Published in final edited form as:

J Mol Biol. 2009 May 15; 388(4): 801–814. doi:10.1016/j.jmb.2009.03.046.

Structural and dynamic implications of an effector-induced backbone amide *cis-trans* isomerization in cytochrome P450_{cam}

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Abstract

Experimental evidence has been provided for a functionally relevant *cis-trans* isomerization of the Ile 88-Pro 89 peptide bond in cytochrome P450_{cam} (CYP101). The isomerization is proposed to be a key element of the structural reorganization leading to the catalytically competent form of CYP101 upon binding of the effector protein putidaredoxin (Pdx). A detailed comparison of the results of molecular dynamics simulations on the *cis* and *trans* conformations of substrate- and carbonmonoxy-bound ferrous CYP101 with sequence-specific Pdx-induced structural perturbations identified by nuclear magnetic resonance is presented, providing insight into the structural and dynamic consequences of the isomerization. The mechanical coupling between the Pdx binding site on the proximal face of CYP101 and the site of isomerization is described.

Keywords

electron transfer; NMR; TROSY; protein dynamics; molecular dynamics; molecular recognition; substrate binding; monooxygenase; proline isomerization

Introduction

Cytochromes P450 catalyze the selective oxidation of unactivated C-H bonds by molecular oxygen, one of the most mechanistically complex and difficult chemical reactions that occur in living organisms. P450 enzymes are involved in a multitude of biosynthetic and catabolic processes, and Entrez Gene currently lists over 10,000 sequences encoding putative P450 enzymes. Despite this diversity, it appears that most P450 enzymes share a common reaction pathway and similar core structural features. Members of this superfamily are characterized by an iron-protoporphyrin IX prosthetic group with a cysteine thiolate axial ligand for the iron.

The best characterized member of the P450 superfamily in terms of structure and mechanism is cytochrome P450_{cam} (CYP101). CYP101 catalyzes the 5-*exo*-hydroxylation of camphor, the first step of camphor catabolism by the soil bacterium *Pseudomonas putida*.¹ Much of what is

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known or suspected regarding P450 function has been learned by examination of this enzyme. However, many questions remain concerning CYP101 function, including the role of effectors in the stimulation of catalytic activity in CYP101. It is well known that the physiological reductant of CYP101, the Fe_2S_2 ferredoxin putidaredoxin (Pdx), is an effector and required component of the catalytically competent CYP101 enzyme system.² In the absence of Pdx, no product formation is observed under standard assay conditions, even if a reductant of appropriate potential is present. Other P450s also make use of effectors: CYP11A1 (P450_{scc}) and CYP27B1, mammalian enzymes involved in biosynthetic pathway leading to pregnenolone and vitamin D, respectively, are activated by adrenodoxin.^{3,4} Cytochrome b_5 has been shown to markedly improve product yields and turnover rates in reconstituted systems of CYP2B4,^{5,6} and P450 reductase shows some ability to act as an effector for this enzyme as well. We have probed the structural basis of effector activity in the CYP101 system, making use of multidimensional NMR methods combined with extensive sequence-specific backbone resonance assignments to follow structural perturbations in the CYP101 structure upon addition of effector (Pdx).⁷⁻⁹ We found that structural perturbations in reduced (Fe^{+2}) CO- and camphor (1)-bound form of CYP101 (CYP-1-CO) upon addition of reduced Pdx (Pdx^r) are not confined to the proximal face of the enzyme where Pdx is presumed to bind,^{10,11} but are transmitted via mechanical coupling to the distal side of the enzyme, which includes structural features adjacent to the active site.⁸ In this context, “distal” refers to the active site face of the heme, while “proximal” refers to the heme face at which the axial thiolate ligand, Cys 357, binds to the heme iron. Based on our observations, we proposed a model for effector activity in which binding of Pdx forces selection of a closed, catalytically active conformation of the enzyme, in which substrates and/or intermediates are blocked from leaving the active site and placed in the correct orientation for catalysis.⁸ Support for this hypothesis was gained when we detected a slow ($\sim 180 \text{ s}^{-1}$) conformational change that takes place in the CYP101 structure upon binding of Pdx.⁷ This conformational change reorients the substrate (camphor) in the enzyme active site appropriately for the observed 5-*exo* hydroxylation reaction.

The discrete nature of the conformational changes observed upon Pdx binding suggested to us that an identifiable structural switch exists between the open and closed (enzymatically competent) conformations of CYP101, and we recently described spectroscopic and mutagenic evidence for the isomerization of a single X-proline amide linkage in CYP101 that occurs upon binding of Pdx.¹² We proposed that this isomerization, specifically that of the amide bond between Ile 88 and Pro 89, is primarily responsible for the Pdx-induced conformational changes and substrate reorientation observed in and around the CYP101 active site.¹² While the Ile 88-Pro 89 bond is found in the *cis* conformation in all published crystallographic structures of CYP101, evidence from NMR and mutagenesis suggests that, while the *cis* conformation is favored upon Pdx binding, a *trans* or distorted *trans* conformation of the Ile 88-Pro 89 bond is most populated in solution. Indeed, mutation of Pro 89 to Ile resulted in both conformers being populated in $\sim 50:50$ ratio, and both camphor conformations, that observed in absence of Pdx and the one observed in the Pdx-bound form, are observed simultaneously in the P89I mutant. Other experimental support for the proline switch was provided by mutation of Tyr 29 to Phe. The phenolic hydroxyl of Tyr 29 stabilizes the *cis* conformer of the Ile 88-Pro 89 bond by hydrogen bonding to the carbonyl oxygen of Ile 88. This interaction is not available to the *trans* conformer (*vide infra*), and while little change was observed in the TROSY-HSQC fingerprint of the Y29F mutant relative to WT CYP-1-CO (as expected if the *trans* form is predominant in solution in the absence of Pdx), the K_d of the Pdx^r-CYP-1-CO complex increases by a factor of ~ 10 ($32 \pm 10 \mu\text{M}$ for WT vs. $441 \pm 202 \mu\text{M}$ for Y29F), as expected for the loss of a hydrogen bond stabilizing the Pdx-bound (*cis*) form.¹²

