Structural and Mechanistic Insights into the Interaction of Cytochrome P4503A4 with Bromoergocryptine, a Type I Ligand

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Background: Human CYP3A4 metabolizes the majority of administered drugs including bromoergocryptine (BEC), a dopamine receptor agonist.

Results: Crystallographic and experimental data suggest the importance of Arg212 and Thr224 in BEC binding.

Conclusion: H-bonding interactions with Thr224 and conformational adjustments modulated by Arg212 are critical for the productive orientation of BEC.

Significance: Mechanistic insights on the CYP3A4-BEC interaction may help develop new and safer pharmaceuticals.

Cytochrome P4503A4 (CYP3A4), a major human drug-metabolizing enzyme, is responsible for the oxidation and clearance of the majority of administered drugs. One of the CYP3A4 substrates is bromoergocryptine (BEC), a dopamine receptor agonist prescribed for the inhibition of prolactin secretion and treatment of Parkinson disease, type 2 diabetes, and several other pathological conditions. Here we present a 2.15 Å crystal structure of the CYP3A4-BEC complex in which the drug, a type I heme ligand, is bound in a productive mode. The manner of BEC binding is consistent with the in vivo metabolite analysis and identifies the 8′ and 9′ carbons of the proline ring as the primary sites of oxidation. The crystal structure predicts the importance of Arg212 and Thr224 for binding of the tripeptide and lysergic moieties of BEC, respectively, which we confirmed experimentally. Our data support a three-step BEC binding model according to which the drug binds first at a peripheral site without perturbing the heme spectrum and then translocates into the active site cavity, where formation of a hydrogen bond between Thr224 and the N1 atom of the lysergic moiety is followed by a slower conformational readjustment of the tripeptide group modulated by Arg212.

Cytochrome P450 enzymes are heme-thiolate proteins that catalyze a wide variety of monooxygenation reactions including hydroxylation, epoxidation, and heteroatom dealkylations (1). Among 57 human P450s, the 3A4 isoform (CYP3A4) is one of the most abundant and important because, in addition to oxidation, it catalyzes a wide variety of functional groups on xenobiotics. CYP3A4 is the ability to accommodate more than one molecule in the substrate-binding pocket, where one molecule serves as a substrate while another acts as a modulator of substrate metabolism. Among the substrates that exhibit binding cooperativity with CYP3A4 are testosterone, progesterone, diazepam, α-naphthoflavone, and several others (6–8).

A CYP3A4 substrate that does not exhibit binding cooperativity is bromoergocryptine (BEC2; also known as bromocriptine) (9–11). BEC is an ergot alkaloid that acts as a dopamine receptor agonist. Currently, BEC is prescribed for inhibition of prolactin secretion, the treatment of Parkinson disease, type 2 diabetes, migraines, and pituitary tumors, and correction of abnormal secretion of growth hormone. BEC is one of the largest CYP3A4 substrates (molecular mass of 655) and consists of a lysergic acid and a proline-containing cyclic tripeptide linked by an amide bond (Fig. 1). The tripeptide group is critical for the interaction with P450s of the 3A family (12). BEC has a high affinity for microsomal and recombinant CYP3A4 (Kd of 0.3–1 μM) and upon binding induces type I spectral changes (a low-to-high spin shift) accompanied by a 80-mV increase in the heme redox potential (11–17). In vivo metabolite analysis has revealed that BEC is oxidized primarily by CYP3A4 at the pyrrolidine moiety, with the 8′-mono- and 8′,9′-dihydroxy derivatives being the major products (Fig. 1) (18, 19).

Although determination of the 2.7 Å crystal structure of BEC-bound CYP3A4 has been reported at a scientific meeting (20), the atomic coordinates are still unavailable. Moreover, to date, there is no structural information on any other type I ligand bound to CYP3A4 in a productive mode. To obtain insights into the mechanism of substrate binding to CYP3A4, we determined and report here the 2.15 Å crystal structure of the CYP3A4–BEC complex. The x-ray data suggest the importance of Arg212 and Thr224 for optimal BEC binding, which we...
confirmed experimentally using mutagenesis, spectroscopic, and kinetic techniques.

