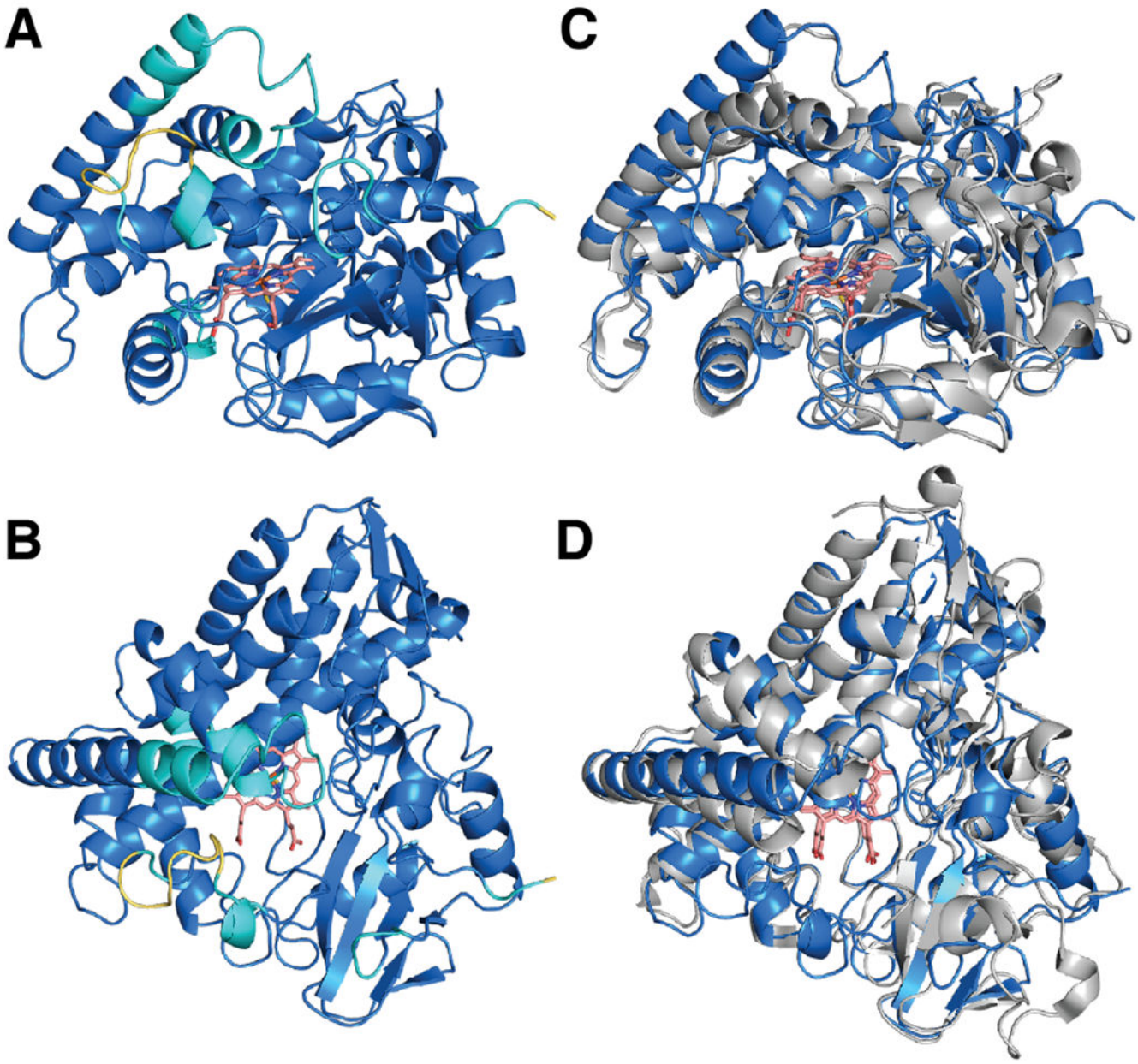


Figure 4.

Structural alignment of P450cam in grey (PDB 3L62) and P450lin in blue (AlphaFold). (A) P450lin Alphafold structure. Regions of high confidence are shown in blue, average confidence shown in cyan, and low confidence shown in yellow. The heme of the Alphafold structure was added in Coot based on the structure of P450terp (1CPT) and is shown in pink. The heme of P450terp was chosen as the orientations of the heme propionates are more common than those found in P450cam. (B) P450lin Alphafold structure rotated ~ 60°. (C) P450lin Alphafold structure aligned to P450cam open structure and (D) rotated ~ 60°.