Pro 89 initiates the B' helix, a structural feature of CYP101 that has been implicated in gating substrate access to the active site and is the distal structural feature of CYP101 that is most uniformly perturbed upon addition of Pdx (Fig. 1). If *cis-trans* isomerization of the Ile 88-Pro

89 amide bond is indeed the structural switch responsible for the observed changes that occur in CYP101 upon binding of Pdx, comparison of the structures of the *cis* and *trans* conformers should show perturbations corresponding to those observed spectroscopically. As noted above, all of the published structures of CYP101 show the Ile 88-Pro 89 bond in the *cis* conformation, so in order to make this comparison, we have performed a series of molecular dynamics simulations on CYP-1-CO with the Ile 88-Pro 89 amide bond in either the *cis* and *trans* conformation. Herein, we compare the results of those simulations with sequence-specific structural perturbations detected by NMR. Finally, we interpret the structural and spectroscopic perturbations in terms of a mechanism for the effector activity of Pdx.

Results

The experimental and computational work described in this paper focuses on the reduced carbon monoxide- and camphor (**1**)-bound form of CYP101 (CYP-1-CO). CYP-1-CO is iso-electronic with the physiologically relevant reduced camphor and O₂-bound CYP101 (CYP-1-O₂) upon which effectors act, but is more convenient for equilibrium NMR studies in that it does not lead to product formation. We have previously described the methodology used for making sequence-specific resonance assignments for CYP-1-CO.^{8,9,12,13} ¹H, ¹⁵N and ¹³C resonance assignments for CYP-1-CO are provided as Supplementary Material.

Backbone perturbations in CYP-1-CO were monitored as a function of Pdx^r addition using a combination of two-dimensional (2D) ¹H, ¹⁵N TROSY-HSQC and three-dimensional (3D) ¹H, ¹³C, ¹⁵N-HNCO experiments for detection of assigned amide ¹H, ¹⁵N correlations. The results obtained using 2D methods have been described previously,^{8,9,12,13} but the use of 3D HNCO experiments to monitor the course of the titrations provides greater detail in overlapped regions of the spectrum. In particular, more details of the effects of Pdx titration on residues in the B, C, I and L helices were obtained using HNCO to monitor the titration. Ile side chain resonances were assigned using selectively ¹³C-Ile, Pro-labeled samples of CYP-1-CO for ¹³C-edited NOESY and TOCSY experiments. Pdx-induced perturbations of Ile side chains were monitored via constant time ¹H, ¹³C HSQC experiments. The assignments of ¹H and ¹³C methyl resonances for enzyme-bound camphor **1** were described previously.⁷

Distribution and time scales of Pdx-induced structural perturbations in CYP101 detected by NMR

Spectral perturbations observed in CYP-1-CO upon addition of Pdx^r can be divided into slow and fast exchange regimes. In the slow exchange regime, the ¹H chemical shift of a correlation changes $\Delta\delta_{\text{max}} > 180$ Hz between the Pdx-free and Pdx-saturated CYP-1-CO. These correlations can only be clearly identified in spectra at the extremes of the titration (i.e., no Pdx^r or Pdx^r-saturated CYP-1-CO, ~ 4 equivalents Pdx^r per equivalent of CYP-1-CO). Correlations in the fast exchange regime show $\Delta\delta_{\text{max}} < 160$ Hz at saturating Pdx^r concentrations, and give rise to distinguishable signals at all intermediate points in the titration. The observed perturbations are shown as a function of structural distribution in Fig. 1. Qualitatively, correlations showing slow exchange behavior are expected to represent larger environmental changes for the residues on which they report, and give some sense of the regions that are most perturbed by Pdx^r binding. Examples of all three classes are provided in Supplementary Material.

Not surprisingly, many correlations assigned to residues in or near the proposed Pdx binding site on the proximal side of the enzyme exhibit slow exchange behavior in the presence of Pdx^r. These include residues on the C and L helices and the proximal loop containing Cys 357, the axial heme iron ligand. However, slow-exchange perturbations are not restricted to the proximal face of CYP101, but are also found in a number of regions on the distal side of the protein (Fig. 1). In particular, residues 247–258 in the region of the I helix that forms one wall

of the active site are among the residues most strongly perturbed by Pdx binding. The I helix is the longest helix in CYP101, and provides a “backbone” to the molecule around which other secondary structural features are arranged. Other slow-exchange perturbations are observed in the distal B helix, the B–B’ loop, the loop connecting the B’ and C helices, and residues on the F helix where it crosses the I helix.