MATERIALS AND METHODS

Protein Expression, Purification, and Mutagenesis—R212A and T224A mutations were introduced to the CYP3A4 (human). The wild type (WT) and mutants of CYP3A4 were expressed and purified as described previously (21).

Crystallization and Structure Determination—The CYP3A4-BEC complex was crystallized by a micro batch method under oil. Half a microliter of the BEC-bound protein (20–25 mg/ml) in 20 mM phosphate, pH 7.7, 20% glycerol, and 100 mM NaCl was mixed with 0.5 µl of 4% tacsimate, pH 5.0, and 12% polyethylene glycol 3350 (solution No. 11 from the Hampton Research PEG/Ion 2 kit) and then covered with 10 µl of paraffin oil. On the next day crystals were harvested and frozen in Paratone oil, used as a cryoprotectant. X-ray diffraction data were collected at the Stanford Synchrotron Radiation Laboratory beamline 7-1. The structure was solved by molecular replacement with PHASER (22) using ligand-free CYP3A4 (Protein Data Bank ID code 1TQN) as a search model. The initial model was rebuilt and refined with COOT (23) and REFMAC (22). The N and C termini as well as residues 266–268 and 280–286 are not present in the final structure because of conformational disorder. Data collection and refinement statistics are given in Table 1. The atomic coordinates have been deposited to the Protein Data Bank with the ID code 3UA1.

Spectral Binding Titrations—Binding of BEC (Sigma) to the WT and mutants of CYP3A4 was monitored in 50 mM phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM dithiothreitol. Spectra were recorded after the addition of small amounts of BEC dissolved in dimethyl sulfoxide; the total volume of the solvent added was <2% (v/v). The difference in absorbance between the wavelength maximum and minimum was plotted against the concentration of BEC, and the spectral dissociation constant ($K_s$) was calculated using quadratic nonlinear regression analysis as described elsewhere (15).

Kinetics of Ligand Binding—The kinetics of BEC binding to CYP3A4 was monitored at ambient temperature in a SX.18MV stopped flow apparatus (Applied Photophysics) in the absence and presence of Emulgen 913 (Kao Chemicals, Japan), IGEPAL CA-630 (Sigma) and CHAPS (Sigma). CYP3A4 solutions (6 µM) in 50 mM phosphate buffer, pH 7.4, were mixed with 0.125–36 µM BEC to follow absorbance changes at 417 nm. Owing to low solubility of BEC, 36 µM was the maximal concentration that could be reached under our experimental conditions. In a separate experiment, 2 µM CYP3A4 was mixed with 2–36 µM BEC to confirm that the rate constant for BEC ligation starts to level off when the BEC:CYP3A4 ratio exceeds 2. Kinetic data were analyzed using the Igor program (WaveMetrics, Inc.).

RESULTS

Soluble CYP3A4 Has High Affinity for BEC—As interaction of truncated human CYP3A4Δ3–22 with BEC had not been investigated previously, we performed equilibrium titrations to determine a spectral dissociation constant ($K_s$) for the drug. As seen in Fig. 2, the addition of a 2-fold excess of BEC to soluble CYP3A4 leads to a nearly complete 417 to 387-nm shift in the absorbance maximum (type I spectral changes), indicative of CYP3A4 leads to a nearly complete 417 to 387-nm shift in the absorbance maximum (type I spectral changes), indicative of formation of the heme iron to a high spin form. The estimated $K_s$ value (0.37 ± 0.02 µM) suggests that BEC binds to the truncated form of CYP3A4 as tightly as to the full-length hemoprotein, whose dissociation constant for BEC ranges from 0.3 to 1 µM depending on the determination method and protein source (11–13, 15, 16).

