ABSTRACT: The camphor hydroxylase cytochrome P450<sub>cam</sub> (CYP101) catalyzes the 5-exo hydroxylation of camphor in the first step of camphor metabolism by <i>Pseudomonas putida</i>. CYP101 forms a specific electron transfer complex with its physiological reductant, the Cys<sub>4</sub>Fe<sub>2</sub>S<sub>2</sub> ferredoxin putidaredoxin (Pdx). Pdx, along with other proteins and small molecules, has also been shown to be an effector for turnover by CYP101. Multidimensional nuclear magnetic resonance (NMR) techniques have been used to make extensive sequential <sup>1</sup>H, <sup>15</sup>N, and <sup>13</sup>C resonance assignments in CYP101 that permit a more complete characterization of the complex formed by CYP101 and Pdx. NMR-detected perturbations in CYP101 upon Pdx binding encompass regions of the CYP101 remote from the putative Pdx binding site, including in particular a region of the CYP101 molecule that has been implicated in substrate access to the active site via dynamical processes. A model for effector activity is proposed in which the primary role of the effector is to prevent uncoupling (formation of reduced oxo species without formation of hydroxycamphor) by enforcing conformations of CYP101 that prevent loss of substrate and/or intermediates prior to turnover. A secondary role could also be to enforce conformations that permit efficient proton transfer into the active site for coupled proton/electron transfer.

Cytochrome P450s are heme-containing enzymes that catalyze the insertion of one oxygen atom from O<sub>2</sub> into unactivated C–H bonds with concomitant two-electron reduction of the other oxygen atom to water (Figure 1). Cytochrome P450<sub>cam</sub> (CYP101)<sup>1</sup> catalyzes the 5-exo hydroxylation of camphor, the first step of camphor catabolism in <i>Pseudomonas putida</i> (<i>P. putida</i>). The two electrons required for camphor hydroxylation by CYP101 come from the oxidation of NADH, which is catalyzed by putidaredoxin reductase (PdR). These electrons are shuttled from PdR to CYP101 by a Cys<sub>4</sub>Fe<sub>2</sub>S<sub>2</sub> ferredoxin (Pdx). While the first reduction of CYP101 [from Fe(III)S to Fe(II)S, Figure 1] can be accomplished by any reducing agent with a suitable reduction potential, the second reduction requires the presence of an effector in order to result in the formation of a hydroxycamphor product (<i>2</i>). It has been demonstrated kinetically that a complex between O<sub>2</sub>Fe(II)S and an effector (Ef) is the catalytically competent species (<i>2</i>). In the absence of such a complex, the O<sub>2</sub>Fe(II)S complex autoxidizes (<i>k = 0.015 s<sup>−1</sup></i> at 295 K) to yield ferric CYP101 [Fe(III)S] and superoxide (O<sub>2</sub>·−) (<i>3</i>). Although a number of small molecules and negatively charged proteins are effectors of CYP101, the most potent and specific effectors are small molecules that can function as putidaredoxin (Pdx) analogues.
for CYP101, the physiological reductant Pdx is the in vivo effector (2).

The interactions between Pdx and CYP101 are quite specific. Homologous ferredoxins adrenodoxin (Adx) and terpredoxin (Tdx) do not support turnover in the CYP101 system (2, 4). Modeling and experiment have provided insight into the nature of the specific interactions between Pdx and CYP101 (5–8). The binding of Pdx to CYP101 results in spectral perturbations to heme-linked transitions in CYP101 (9–11). Resonance Raman spectra show that the binding of reduced Pdx (Pdxr) to reduced CO- and NO-bound Fe(II)S results in changes in the electronic and/or structural environment of the proximal cysteine ligand and the porphyrin ring (12). It has been suggested that these perturbations are related to the effector activity of Pdx and may alter the electron-donating nature of the axial thiolate ligand (12, 13). Still, a number of observations argue against a hemelocalized model of effector activity or one that directly affects electron transfer rates. Cytochrome bs, which is an effector for CYP101 turnover, does not cause the same types of spectral changes in CYP101 that are associated with Pdx binding (12). This suggests that the localized changes in the heme environment observed upon binding of Pdx are not critical for effector activity. Also, the effectors identified for CYP101 turnover besides Pdx are by and large not capable of donating electrons to that enzyme; either their redox potentials are not appropriate or they contain no redox center (14). As such, effector activity must be separate from electron transfer, and perturbations in the active site associated with Pdx binding, while perhaps advantageous for rapid electron transfer, seem unlikely to be the primary cause of effector activity. Taken together, these observations suggest that the origins of effector activity are as yet unknown.