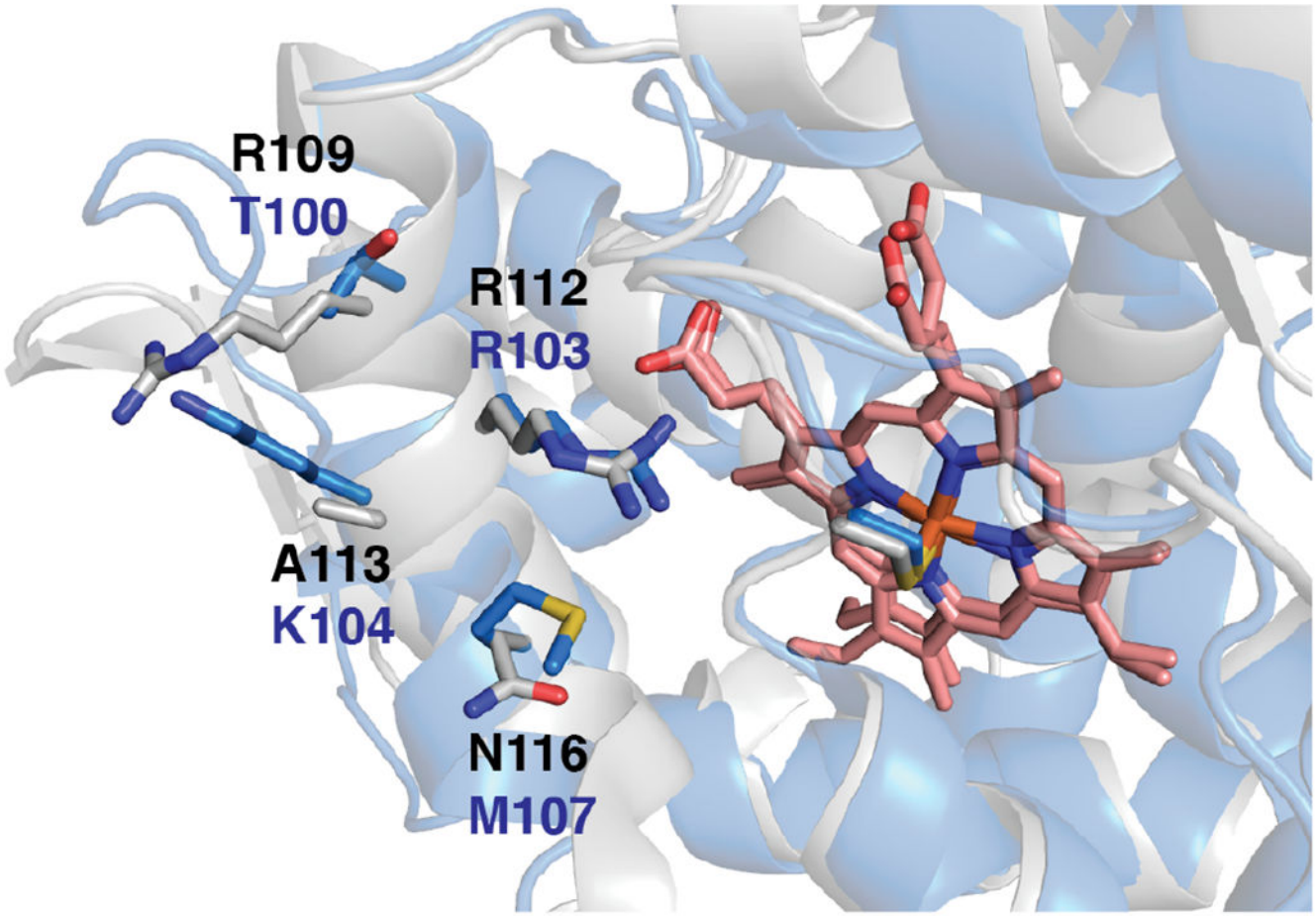
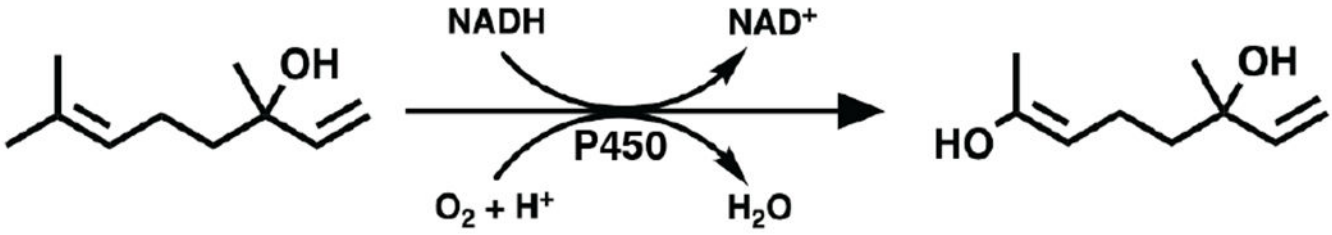


Figure 5.

Structural alignment of residues involved in ferredoxin binding on C helices of P450cam (grey) and P450lin (blue). The heme is shown in pink. Residue labels are shown in black for P450cam and blue for P450lin.



Scheme 1.
The hydroxylation of linalool to 8-oxo-linalool as catalyzed by the P450lin system.

Table 1.

NADH consumption assay rates. Rates are given in mM^{-1} NADH μM^{-1} P450 min^{-1}.

P450	Arx	Pdx WT	Pdx Δ106	Pdx D38L	Pdx D38L/Δ106
lin	179 ± 17	5.44 ± 0.45	8.67 ± 0.17	40.3 ± 3.3	198 ± 1
cam	− [9]	~1000 [24]	− [22]	–	–

Table 2.

P450lin oxycomplex decay rates from stopped flow kinetics.

Wavelength (nm)	Ferredoxin	k_1 (s^{-1})	k_2 (s^{-1})	Fold Increase
431.5	–	0.0584 ± 0.0007	–	1
431.5	Pdx WT	0.274 ± 0.007	0.0663 ± 0.001	4.69
431.5	Arx	1.17 ± 0.16	0.158 ± 0.031	20.0
431.5	Pdx D38L/Δ106	9.61 ± 0.14	1.28 ± 0.07	165
388.8	–	0.0590 ± 0.0005	–	1
388.8	Pdx WT	0.310 ± 0.005	0.0795 ± 0.0006	5.25
388.8	Arx	0.881 ± 0.017	0.136 ± 0.003	14.9
388.8	Pdx D38L/Δ106	9.10 ± 0.24	1.31 ± 0.09	154