Comparison of molecular dynamics (MD) results for *cis* and *trans*

conformations of CYP-1-CO—The ‘*cis*’ and ‘*trans*’ designations for the two forms of CYP101 refer to whether the dihedral angle ω of the Ile 88–Pro 89 peptide bond is $\sim 0^\circ$ and $\sim 180^\circ$, respectively. Differences between the *cis* and *trans* structures were calculated from the simulations by measuring the root mean square deviation (RMSD) of each residue in the *trans* structure against the MD average of the *cis* structure. Six regions can be identified as showing significant differences between the two conformers (Figure 2). The most pronounced differences are observed in the B helix region followed by a smaller peak at the B’–C loop. The F, H and I helices also differ between *cis* and *trans*. The rest of the protein shows deviations less than 0.2 Å between *cis* and *trans*, which are likely due to dynamic fluctuations. We noted that even in the equilibrated *trans* structure, the ω dihedral angle between Ile 88 and Pro 89 is somewhat distorted from 180° , fluctuating around a mean value of 162° (-198°). This was reproduced in multiple runs, even in the presence of a dihedral restraint, so we do not consider it an artifact of the simulations, but a reasonable representation of the *trans* structure. As discussed below, such a distortion has been observed in transition state analogs bound to prolyl peptide isomerases, and may be critical in lowering the barrier to isomerization upon Pdx binding.

As expected, the B’ helix, which begins at Pro 89 and is the distal secondary structural feature most uniformly perturbed by Pdx binding from NMR data, is displaced in the *trans* conformer towards the proximal Pdx binding site (Figure 3). The B’–C loop (residues 97–106) which is relatively rigid due to the presence of three prolines, Pro 100, Pro 105 and Pro 106, is also shifted by this displacement, resulting in an outward movement of the N-terminal portion of the proximal C helix, which is a primary contact for Pdx binding. This sets up a “pressure switch”, that is, binding of Pdx moves the C helix inward, driving the B’–C loop and B’ helix distally and forcing the *cis* conformation of the Ile 88-Pro 89 bond (Fig. 3).

An unexpected consequence of the proximal displacement of the B’ helix in the *trans* conformer is a regularization of hydrogen bonding in the I helix between Gly 248 and Thr 252. In all published CYP101 structures (and most other P450 structures as well), the I helix exhibits a significant “kink” that interrupts regular α -helical structure of the I helix near the position of dioxygen binding in the active site of the enzyme. In CYP101 structures, this kink is found between Gly 248 and Thr 252 in the I helix. Poulos and co-workers noted that this distortion permits the side chain hydroxyl Thr 252 $O_\gamma H$ to act as a hydrogen bond donor to the carbonyl of Gly 248, replacing the *i*, *i*+4 hydrogen bond from the Gly 248 carbonyl to the NH of Thr 252 expected in α -helical structures. The resulting gap in the I helix provides a pocket to accommodate the Fe-bound O_2 in the appropriate orientation for chemistry.¹⁴ The Thr 252 $O_\gamma H \cdots O=C$ Gly 248 hydrogen bond was stable in our simulations of the *cis* form, but is lost in the *trans* form, and replaced by the more typical *i*, *i*+4 α -helical hydrogen bond between Gly 248 C=O and HN Thr 252 (Figure 4). While this change does not result in a large RMS displacement in the immediate vicinity of the kink (see Fig. 2), the change is amplified over the length of the I helix, so that the C-terminal end of the helix (residues 264–267) along with the beginning of the J helix show among the largest RMS displacements between *cis* and *trans* conformations in the protein. The displacement of the B’ helix and the B’–C loop in the *trans* conformer is coupled to these changes in the I helix by interactions between the side chains of Phe 98 and Ile 99 in the B’–C loop with the backbone and side chain of Leu 244 and side chain of Met 241 in the I helix, respectively. The side chain methyl groups of Ile 99 show

the largest perturbations in chemical shift of any Ile residue in CYP-1-CO upon addition of Pdx^r, likely due to changes in the relative positions of the Ile 99 side chain and the benzyl group of Phe 111 on the C helix (see Supplementary Material).

The B–B' loop, which is connected to the B' helix by the Ile 88-Pro 89 bond, is another region that shows pronounced differences between the two conformers, in particular at residues Pro 78 and Arg 79, which lie at the C-terminal end of the B helix. We observe that Pro 78 and Arg 79 are oriented differently in the two conformers in simulation. As the *trans* conformation relaxes in the simulation, changes in Pro 78 and Arg 79 appear to be coupled with an inward movement of the B helix (Figure 5).

An additional structural difference near the B' helix is that in crystallographic (*cis*) structures, the phenolic hydroxyl group of Tyr 29 is hydrogen bonded to the carbonyl oxygen of Ile 88, stabilizing the *cis* conformer of the Ile 88-Pro 89 bond (Figure 6). This hydrogen bond is lost in the *trans* conformer, and is not replaced by any other hydrogen bond donor to the Ile 88 carbonyl. This destabilizes resonance delocalization of the Ile 88-Pro 89 amide in the *trans* conformer, and would be expected to increase the “ketone” character of the Ile 88 carbonyl (vide infra).