To obtain a more detailed picture of the interactions between Pdx and CYP101, we have applied multidimensional nuclear magnetic resonance (NMR) methods to the characterization of the CYP101/Pdx complex. NMR methods have been previously used to investigate this complex from the point of view of Pdx (6, 15), but CYP101 is a bigger challenge for the spectrosocist (16–18). Until the advent of TROSY-based NMR methods designed for use with large proteins (19) it would have been difficult to imagine tackling the problem of sequence-specific assignments in the monomeric 414-residue CYP101. However, using a series of perdeuterated uniformly and selectively 13C- and 15N-labeled samples of CYP101, we have now made extensive sequential 1H, 15N, and 13C resonance assignments in CYP101. Using these assignments, we have monitored perturbations in the CYP101 structure upon addition of Pdx, and we propose a model for the effector activity of Pdx consistent with our own and other published experimental observations on the CYP101/Pdx couple.

MATERIALS AND METHODS

Protein Expression and Purification. Perdeuterated [U-13C,15N]CYP101 was expressed in Escherichia coli strain NC5353 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 (20). 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 15NH4Cl. Protein expression was induced with 1 mM IPTG when the OD600 reached 1.0 and harvested after 24 h.

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 (21). Bovine Adx was expressed and purified according to published methods (22). The bacterially expressed soluble fragment of rat cytochrome bs was obtained in purified form from Prof. J. T. J. LeComte (Pennsylvania State University). The purities of CYP101, Pdx, and Adx were determined by measuring absorption ratios at A390/A275, A225/A280, and A414/A276, respectively, on a Hitachi U-2000 UV–visible spectrometer. CYP101 (0.5 mM) of 1.4 absorption ratio, Pdx (3.5 mM) of 0.55 absorption ratio, and Adx (1.8 mM) of 0.6 absorption ratio were used for NMR experiments. The level of deuteration of CYP101 was ca. 85% as measured by mass spectrometry. NMR samples were prepared by concentrating samples using Amicon (Millipore) centrifugal concentrators and then passing samples through buffer exchange resins preequilibrated with NMR buffer.

Reduction of CYP101, Pdx, Adx, and Rat Cytochrome bs. Samples of reduced CO-bound CYP101 [CO–Fe(II)S] were prepared as follows. Two hundred microliters of 0.5 mM [U-15N]CYP101 was placed in a CO atmosphere for 15 min and then reduced with 8 µl of 0.25 M Na2S2O4 prepared in degassed 1 M Tris-HCl (pH 8.0). The reduced sample remained under CO for an additional 2 min before anaerobic transfer to an NMR sample tube (Shigemi). Reduced samples of Pdx were prepared as follows. Two hundred fifty microliters of Pdx was concentrated to 3.95 mM in an anaerobic chamber and then reduced with 10 µl of 1 M Na2S2O4 prepared as described above. Bovine Adx and soluble rat cytochrome bs samples were concentrated and reduced in a similar fashion, using 20 and 10 µl of reducing agent, respectively.

NMR Spectroscopy. CYP101 samples for all NMR experiments were 0.2–0.65 mM in 90% H2O/10% D2O, pH 7.4, 50 mM Tris-d-HCl, 150 mM KCl, and 1 mM d-camphor. All NMR experiments were performed on a Varian Inova 600 MHz spectrometer, except for the two-dimensional HNCo experiment which was performed on a Varian Inova 500 MHz spectrometer. The Inova 600 operates at 599.702, 150.812, and 60.774 MHz for 1H, 13C, and 15N, respectively, and is equipped with a dedicated 1H RF channel (92.06 MHz) for 1H decoupling, a pulsed field gradient amplifier, and a 5 mm 1H(13C,15N) triple resonance PFG probe. The Inova 500 (operating at 499.708, 125.67, and 50.68 MHz) has a similar configuration. All NMR experiments were performed at 35 °C except for the titration with Pdx, which was performed at 17 °C to avoid thermal decomposition of the Pdx.