Crystal Structure of the CYP3A4-BEC Complex—Owing to the high affinity of the CYP3A4-BEC complex, it was possible to maintain CYP3A4 in the BEC-bound form during crystallization. The deep brown color of the crystals was the first indication that crystalline CYP3A4 forms a high spin complex with BEC (Fig. 3A). The well defined electron density of the BEC molecule shows that the drug is bound in the active site in an extended conformation, with an angle between the tripeptide

![Figure 1. Structure of BEC. Two primary hydroxylation sites are indicated by arrows.](image-url)

![Cyclic tripeptide](image-url)

![Lysergic acid](image-url)

**TABLE 1**

| Data collection and refinement statistics |
|-----------------------------------------|
| **Data statistics**                      |
| Space group                              |
| Unit cell parameters                      |
| Resolution range                         |
| Total reflections                        |
| Unique reflections                       |
| Redundancy                               |
| Completeness                             |
| Average I/σ                            |
| $R_{merge}$                              |
| $R_{free}$                               |
| **Refinement statistics**                |
| Molecules per asymmetric unit            |
| $R/R_{free}$                             |
| Average B-factor (Å²)                    |
| Root mean square deviations              |
| Bond lengths (Å)                         |
| Bond angles (°)                          |

*All values in parentheses are for the highest resolution shell.

$R_{merge}$ was calculated from a subset of 5% of the data that were excluded during refinement.
and lysergic groups of 130° (Fig. 3, B and C). As metabolic analyses have predicted (12, 18, 19), BEC approaches the heme via the tripeptide moiety (Fig. 3, C and D), with the primary sites of oxidation, the 8th and 9th carbons of the proline ring (Fig. 1), being 4.1 and 3.7 Å, respectively, away from the iron. Thus, in the crystal structure BEC is bound in a productive mode. The tripeptide group does not establish any specific polar or electrostatic interactions but makes extensive van der Waals contacts with Ile301, Phe304, and Ala305 from the l-helix and with the side and main chains of Arg105, Arg212, Ala370, and Arg372. To allow the tripeptide moiety to approach the heme iron, Arg212 adopts a new rotamer relative to the ligand-free structure (29). This conformation is stabilized by two hydrogen bonds formed between the Arg212 guanidinium group, the carboxyl of Glu308, and the carbonyl oxygen of Ile309 (Fig. 3E). The lysergic moiety of BEC is sandwiched between the parallel Arg106 and Phe215 side chains and H-bonded to Thr224 from the G'-helix via the N1 atom (Fig. 3, C and D). To accommodate this bulky group, the Pro107–Gly109 peptide shifts aside by 2.4 Å. The fact that the drug establishes only one hydrogen bond and there are no well defined waters or water-mediated contacts in the active site allows us to conclude that the CYP3A4–BEC interactions are predominantly non-polar.

Rationale for Mutagenesis—Despite the large size and complex chemical structure of BEC, only small changes in the CYP3A4 conformation are needed to position the substrate optimally for oxidation. As mentioned, one notable rearrange-
spent is in the Arg212 side chain but not the associated peptide backbone. This is in contrast to other large ligands such as ritonavir, ketoconazole, and erythromycin, where a major conformational change in the Arg212-containing F2’-loop occurs upon binding (Fig. 3E) (4, 21). Because the tripeptide moiety of BEC binds next to Arg212, we anticipated that elimination of the Arg212 side chain would increase the volume of the active site cavity near the heme iron and, hence, should affect the affinity and/or binding kinetics of the drug. Thr224, on the other hand, assists BEC binding by H-bonding with the lysergic group (Fig. 3, C and D). Elimination of this hydrogen bond could increase motional freedom and prevent proper positioning of the drug, which could be manifested through changes in the extent and kinetics of a spin shift in CYP3A4. To test these predictions, we replaced Arg212 and Thr224 with alanine to determine how single and double mutations affect the CYP3A4-BEC interaction.