All the structural differences observed point to a more compact active site in the *cis* conformer relative to the *trans*. Representative distances that are directly related to the volume of the active site are shown in Table 1. Comparison of inter-residue distances in the two conformers indicate that active site volume in the *trans* conformer is ~30% greater than in the *cis* conformer. This is in agreement with our original solvent-free dynamics simulations that were restrained by chemical shifts of bound camphor, which indicated that the active site must expand in order to accommodate the camphor orientation seen in the Pdx-free conformation.⁷

Camphor reorientation in the CYP101 active site upon binding of Pdx—We previously noted that the ¹H shifts of the methyl groups of CYP-1-CO-bound camphor **1** in the presence of saturating Pdx^r match better with those predicted from crystallographic structures than those observed in the Pdx-free CYP-1-CO.⁷ We took this as additional evidence that the crystallographic (*cis* Ile 88-Pro 89) structures of CYP101 represent the closed conformation of the enzyme. Using the ring current shifts as constraints in a vacuum molecular dynamics simulation of the CYP101 starting from the 3CPP crystal structure, the lowest shift violations were obtained for a conformation in which the camphor rotates ~20° around a vector between the 1-C and 5-CH₂ of the camphor, leaving the 10-CH₃ roughly unchanged in position, while the 8-CH₃ moves into a position more directly over the heme ring. The 9-CH₃ remains at approximately the same distance from the heme plane, but moves closer to the heme normal.⁷

To quantify the camphor displacements and/or rotations in the *trans* simulation we measured the distances of 8-CH₃, 9-CH₃ and 10-CH₃ from the heme iron (Fig. 7a). As expected, the 10-CH₃ remains at about the same distance from the heme iron in both simulations. In the *trans* simulation the 8-CH₃ moves over the heme ring (as expected from the observed upfield shift in the *trans* conformer), while in the *cis* simulation, it oscillates between 6 Å and 8 Å from the Fe at the heme ring while 9-CH₃ and 10-CH₃ remain at a constant distance, ~ 5.5 Å. The orientation of camphor with respect to the heme plane also changed during the *trans* simulation (Fig. 7c). The 9-CH₃ rotates with respect to the heme plane normal, the angle formed between 9-CH₃ and the vector joining Fe with C at CO changes from ~125° to ~155° (Figs. 7b and 7d). It was found that 10-CH₃ also moves through this plane although its fluctuations are less pronounced than is observed for the 9-CH₃. The disruption of the crystallographically observed hydrogen bond between the camphor 2-O and Tyr 96 hydroxyl observed in our original simulations using chemical shift restraints⁷ is also seen here in the *trans* simulations.

Discussion

Comparison of NMR and MD results

For the most part, regions of the CYP101 structure that are most perturbed upon isomerization of the Ile 88-Pro 89 amide bond in simulation are also observed experimentally to be the most affected by Pdx titration, supporting the idea that this isomerization is the primary driver of structural changes upon binding of Pdx. For example, large effects are seen both in the simulations and experimentally for the B, B' and C helices, as well as the loops between these helices. However, it is important to realize that large RMSD displacements do not necessarily reflect major environmental changes (to which NMR is more sensitive). A good example of this is found in the I helix. Residues adjacent to and including the “kink” between Gly 248 and Thr 252 in the I helix (Val 247-Ser 258) all show chemical shift changes > 160 Hz (see Fig. 1), indicating that these residues undergo significant environmental changes upon Pdx binding, resulting from changing hydrogen bond lengths and ring current shifts from the heme. Indeed, the regularization of the I helix structure between Gly 248 and Thr 252 observed in the *trans* simulation is a substantial change in the local environment, even though it does not result in large RMS displacement differences between the *cis* and *trans* conformers in this region. However, where larger RMSD differences are observed, near the C-terminal of the I helix, chemical shift changes are smaller, since the displacements do not result in changes in local hydrogen bonding patterns or ring current shifts.

Another region in which large RMS displacements occur in the *trans* relative to the *cis* simulations is at the N-terminus. In particular, in the last 200 ps of the *trans* simulation, this deviation becomes more pronounced. No coordinates are reported for the first eight residues of CYP101 in the 3CPP structure due to disorder, and we did not include these residues in either simulation. Furthermore, narrow NMR line widths and lack of significant chemical shift dispersion indicate that in CYP-1-CO, the N-terminal disorder extends at least through the backbone atoms of Leu 11. As such, we expect that the RMS differences between *cis* and *trans* conformers seen at the N-terminus, if not an artifact, are indications of weak structural constraints on this region of the polypeptide and do not reflect intrinsic differences resulting from the isomerization. To insure that the conclusions we reach regarding structural differences between the two conformations of CYP-1-CO are based on structural changes induced by the isomerization and not due to instabilities in the simulation, we divided the data into four different blocks: (0–500 ps, 500–1000 ps, 1000–1500 ps and 1500–2000 ps) and compared RMSD between *cis* and *trans* for each block. The RMS differences between *cis* and *trans* shown in Fig. 2 to which we refer in forming conclusions about the structural consequences of isomerization are present in all four blocks.

Role of the effector in CYP101 turnover

Based on previous and current observations, we can now propose a mechanism by which effector binding drives population of the catalytically competent conformation of CYP101. Binding of the effector on the proximal side of CYP101 exerts a lateral force on the C helix and the relatively rigid B'–C turn-loop. This force is transmitted mechanically via the B' helix, which is displaced distally approximately along the helical axis, resulting in isomerization of the Ile 88-Pro 89 bond from a distorted *trans* conformation in the absence of Pdx to the crystallographically observed *cis* conformer in the Pdx-bound form. This mechanism is depicted in Figure 3. The isomerization also drives reorientation of the substrate as described in the Results section.