Oxidized Camphor-Bound CYP101 [Fe(III)S]. A [15N,13C,2H]-labeled Fe(III)S sample was used to acquire 3D TROSY-based HNCO, HN(CO)CA, HNCA, and HN(CACB) spectra. Experiments were run with 16 scans per t1 point, 512 (1H) × 32 (13C) × 32 (15N) complex points, and 1 s recycle delays. Spectral widths were 10000 Hz (1H) and 2600 Hz (15N). 13C
spectral widths were 4524 Hz [HNCA and HN(CO)CA], 12064 Hz (HNCACB), and 3000 Hz (HNCO). The implementations of triple resonance experiments used were sensitivity-enhanced gradient coherence selection versions described by Kay et al. (23).

A 3D $^1$H, $^{15}$N TROSY-NOESY spectrum and 2D $^1$H, $^{15}$N TROSY-HSQC spectrum were acquired with a $^3$H, $^{15}$N-labeled Fe(II)S sample. The HSQC was acquired with spectral widths of 8000 Hz ($^1$H) and 2200 Hz ($^{15}$N), 640 complex points, sweep widths of 8000 and 3000 Hz, and 256 scans per point, an and a 1 s recycle delay. The spectral widths for the 3D NOESY experiment were 10000 Hz ($^1$H), 10000 Hz ($^1$H), and 2000 Hz ($^{15}$N), with 512 × 128 × 32 complex points, respectively. The mixing time was 150 ms, the recycle delay was 1 s, and 16 scans per $t_1$ point were acquired.

**Reduced Carbon Monoxide- and Camphor-Bound CYP101 [CO—Fe(II)S].** A $^{15}$N, $^3$C, $^2$H-labeled CO—Fe(II)S sample was used to acquire 3D TROSY-based HNCA and HNCACB spectra. The same experimental parameters were used as detailed for the Fe(III)S sample, except that the recycle time was 2.1 s. The 2D $^1$H, $^{15}$N TROSY-HSQC and 3D TROSY-NOESY spectra were also acquired using a $^3$H, $^{15}$N-labeled CO—Fe(II)S sample. Experimental parameters were the same as for the Fe(III)S sample except the recycle times were 2 s (2D) and 1.3 s (3D).

**Selectively Labeled CYP101 Samples.** Perdeuterated samples $^{15}$N-labeled at one amino acid (leucine, valine, alanine, glycine, threonine, lysine, tyrosine, and glutamate) were used to acquire $^1$H, $^{15}$N TROSY-HSQC spectra of the Fe(II)S and CO—Fe(II)S forms. Scrambling of $^{15}$N labels was observed with the $[^{15}$N]glutamate, $[^{15}$N]tyrosine, $[^{15}$N]valine, and $[^{15}$N]leucine samples. Experiments were run with 512 × 64 complex points, sweep widths of 8000 and 2200 Hz, and 2.7 s recycle times. The number of transients per $t_1$ point varied between 32 and 256, depending on the concentration of the selectively labeled sample.

A two-dimensional HNCO experiment was performed using a uniformly $^{15}$N-labeled and perdeuterated sample of CYP101 in which only the proline carbonyl carbons were labeled with $^{13}$C. For this experiment, the $^1$H and $^{15}$N signals evolved in independent time domains, while the carbonyl $^{13}$C coherence transfer was used to remove signals of NH groups not bonded to proline carbonyls. The experiment was acquired with 512 × 64 complex points, sweep widths of 8000 and 3000 Hz, and 256 scans per $t_1$ point.

**Titration of CYP101 with Pdx’, Adx’, and Rat Cytochrome b$_5$.** A series of $^1$H, $^{15}$N TROSY-HSQC spectra of a $^3$H,$^{15}$N-labeled CO—Fe(II)S sample were acquired at 31, 25, 20, and 17 °C to allow the correlation of our sequential assignments (data acquired at 35 °C) and the titration results (acquired at 17 °C). The titration of CO—Fe(II)S with Pdx' was accomplished by anaerobic addition of aliquots of Pdx' to the CO—Fe(II)S NMR sample. After careful mixing and temperature equilibration at 17 °C in the NMR probe, two $^{15}$N, $^1$H TROSY-HSQC experiments were acquired at each titration point. One was acquired with a standard $^{15}$N spectral width (2200 Hz), and the other was acquired with a wider $^{15}$N spectral width (4000 Hz) in order to detect the NH resonances of arginine residues. All spectra were acquired with 596 ($^1$H) × 128 ($^{15}$N) complex points, $^1$H sweep widths of 8000 Hz, 8 scans per $t_1$ point, and 2.7 s recycle times. Five titration points were used, with final CYP101/Pdx' concentrations as follows (in mMs): 0.64/0, 0.618/0.134, 0.588/0.319, 0.544/0.591, and 0.474/1.03. Figure 2 shows comparisons of titration data points in the region of the spectrum showing correlations for glycine residues.