Arg212 and Thr224 Define Affinity and Facilitate BEC Binding to CYP3A4—Equilibrium titrations show (Fig. 4) that, at saturating BEC levels, there is only a partial conversion of the mutants to a high spin form: 56, 60, and 52% for CYP3A4 R212A, T224A, and R212A/T224A, respectively, as opposed to ~90% for the WT. The $K_s$ value calculated for the T224A variant was close to that of the WT, whereas the corresponding constants for the R212A and double mutants were 6-fold higher (Table 2). This implies that during equilibrium binding, Arg212 is more critical for the BEC binding than Thr224.

To determine the mutational effects on the kinetics of BEC association, we monitored the disappearance of the low spin form as an absorbance decrease at 417 nm after mixing CYP3A4 and BEC in a stopped flow spectrophotometer. Consistent with the incomplete shift from low to high spin when BEC was added to the mutants, the change in amplitude at 417 nm was less for both mutants: 33% for CYP3A4 R212A, and 63% for the T224A and double mutants compared with the WT (Fig. 5, A–D). In addition, the kinetics of BEC binding to the WT and variants was biphasic under all studied conditions. The rate constants for the fast phase of BEC binding ($k_{fast}$) were independent of the BEC concentration at subequimolar BEC:CYP3A4 ratios (<0.5) and then gradually increased and remained unchanged after the BEC:CYP3A4 ratio exceeded 2 (Fig. 5E). A comparison of the $k_{fast}$ values calculated at saturating levels of BEC (Table 2) indicates that BEC binds faster to the R212A mutant (~32% increase in $k_{fast}$), whereas the T224A mutation slows down the spin conversion by 30%.

For CYP3A4 T224A, the rate constant for the slow phase ($k_{slow}$) was independent of BEC concentration (Fig. 5F). For other proteins, $k_{slow}$ decreased to a different extent until the BEC:CYP3A4 ratio reached 2; this remained unchanged at higher drug concentrations. The most notable changes in the slow phase were observed for the R212A and double mutants. Stopped flow measurements also revealed that regardless of whether or not mutations were present, the percentage of the slow phase changed sharply from ~30–35% to 50–55% when the BEC:protein ratio exceeded unity (Fig. 5G). Because the latter effect could be due to conformational heterogeneity of CYP3A4 (14), we checked whether the biphasicity of the BEC binding reaction could be eliminated in the presence of detergents.

**TABLE 2**

|          | WT       | R212A   | T224A   | R212A/T224A |
|----------|----------|---------|---------|-------------|
| $K_s$ ($\mu$M) | 0.37 ± 0.02  | 2.3 ± 0.3  | 0.44 ± 0.03  | 2.5 ± 0.2    |
| $k_{fast}$ (s$^{-1}$) | 0.66 ± 0.02  | 0.87 ± 0.03  | 0.46 ± 0.03  | 0.56 ± 0.02  |
| $K_m$ ($\mu$M) | 0.035 ± 0.002  | 0.034 ± 0.002  | 0.039 ± 0.003  | 0.074 ± 0.002  |

$^a$ Spectroscopic dissociation constant.

$^b$ Rate constant for ligand binding in the fast phase, determined at BEC:CYP3A4 = 5.

$^c$ Rate constant for ligand binding in the slow phase, determined at BEC:CYP3A4 = 5.

**BEC Binding Kinetics Remains Biphasic in the Presence of Detergents**—The kinetics of BEC binding to WT CYP3A4 was measured in the presence of two non-ionic detergents, Emul-
gen 913 and IGEPAL CA-630, as well as zwitterionic CHAPS. All detergents were used at a final concentration of 0.1–0.12%, which is lower than the critical micelle concentration and sufficient to dissociate aggregates of full-length CYP1A2 and 2B4 to catalytically active dimers (24).