Mechanical linkage between the I helix and the B'–C loop is provided by contacts between the side chains of Phe 98 with Leu 244 and Ile 99 with Met 241. The distal displacement of the B'–C loop upon effector binding is coupled to distortions in the I helix by these interactions.

This model is consistent with the observation of product evolution in the absence of effector in crystalline CYP101.¹⁵ It is likely that crystal packing forces exert a restraint similar to that exerted by effectors on conformational selection in CYP101, so that only the more compact *cis* conformer is observed in crystallographic studies.

It is worth noting that Morishima et al. reported a mutant of CYP101, L358P, which, in the absence of Pdx, spectrally resembles Pdx-bound WT CYP-1-CO.¹⁶ We have found that the camphor orientation in L358P CYP-1-CO is similar, but not identical to that found in the Pdx-bound WT CYP-1-CO (or in the *cis* conformer of the P89I mutant). Furthermore, there are a large number of distal NH correlations in the HSQC spectrum of L358P which show splitting, indicating the presence of non-native conformations.¹³ Therefore, while it is likely that the *cis* conformation of Ile 88-Pro 89 bond is favored in Pdx-free L358P CYP-1-CO, we cannot draw any conclusions as to the origin of this preference based on current results.

Role of the local environment of the Ile 88-Pro 89 bond in the isomerization

In our previous publication,¹² we proposed that the isomerization of the Ile 88-Pro 89 peptide bond ($\sim 160 \text{ s}^{-1}$ at half-saturation with Pdx) is catalyzed by local structural features, in analogy with the active sites of prolyl peptide isomerases (PPIases), which can increase X-Pro isomerization rates up to six orders of magnitude relative to the uncatalyzed reaction.¹⁷ Typically, these enzymes provide a hydrophobic environment for the carbonyl of the target amide that destabilizes the $^-\text{O}=\text{C}=\text{N}^+$ resonance structure that imparts double-bond character to the isomerizable C-N bond. They also provide a specific hydrogen bond donor that stabilizes the developing sp^3 hybridization on N in the transition state by hydrogen bonding to the nitrogen lone pair. Finally, there is evidence that the ground state of the *trans* form of the target peptide bond is distorted from planarity in the PPIase active site by a combination of steric interactions and destabilization of the charge-separated resonance structure mentioned above.^{18,19}

All of these factors are present in the CYP101 structure near the Ile 88-Pro 89 amide bond. We noted that, with the exception of the Tyr 29 OH---O=C Ile 88 hydrogen bond in the *cis* conformer, the Ile 88-Pro 89 amide is in a hydrophobic environment, with the nearest side chains being those of Met 28, Tyr 29, Phe 87, Ile 88, Pro 89 and Ile 395. There are no water molecules within hydrogen bonding distance of the Ile 88-Pro 89 bond in the 3CPP crystal structure, or in either the equilibrated *cis* and *trans* dynamics-derived structures. Secondly, besides stabilizing the *cis* conformer of the amide, the hydroxyl group of Tyr 29 appears to be positioned such that it could provide a hydrogen bond to stabilize the developing sp^3 hybridization of the Pro 89 N in the transition state: A tyrosine hydroxyl group is proposed to function in this manner in the active site of the PPIase FKBP.¹⁷ Finally, the equilibrated dihedral angle ω of the Ile 88-Pro 89 bond fluctuates around 162° in our simulations of the *trans* conformer, even in the presence of an active dihedral restraint to keep the angle at 180° . This suggests to us that steric restraints favor a distorted conformation for the Ile 88-Pro 89 amide bond, rather than a true planar *trans* ($\omega = 180^\circ$) arrangement. Other workers have previously described evidence for intramolecular catalysis of Xxx-Pro isomerization in the context of protein folding.^{20,21}

The *trans* conformation of the Ile 88-Pro 89 bond opens substrate access/product egress channels

An important feature of the *cis/trans* isomerization of the Ile 88/Pro 89 bond is that, besides controlling the orientation of substrate in the active site, the isomerization provides a simple gating mechanism for substrate access and product egress to and from the CYP101 active site. A careful series of simulations by Ludemann et al.^{22–24} was used to identify potential substrate/product ingress/egress pathways in the *cis* conformer and rank them according to relative

activation energy. The lowest activation barriers and smallest displacements were identified for routes involving residues that are for the most part either adjacent to or are perturbed by the Ile 88/Pro 89 isomerization: Phe 87, Ile 88 and Ala 92 showed the largest RMS deviations for a likely egress path those authors designated as 2a, with smaller perturbations occurring for Asp 297, Val 396, Ile 395, Tyr 96, Gly 248, Leu 244, Ala 296 and Gly 298.

We find that in our simulations the *trans* conformation results in considerably more solvent exposure for bound camphor than the *cis*, as shown in Figure 8. The gap exposing the camphor is lined by Phe 87 and Pro 89 in the B–B' loop, Thr 185 (F–G loop), Phe 193 (G helix) and Ile 395 (β 5 strand). Clearly, less distortion of the structure is required to allow access to the active site in the *trans* as opposed to the *cis* conformer via several potential pathways described by Ludemann et al. Combined with the increased volume of the active site in *trans* relative to *cis* discussed above, these observations strongly support our previous proposal that the conformation of CYP101 in the absence of Pdx represents an “open” form that, while not catalytically competent, provides ready access to the active site from solvent.