The titration of CO—Fe(II)S with Adx' followed a similar procedure, using an initial Adx' concentration of 1.8 mM. The Adx' was added in two aliquots to 160 μL of a 0.2 mM CO—Fe(II)S NMR sample, resulting in 2:1 and 5:1 molar ratios of Adx':CYP101. Cytochrome $b_5$ was added in reduced form to ensure that no unwanted redox processes would take place.

**Figure 2:** (A) Comparison of corresponding regions of $^{15}$N, $^1$H TROSY-HSQC correlation spectra obtained at 600 MHz $^1$H, 308 K, for oxidized camphor-bound CYP101, Fe(II)S, in blue, and reduced camphor- and CO-bound CYP101, CO—Fe(II)S, in red. Sequential assignments are labeled. Vertical axes are $^{15}$N chemical shifts (ppm); horizontal axes are $^1$H shifts (ppm). Note that some peaks move upon reduction, e.g., Gly 398 (G398), while others, including Gly 248 (G248), are only observed in CO—Fe(II)S. (B) Region of $^{15}$N, $^1$H TROSY-HSQC correlation spectra obtained at 600 MHz $^1$H, 290 K, showing effects of titration of CO—Fe(II)S with Pdx', upon glycine residues. Peaks are color coded: green corresponds to no Pdx, purple to ~1:5 Pdx:CYP101, and blue to ~2:1 Pdx:CYP101. Resonances corresponding to Gly 60 and Gly 309 are unperturbed by Pdx and Gly 298 and Gly 398 are shifted upon Pdx addition, while Gly 248 is broadened to invisibility after the first addition of Pdx.
place in the course of the titration. The cytochrome $b_5$
solution was originally 3 mM in concentration and was added
in two aliquots to 200 $\mu$L of a 0.2 mM CO$-$Fe(II)S NMR
sample, resulting in $\sim$5:1 and $\sim$10:1 molar ratios of cyt $b_5$
CYP101. Experimental conditions were as for the other
titrations.

Processing of NMR Experiments. All data was processed
using the Bruker XWIN NMR program (version 2.5). XEASY (24)
was used to assist in the sequential assignments and analysis of the
titration data.

Estimation of $K_d$ and Line Shape Analysis for the Pdx/CO
-Fe(II)S Complex. For five NH resonances that show
significant perturbation on the chemical shift time scale and
for which resonances are well resolved in all five HSQC
spectra, the $^1$H chemical shifts $\delta$ were fitted to the following
standard equation using the nonlinear regression analysis
package of Mathematica 3.0:

$$\delta = \delta_0 + (\delta_{\text{max}} - \delta_0)K_d + m_0 + p_0 -
(K_d + m_0 + p_0)^2 - 4m_0p_0^{1/2}/2m_0,$$

where $\delta_0$ and $\delta_{\text{max}}$ are the chemical shifts of the free
and complexed forms of CYP101, respectively, and $m_0$ and $p_0$
are the nominal concentrations of CYP101 and Pdx, respectively.
The fits yielded values for $\delta_{\text{max}}$ and $K_d$. The reported
value of $K_d$ in the text is the average value obtained from
the fits, with the error representing the extrema of the values.

Line widths used to estimate state lifetimes were obtained
using a Lorentzian deconvolution and line fitting available
in XWIN NMR. $T_{1 \text{e}}$ for line fitting was estimated from line
width measurements and from ratios of relative peak heights
after adjustment for concentration changes.

RESULTS

Sequential $^1$H, $^{15}$N, and $^{13}$C Backbone Resonance Assign-
ments for Oxidized Camphor-Bound CYP101 [Fe(III)S]. The extent of sequential resonance assignments in CYP101 is
shown in relationship to the structure in Figure 3. Sequential
assignments of CYP101 were made using a combination of
$^{15}$N-edited NOESY, HNCA, HN(CO)CA, and HNCACB