Spectral measurements showed that the addition of CHAPS, Emulgen 913, and IGEPAL CA-630 to CYP3A4 caused partial low-to-high spin shifts (53, 14, and 27%, respectively (Fig. 6A)). This means that each compound binds near the heme and displaces the distal water ligand or partially stabilizes a conformer that favors water ligand displacement. When CHAPS-bound CYP3A4 was mixed with BEC, a large decrease in 417-nm absorbance was detected (Fig. 6B). Further, as observed in a detergent-free buffer, the kinetics of CYP3A4-BEC complex formation in the presence of CHAPS was biphasic, with $k_{\text{fast}}$ and $k_{\text{slow}}$ values of 0.66 and 0.06 s$^{-1}$, respectively, and the percentage of the slow phase was ~50%. According to an absorbance spectrum recorded at the end of the stopped flow experiment (Fig. 6C), the conversion of CYP3A4 to a high spin form was near completion (~100%) at saturating BEC levels.

When Emulgen 913 or IGEPAL CA-630 was present in the medium, the BEC-induced absorbance changes were very small (Fig. 6B). Nevertheless, the reaction remained biphasic at BEC: CYP3A4 ratios of >1 ($k_{\text{fast}}$ and $k_{\text{slow}}$ of 5–8 and 0.02–0.04 s$^{-1}$, respectively), with the fast phase accounting for 90% of the total absorbance change. At subequimolar ratios, the ligation of BEC was slow (0.01–0.05 s$^{-1}$) and monophasic. A BEC-dependent increase in the high spin content reached only 21 and 7% for Emulgen- and IGEPAL-bound CYP3A4, respectively (Fig. 6C). Thus, non-ionic detergents significantly interfere with the BEC binding and, similar to CHAPS, do not eliminate the biphasicity of the reaction.

**DISCUSSION**

Co-crystallization of CYP3A4 with substrates/type I ligands is very challenging because most of these have low affinity ($K_d$ of 5–150 μM) and low solubility in aqueous solutions. In the two currently available substrate-bound structures of CYP3A4, erythromycin is bound in a nonproductive mode (4), whereas progesterone is docked outside of the active site pocket (25). The present CYP3A4-BEC complex is the first in which the substrate is bound in a mode suitable for oxidation.

Surprisingly, no major conformational changes in CYP3A4 were required to accommodate a large BEC molecule (the root mean square deviation between the ligand-free and BEC-bound structures is only 0.32 Å). The crystal structure shows that two residues, Arg$^{212}$ and Thr$^{224}$, may be important for association and optimal orientation of the drug. Arg$^{212}$, part of the FF’loop (residues 210–214), is thought to be actively involved in substrate binding and the mediation of cooperativity in steroid hydroxylation reactions (26, 27). Although Arg$^{212}$ plays no significant role in the hydroxylation of relatively small steroids (26, 27), it is predicted to influence the action of the effector molecule and control the substrate orientation via interactions with Phe$^{304}$, supposedly located at the interface between the active and the effector binding sites (28).

In our structure, Arg$^{212}$ is positioned strategically near the tripeptide group of BEC and establishes interactions with Glu$^{308}$ and Ile$^{369}$. This led us to hypothesize that Arg$^{212}$, as well as the H-bond-forming Thr$^{224}$, could be important for the affinity and kinetics of BEC binding. Indeed, our experimental data show that substitution of Arg$^{212}$ or/and Thr$^{224}$ with alanine alters both the extent of the low-to-high spin shift and the rate of BEC binding. The reaction of BEC ligation to the WT or mutants of CYP3A4 was biphasic, with non-hyperbolic dependence of $k_{\text{fast}}$ on BEC concentration. As the drug is known to bind to CYP3A4 stoichiometrically and non-cooperatively (11, 15), such kinetics cannot be explained by the allosteric properties of the enzyme.