Conclusions

Our previous mutagenesis and NMR results allowed us to conclude that binding of the effector Pdx^r to CYP-1-CO forces isomerization of a single amide bond between Ile 88 and Pro 89 from a *trans* or distorted *trans* conformation to *cis* in the Pdx-CYP-1-CO complex, and that the *cis* conformer is the catalytically competent form of the enzyme.¹² In the present paper, we compare NMR results from titration of CYP-1-CO with Pdx^r with molecular dynamics simulations of the *cis* and *trans* conformers of CYP-1-CO. Based on this comparison, we predict the structural consequences of the isomerization, and provide a mechanism for the effector activity of Pdx.

We conclude that the Ile 88-Pro 89 amide at the N-terminal end of the B' helix is in a distorted *trans* conformation in the Pdx-free form of CYP-1-CO. The *trans* conformer is conducive to substrate binding and product release, due to increased solvent exposure of bound substrate and residues lining the putative active site entry path. Binding of Pdx on the proximal face of CYP101 forces isomerization of the Ile 88-Pro 89 bond from *trans* to *cis* via a mechanical linkage provided by the C helix, the B'–C loop and the B' helix. The isomerization moves bound substrate into the correct orientation for subsequent hydroxylation, and furthermore closes the active site to substrate and/or solvent access. As a result of Pdx-enforced displacements in the B'–C loop, the I helix undergoes a distortion resulting in formation/widening of a “kink” between Gly 248 and Thr 252. This kink is observed in the (*cis*) crystallographic structure of CYP101, and provides a cleft for bound dioxygen in the reactive complex. Local structural features surrounding the Ile 88-Pro 89 amide bond, including hydrophobic nearest neighbors and a specific hydrogen bond donor to stabilize the transition state of the isomerization, lower the activation barrier to formation of the *cis* conformer upon Pdx binding.

Materials and Methods

NMR sample preparation

Perdeuterated U-¹³C, ¹⁵N CYP101 was expressed in *E. coli* strain NCM533 transformed with a plasmid construct encoding the C334A mutant of CYP101 under the control of the *lac* promoter. The C334A mutant has been shown to be spectroscopically and enzymatically identical to wild-type enzyme, but does not form dimers in solution.²⁵ The inoculant was grown in M9 medium supplemented with trace metals (M9+) to an OD₆₀₀ of 1.0. The cells were then transferred into 100% D₂O M9+ media prepared using uniformly ¹³C-labeled glycerol

and $^{15}\text{NH}_4\text{Cl}$. Protein expression was induced with 1mM IPTG when OD_{600} reached 1.0 and harvested after 24 hours.

Purification of CYP101 followed published procedures, with the inclusion of a protamine sulfate nucleic acid precipitation and the exclusion of the ammonium sulfate precipitation. Typically, 1 L of growth medium produces 11 mg of CYP101. Pdx was expressed and purified as described previously. The purities of CYP101 and Pdx were determined by measuring absorption ratios at A_{390}/A_{275} , A_{325}/A_{280} respectively on a Hitachi U-2000 UV-visible spectrometer. Typical concentrations of CYP101 samples used for NMR were ~ 0.2 mM CYP101 of 1.4 absorption ratio, 6 mM Pdx of 0.55 absorption ratio, and were used for NMR experiments. NMR samples were prepared by concentrating samples using Amicon (Millipore) centrifugal concentrators, and then passing samples through buffer exchange resins pre-equilibrated with NMR buffer.

Reduction of CYP101 and Pdx

Samples of CYP-1-CO were prepared as follows. 200 μL of 0.2 mM CYP101 were placed in a CO atmosphere for 15 min, then reduced with 4 μL of 0.25 M $\text{Na}_2\text{S}_2\text{O}_4$ freshly prepared in degassed 1M Tris HCl (pH 8.0). The reduced sample remained under CO for 2 more min before anaerobic transfer to an NMR sample tube (Shigemi). Pdx samples were concentrated to 6 mM in an anaerobic chamber, and then reduced prior to titration experiments with 10 μL of 1M $\text{Na}_2\text{S}_2\text{O}_4$ prepared as described above. Addition of Pdx^{r} to CYP-1-CO for titration experiments were performed anaerobically in the NMR tube using a μL syringe, with subsequent additions of Pdx yielding Pdx:CYP-1-CO ratios of 1/5, 1/2, 1/1, 2/1, and 4/1. Starting concentration of CYP-1-CO was typically 0.2 mM, with Pdx^{r} concentrated to ~6 mM after reduction. Exact concentrations of starting solutions of Pdx^{r} and CYP-1-CO were calculated using by UV-visible spectrophotometry after dilution in appropriate buffers, and Pdx/CYP ratios calculated as described previously.⁸ Reference TROSY-HSQC and HNCOC experiments were obtained prior to addition of Pdx, and both experiments were performed for each titration point using identical acquisition and processing parameters.

NMR Spectroscopy

CYP-1-CO samples for all NMR experiments were 0.2 mM in 90% $\text{H}_2\text{O}/10\%$ D_2O , pH 7.4, 50 mM D-TrisCl, 100 mM KCl and 2 mM *d*-camphor. All NMR experiments were performed on a Bruker Avance 800 MHz spectrometer operating at 800.13, 201.2 and 81.08 MHz for ^1H , ^{13}C and ^{15}N respectively, and is equipped with a dedicated ^2H RF channel (92.06 MHz) for ^2H -decoupling, a pulsed field gradient amplifier and a 5 mm $^1\text{H}(^{13}\text{C}, ^{15}\text{N})$ TCI cryoprobe. All NMR experiments were performed at 25 $^{\circ}\text{C}$.