\[ \text{Figure 3: Amino acid sequence of CYP101, annotated for secondary structural features, NMR chemical shift assignments, and perturbations due to Pdx addition. Sequence numbers are noted at the beginning and end of each line. Wavy red lines indicate helices, and straight purple lines indicate } \beta\text{-sheets. Secondary structural features are named above the sequence according to the nomenclature of Raag and Poulos (41). Residues for which resonance assignments have been made are backgrounded in light blue for Fe(III)S and in yellow if assigned only in CO$-$Fe(III). Dark blue borders indicate resonances that are unperturbed by Pdx addition, which are bordered in orange are shifted upon addition of Pdx, and those bordered in red are broadened to invisibility upon the first addition of Pdx.} \]
$^{13}C$ shifts were usually sufficient to identify such scrambling when it occurred. CYP101 also contains 30 proline residues, which interrupt sequential connectivity by their lack of an NH proton. A perdeuterated sample of CYP101 was prepared, selectively labeled with $^{13}CO$-proline and uniformly labeled with $^{15}N$. A two-dimensional HNCO experiment performed with this sample provided unambiguous identification of NH resonances that are $i+1$ relative to prolines.

We believe that the current assignments are the most complete possible using current levels of sample deuteration and magnetic field strengths. All of the data used for these assignments were collected at 14 T (600 MHz $^1H$) with sample deuteration levels of $\sim$85%. Even with this level of deuteration, $^{13}C$($^1H$) dipolar cross-relaxation is efficient enough to degrade multiple-step coherence transfer important for the experiments used for sequential assignments. We are currently preparing samples with more complete deuteration (using perdeuterated $^{13}C$ sources) which should allow a more complete set of assignments to be obtained.

**Resonance Assignments for Reduced Carbon Monoxide- and Camphor-Bound CYP101 [CO–Fe(II)S]** Reduction of CYP101 with sodium dithionite followed by treatment with carbon monoxide (CO) produces the stable diamagnetic Fe(II)S with sodium dithionite followed by treatment with $^{15}N$. A two-dimensional HSQC experiment performed with this sample provided unambiguous identification of NH resonances that are $i+1$ relative to prolines.

**Spectral Changes Observed upon Addition of Pdx to CYP101.** The titration of Fe(III)S with Pdx does not result in any obvious large changes to the $^1H$, $^{15}N$ HSQC spectrum of CYP101 [Fe(III)S], as expected, since this nonphysiological complex is relatively weak ($\Delta\delta_{\text{max}} > \kappa_{\text{ex}}$). However, the titration of CO–Fe(II)S with Pdx results in considerable spectral perturbation. The response of $^1H$, $^{15}N$ correlations for CO–Fe(II)S to Pdx addition can be divided into three categories. The first category consists of resonances that do not show appreciable chemical shift perturbation ($\Delta\delta_{\text{max}} < 10$ Hz) or line width changes upon addition of Pdx. The second category consists of resonances that show both chemical shift and line width changes as Pdx is added. The perturbations in this category are in fast exchange ($\Delta\delta_{\text{max}} < \kappa_{\text{ex}}$), although considerable line broadening is observed. The third class includes those resonances that abruptly disappear upon the first addition of Pdx. These represent perturbations that are large enough to fall into the slow-exchange regime ($\Delta\delta_{\text{max}} > \kappa_{\text{ex}}$). Most of the residues in this class are either in the active site or in close proximity to the heme. The NH protons of Arg 112 and Arg 299, residues that form salt bridges with the heme propionates in CYP101, also fall into this category.

As controls, titrations of CO–Fe(II)S with reduced bovine adrenodoxin and reduced rat cytochrome bs were performed. Adrenodoxin, while capable of transferring the first electron to CYP101, is not an effector for CYP101 turnover (14). Cytochrome bs, on the other hand, has effector activity for CYP101, although concentrations required for effector activity are $\sim$20-fold higher than for Pdx (2). Perturbations of the CO–Fe(II)S spectrum by Adx are within experimental error and do not include any of the marked changes observed upon Pdx addition. However, cytochrome bs addition perturbs many of the same resonances in CO–Fe(II)S that Pdx does, strongly suggesting that the perturbations observed upon Pdx addition are directly related to effector activity. A more complete study of the interactions between CYP101 and cytochrome bs is underway.