2D $^1\text{H}, ^{15}\text{N}$ TROSY-HSQC experiments were run with 16 scans per t_1 point, $512 (^1\text{H}) \times 64 (^{15}\text{N})$ complex points, and 1 second recycle delays. Spectral widths were 14 367 Hz (^1H) and 3243 Hz (^{15}N). 2D constant-time $^1\text{H}, ^{13}\text{C}$ HSQC experiments were performed using selectively ^{13}C -Ile, ^{13}C -Pro labeled and otherwise perdeuterated and ^{15}N -labeled CYP-1-CO samples, with spectral widths of 14 367 Hz (^1H) and 4 428 (^{13}C). 3D ^{13}C -edited NOESY ($t_{\text{mix}} = 150$ ms) and HCCH-TOCSY²⁶ ($t_{\text{spin-lock}} = 10$ ms) datasets were obtained for the same samples using spectral widths of 11,160 Hz in both ^1H dimensions and 3 081 Hz in ^{13}C . Digital resolutions for each dimension were 2048 complex points for ^1H (direct dimension), 24 complex points (^{13}C) and 128 complex points (^1H , indirect dimension). Titrations of CYP-1-CO with Pdx^{r} were also monitored with 3D $^1\text{H}, ^{15}\text{N}$ -TROSY-HNCOC²⁷ experiments, which were acquired with a standard pulse sequence and parameters²⁸ using spectral widths of 14 367 Hz (^1H), 3 081 Hz (^{15}N) and 4 428 Hz (^{13}C), with digital resolutions of 2048, 64, and 64 points for each dimension respectively. All NMR data was processed using TopSpin (Bruker Biospin Inc.). Data analysis was also performed using TopSpin.

Molecular dynamics simulations

All simulations were performed using the Amber 9 package.²⁹ The parameters of the system were described using the Amber ff03 force field, except the heme-CO complex and the camphor. The heme and CO were represented using parameters developed and tested by Estrin.^{30,31} Camphor parameters were generated using the Antechamber utility included in the Amber 9 package.

The starting structures were constructed using the structure of camphor-bound carbonmonooxygenase CYP101 (PDB ID 3CPP).³² A covalent bond was created between the Fe atom at the coordination site of heme and the proximal cysteine, Cys 348, with a Fe-S distance of 2.45 Å, as computed by Freindorf et al.³³ using QM/MM. WAT 515 in the 3CPP structure (proposed to correspond to the K⁺ bound between the B-B' loop and B' helix) was replaced with a potassium ion.³⁴ Protonation states for histidine residues were assigned by examination of the crystal structure. All His residues were held protonated at N_ε (Amber residue type HIE) with the exception of His 355, which was assigned N_δ protonation (HID). Seventeen additional K⁺ were added to neutralize the system. The system was solvated with 16931 TIP3P water molecules. Finally, 25 K⁺ and 43 Cl⁻ were added to maintain electroneutrality and to generate a CYP-1-CO final 0.1 M KCl concentration, which is the typical concentration of KCl used to stabilize in NMR experiments.

The *trans* configuration was generated starting from the minimized *cis* configuration and performing a series of restrained molecular dynamics runs, varying the Ile 88–Pro 89 torsion angle ω by 5° at 350 K, using a torsional restraint with a force constant set to 50 kcal/mol, and relaxing the system at 300 K after each run until the *trans* conformer was obtained.

For production runs, the systems were heated to 300 K and equilibrated for 500 ps. The production runs were performed using a 1 fs time step at 1 atm and 300 K. Temperature and pressure were kept constant through a weak coupling with a 2 ps pressure relaxation time and Langevin dynamics with a collision frequency of 10 ps⁻¹. Periodic boundary conditions were used with a Particle Mesh Ewald implementation of the Ewald sum for the description of long-range electrostatic interactions.³⁵ A spherical cutoff of 8 Å was used for the Lennard-Jones nonbonded potentials. Bonds involving hydrogen atoms were constrained to their equilibrium length using the SHAKE routine. For both structures (*cis* and *trans*) three independent runs, each 3 ns long, were performed. The first 500 ps of each run were not considered as part of the production run in order to guarantee statistics performed on completely equilibrated data. Each run was analyzed using the *ptraj* module included in the AMBER 9 program. Root mean square deviations (RMSD) were calculated to test the stability of the systems and to identify the differences between the *cis* and *trans* configurations.

RMSD against the starting structure (PDB entry 3CPP) is shown in Figure 9, for both the *cis* and *trans* conformations. The *cis* conformer is quite stable after the long equilibration of 500 ps, having an average RMSD value of 0.5 Å. As expected, the *trans* conformation shows more variation from the initial structure. RMSD values for the *cis* conformation converge to a small value (1.2 Å), an indication of a good starting structure. Fig. 9 shows the very small deviations of both *cis* and *trans* structures against their MD average, characteristic of well-equilibrated systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a grant from the National Institutes of Health, GM44191 (T.C.P). J.D.M. thanks the Pittsburgh Supercomputing Center (CHE070042P) and Department of Education grant P116Z080180 for providing generous computing resources. Some of the computations were performed on the SGI-Altix (Pople) system at the Pittsburgh Supercomputing Center. E.K.A. thanks Prof. Dario A. Estrin for the heme partial charges and parameters.

Abbreviations

CYP-1-CO	carbon monoxide and camphor-bound oxidized cytochrome P450 _{cam}
CYP101	cytochrome P450 _{cam}
Hz	cycles per second
IPTG	isopropyl-β-D-thiogalactoside
NMR	nuclear magnetic resonance
HSQC	heteronuclear single quantum correlation
M9	minimal growth medium
mM	millimoles per liter
CYP-1-O ₂	dioxygen- and camphor-bound reduced cytochrome P450 _{cam}
Pdx ^r	reduced putidaredoxin
RMS	root mean square
TPPI	time proportional phase incrementation
TROSY	transverse relaxation optimized spectroscopy
WT	wild type
2D	two dimensional
3D	three dimensional

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Figure 1. Model for the complex between Pdx and CYP101¹⁰ showing spatial distribution of Pdx-induced perturbations in the CYP101 molecule relative to the Pdx binding site. Residues shown in blue are unperturbed, those in carmine are perturbed by between 20 Hz and 160 Hz (i.e., they exhibit titrable or fast-exchange behavior) and those in green are perturbed by greater than 160 Hz (i.e., slow exchange behavior). Figure was generated using PyMOL.³⁶

Figure 2.

RMSD for each residue of the *trans* structure against the MD average of the *cis* structure. The histograms are fitted with smooth lines to emphasize the regions showing larger differences. Sequence numbers are shown along the horizontal axis. With the exception of the N-terminal region, comparison of RMS differences for subdivisions of the dynamics tracks confirm that comparable differences are present in all four of the time quadrants of the simulations (see text).

Figure 3.

Displacement of the C helix upon Ile 88-Pro 89 isomerization. The *trans* conformer is shown in magenta, *cis* conformer in blue. In the *trans* conformer, the N-terminal of the C helix is more exposed to solvent than in the *cis* form. Binding of effector (Pdx) on the proximal side of CYP101 is expected to exert a lateral force on the C helix and the relatively rigid B'-C turn-loop. This force (indicated by dark arrow) is transmitted mechanically to the B' helix, which is displaced distally along the helical axis, forcing isomerization of the Ile 88-Pro 89 bond from a distorted *trans* conformation to the *cis*.

Figure 4.

Regularization of the I helix “kink” in the *trans* conformation of the Ile 88-Pro 89. The I helix in the *trans* form is shown in magenta and in blue for the *cis* form (a). Relative positions of the carbonyl oxygen of Gly 248 and the amide NH of Thr 252 from simulation are shown for *cis* (b) and *trans* (c) conformers. The backbone hydrogen bond between the Gly 248 carbonyl oxygen and Thr 252 amide NH in the *trans* conformer is shown as a dotted line.

Figure 5.
Displacement of Pro 78, Arg 79 (B-B' loop) and the B helix upon Ile 88-Pro 89 isomerization.
The *trans* conformer is represented in magenta, the *cis* conformer in blue.

Figure 6. Stabilization of *cis* conformation of the Ile 88-Pro 89 bond by hydrogen bonding between the phenolic OH of Tyr 29 and the carbonyl oxygen of Ile 88. This hydrogen bond is absent in the *trans* conformer.

Figure 7.

Reorientation of camphor in the *trans* conformer. 8-CH₃, 9-CH₃ and 10-CH₃ distances from the heme Fe are shown in (a) and the CH₃-carbonmonoxy C-Fe angles are plotted in (c). The orientation of camphor in the *cis* and *trans* conformers are shown in (b) and (d), respectively. Note that the 8-CH₃ is more directly over the heme macrocycle in the *trans* form, as expected from the increased ring current shifts observed to that methyl group in the *trans* conformer relative to the *cis* (see text).

Figure 8. Increased active site access from solvent in the *trans* conformer. **Top:** *cis* conformation showing Pro 89 (in orange), Ile 395 (light blue), Thr 185 (dark blue) and bound camphor (magenta). Note the low solvent exposure of camphor. **Bottom:** same view in *trans* conformation. Camphor is more solvent exposed, indicating that the proposed substrate access channel is opened by the isomerization. Figure was generated using PyMOL.³⁶

Figure 9.

RMSD of the *cis* and *trans* conformations against the initial PDB structure (a). Only the data considered for the production run is shown. RMSD against the MD average show that both structures fluctuate less than 1.4 Å around their own average. The fairly large deviation in the last 200 ps of the *trans* from the *cis* average is due primarily to changes at the N-terminus (see text).

Table 1

Inter-atomic distances in average *cis* and *trans* MD structures within and near the active site of CYP-1-CO. Distances to CAM (bound camphor) represent an average of the distance between Tyr 29 C ζ and all CAM carbon atoms.

Inter-residue distance (Å)	<i>cis</i>	<i>trans</i>
Ala 92 N-Val 396 N	13.8	14.5
Ala 92 N-Gly 248 N	17.4	18.3
Ala 92 N-Leu 244 N	16.2	17.1
Ala 92 N-Ala 296 N	14.9	15.6
Tyr 29 C ζ -CAM *	12.0	17.2
heme Fe -Phe87 C ζ	12.1	13.7
heme Fe -Ile88 C(O)	20.1	22.7