The structural distribution of Pdx′-induced perturbations in relation to the proposed complex structure is shown in Figure 4 (6). The most perturbed regions of the protein are the N-terminal end of the B helix (residues 66–70), the B′ helix (residues 91–96), the N-terminal end of the C-helix (residues 107–114), both ends of helix E, portions of the F...
and G helices, most identified residues in the central I helix, and portions of β-sheets 1, 3, and 5. Relatively unperturbed features include most of the N-terminal residues through residue 35, most C-terminal residues from residue 405 to residue 414, residues in the F/G loop, helices J and K, and β-sheet 4. Secondary structural features not specifically noted here either contain a mix of perturbed and unperturbed residues or do not contain enough assigned residues to determine in which category the feature belongs. In either case, these features are color-coded appropriately in Figure 4.

Nonlinear fits of chemical shift changes for a series of affected resonances as a function of Pdx' and CO–Fe(II)S concentration give an estimated $K_d$ of 26 ± 12 μM (at 290 K) for complex formation between Pdx' and CO–Fe(II)S. This agrees well with $K_d$ calculated from published $k_{on}$ and $k_{off}$ rates for Pdx' with O2–Fe(II)S determined by single-turnover kinetics (27). Ratios of these rate constants give a $K_d$ ranging from 4 to 30 μM. The lifetimes of the conformational changes enforced by binding of Pdx' to CO–Fe(II)S can also be estimated from the changes in line width that occur during the course of the titration. If a two-state model (Eff on vs Eff off) is assumed, Lorentzian line fitting for a series of affected resonances yields an estimated bound state lifetime at half-saturation of 3 ms (also at 290 K), which in turn yields an estimate for $k_{on}$ of ~300 s⁻¹. Fits for residues in various parts of the protein gave approximately the same values for $K_d$, $k_{on}$ and state lifetimes, indicating that a two-state model is appropriate for the binding of Pdx' to CO–Fe(II)S.

Assuming that the Pdx-bound state of CO–Fe(II)S is a good model for the physiological Pdx/O2–Fe(II)S complex, the probability of electron transfer (ET) within the complex must be considerably higher than the observed ET rate (from 0.06 to 0.1 transfers per millisecond) (27) in order for ET to take place efficiently during the lifetime of the bound form (~3 ms). However, if the complex geometry in the proposed model is correct, with an intermetal distance of 12 Å (6), one might expect a much higher intrinsic electron transfer rate constant. Biological ETs between centers in nondissociating complexes with similar separations often exhibit rate constants between $10^6$ and $10^8$ s⁻¹ (28). An intrinsically large electron transfer rate will be masked if that transfer must take place in a relatively short-lived complex (29). This would rationalize the observation that, at higher Pdx concentrations, the second electron transfer step is no longer rate limiting (27).

**DISCUSSION**

**Role of the Effector in CYP101 Turnover.** The most important observation made in the current work is that perturbations induced by the binding of Pdx to CYP101 are not confined to the proximal face of CYP101 (the proposed binding site for Pdx) (5–8) but encompass many regions of the CYP101 structure. Furthermore, cytochrome bs (a known effector for CYP101 turnover) perturbs many of the same resonances that Pdx does, while adrenodoxin (which is not an effector) does not, strongly suggesting that the perturbations are related to effector activity. The long-range conformational effects of Pdx binding on CYP101 suggest that effector activity need not be the result of localized perturbations to the proximal ligand or active site (although these do occur). Effector activity could be the result of selection for a particular subset of conformations of the CYP101 molecule upon effector binding (Figure 5). This model for effector activity would be consistent with the observation that other molecules that do not cause localized spectral perturbations in CYP101 near the heme are capable of acting as effectors for substrate turnover in CYP101. Such molecules would only need to force the conformational selection, without necessarily causing perturbations to the active site, to effect turnover. The model is also consistent with the observation of product evolution in the absence of effector in crystalline CYP101: It is possible that crystal packing forces exert a restraint similar to that exerted by effectors on conformational selection in CYP101 (30).

Ascertainment of the role of this conformational selection in allowing turnover is more difficult. However, we note that regions of the protein that have been implicated in substrate access and product egress to and from the active site are perturbed by the binding of Pdx (Figure 4). In particular, the region centered around the B helix (residues 90–96) is strongly affected by Pdx binding. This helix and residues in contact with it have been implicated by mutation (1), structural analysis (31), and dynamics simulations (32, 33) in the control of substrate access into the active site of CYP101. Other evidence suggests that residues in this region are involved in substrate orientation and prevention of uncoupling (34, 35). On the basis of these observations, we propose that the binding of effector restricts the available conformations of CYP101 to those that prevent the loss of substrate and/or intermediate via transiently opened substrate access and egress routes within CYP101. From the observed
patterns of perturbation, it seems likely that the binding of Pdx to the critical residues Arg 109 and Arg 112 causes mechanical displacement of the C helix. This displacement is then transmitted mechanically to the B' helix either through contacts with the β2 sheet and central I helix or directly through the B/C loop.

The proposed role of the effector in CYP101 turnover (to prevent substrate and/or intermediate loss in the course of the reaction cycle) is consistent with other published results. The regions of CYP101 that are affected by Pdx binding are the same that are found to be perturbed in the crystal structure of CYP101 determined with ruthenium-linker substrates. These substrates force a channel opening between the active site and the surface of CYP101 (36). In that work, it was found that the open-channel conformation of CYP101 results in significant displacements of helices G and F, as well as changes in the B' helix. All three of these secondary structural features are perturbed upon binding of Pdx (see Figure 4). Those authors note that, although the F/G loop is displaced in their structures, the conformation of that loop is similar to that of camphor-bound CYP101. This is consistent with our observation of little perturbation in the backbone resonances of the F/G loop.

A mutant of CYP101, D251N, has been described in which the turnover rate is considerably slower than wild type (37). The lower activity of D251N CYP101 is ascribed to the inhibition of proton transfer into the active site required for turnover of the enzyme. Nevertheless, binding of Pdx to D251N CYP101 is accompanied by spectral perturbations similar to what is seen in the wild-type complex (38). Analysis of oxygen vibrational modes observed in resonance Raman spectra of reduced D251N CYP101/Pdx complexes suggests that the oxygen in the O₂−–Fe(II)S/Pdx complex is present as O₂⁻–Fe⁵⁺⁺ (superoxide) rather than the two-electron-reduced species O₂⁻–Fe⁵⁺⁺ (peroxide) (13). Clearly, electron transfer is also slowed in the D251N complex relative to wild type. The results also indicate that the conformational changes detected upon Pdx binding do not in and of themselves promote electron transfer. Furthermore, heterolysis of the Fe→O bond in the unreacted (Fe³⁺→O²⁻) form would lead to superoxide, the product of O₂⁻–Fe(II)S autoxidation that is observed in the absence of effector (2). If effectors act to prevent loss of ligand and/or substrate by preventing the transient opening of access paths to the active site of CYP101, transient dissociation of superoxide from the iron might not automatically result in uncoupling. Instead, the presence of an effector might encourage geminate recombination of transiently dissociated superoxide and eventual turnover.

Another possibility that must be considered is that, if proton and electron transfer are coupled, the effector-enforced conformation might promote efficient proton transfer into the active site. However, we note that neither of the two residues implicated in proton transfer into the active site for which amide resonances have been assigned, Lys 178 and Arg 186 (near the C-terminus of the F helix and within the FG loop, respectively), is perturbed by Pdx binding, despite the fact that some of their near neighbors are perturbed. It is possible that, as our methods report primarily on the effects detected by backbone amides, the side chains of these residues may be affected without perturbing the backbone. Still, while the current data do not rule out a role for effector-induced conformational changes aiding proton transfer, neither do they support it.

Finally, it must be considered that the primary binding mode of Pdx (at the proximal face, as shown in Figure 4) is not the same one that results in effector activity. Multiple binding modes have been suggested for other soluble redox complexes (39). The primary arguments against this are the apparently simple two-state nature of the binding that we observe and the lack of effect of surface mutations in CYP101 remote from the proximal site upon activity (5, 40). Still, it is likely that more mutational and spectroscopic work will be required to rule out the possibility of multiple functional binding geometries.

The model for effector activity in the Pdx/CYP101 couple proposed here is appealing in that it complements our dynamic model for modulation of Pdx binding to CYP101 as a function of the Pdx oxidation state. Reduction of Pdx causes a decrease in the conformational freedom of the ferredoxin and increases the binding affinity for Fe(III)S, presumably by decreasing the conformational entropy of Pdx in a manner consistent with CYP101 binding (6). There is also evidence that the state of CYP101 affects the binding of Pdx. In the absence of substrate, no change is observed in the EPR spectrum of oxidized CYP101 [Fe(III)] upon addition of Pdx (10) nor is there any effect of Pdx upon autooxidation rates of O₂−–Fe(II)S unless a substrate analogue is present (14). Together, these observations suggest that the specific binding between Pdx and CYP101 is not a passive event for either protein, but a delicate balance of structural and dynamic factors that modulate molecular recognition in this system.

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