One possible reason follows from a previous study in which three kinetic phases were distinguished (20, ~1, and 0.009 s$^{-1}$) based on fluorescence and absorbance changes observed during association of BEC to the full-length CYP3A4 (15). The
fastest step, detected only by fluorescence spectroscopy, was proposed to correspond to the peripheral binding of BEC, proceeding without perturbations in the heme spectrum. The two subsequent steps, in turn, are thought to reflect the interaction of the second BEC molecule with the active site and the heme. Our data are consistent with this model and provide further insights into the mechanism of BEC association.

Because BEC has low solubility in aqueous solutions, it was not possible to study the binding kinetics under pseudo-first order conditions over a wide range of BEC concentrations (Fig. 5, E and F). Nonetheless, the range studied was sufficient to estimate the limiting $k_{\text{fast/slow}}$ values, because BEC is a high affinity ligand whose binding rate becomes concentration-independent when the BEC:CYP3A4 ratio exceeds 2. Small changes in binding rates at low BEC concentrations mean that an event taking place remotely from the heme (e.g. binding of BEC to a peripheral site or structural changes within the protein) precedes the spin state change and is rate-limiting. Once this remote site is saturated, the reaction is first order and presumably is limited by movement of BEC from the remote site to the active site, which leads to the low-to-high spin shift.

Another factor that can affect binding is conformational reorganization in the BEC molecule itself. The BEC conformation currently deposited in the DrugBank database (Fig. 1), which we used as a starting model, differs from that in the x-ray model. To fit into the CYP3A4 active site, the tripeptide group of BEC rotates by $180^\circ$ around the amide bond (Fig. 7A). The existence and interconversion of different BEC conformers may complicate the binding process and limit the overall reaction rate.

Our kinetic data did not allow us to differentiate whether binding of BEC to CYP3A4 occurs before or after a conformational change in CYP3A4 and/or BEC takes place (“induced fit” versus “conformational selection” mechanism). However, the structure-based mutagenesis identified two possible events through which the entering BEC molecules may cause a spin shift. The T224A substitution slows down and the R212A speeds up the fast phase of the BEC binding kinetics. The T224A mutation has little effect on the slow phase, whereas the R212A replacement increases the slow phase. Therefore, these residues play a role in the translocation of BEC from the remote site to the heme active site, with Thr$^{224}$ primarily affecting the early stage and Arg$^{212}$ the later stage of binding near the heme. Based on this scenario and data reported previously (15), we propose that upon translocation from the peripheral binding site into the active site cavity, BEC first establishes a hydrogen bond with Thr$^{224}$ via the lysergic head group (Fig. 8). This interaction directs the tripeptide moiety toward the heme and, if the orientation is favorable, leads to partial displacement of the water ligand and low-to-high spin shift (fast kinetic phase). When a different BEC conformer enters the active site, the tripeptide group must rotate prior to heme ligation. The final step could...
Interaction of CYP3A4 with Bromoergocryptine

be positional adjustment of the tripeptide moiety assisted by Arg<sup>212</sup>. A conformational switch in the Arg<sup>212</sup> side chain, stabilized through H-bonding interactions with Glu<sup>308</sup> and Ile<sup>369</sup>, would allow the tripeptide group to come closer to the heme iron, thereby leading to further changes in spin equilibrium.

In addition to the aforementioned factors, the BEC binding reaction may be affected by intrinsic properties of CYP3A4 such as conformational heterogeneity and aggregation. High pressure spectroscopy studies, for instance, have suggested that there are two conformers of full-length CYP3A4 that have distinct spin equilibrium, barotropic properties and reactivity toward BEC (14). The relative content of these conformers, 70 and 30%, was unaffected by BEC concentration but modulated by Emulgen 913. This prompted us to check whether conformational heterogeneity or/and hydrophobic interactions between the CYP3A4 molecules contribute to the multiphasicity of the BEC binding reaction. To do so, we monitored BEC-dependent spin shift in CYP3A4 in the presence of Emulgen 913 and two other detergents frequently used for CYP3A4 purification, zwitterionic CHAPS and non-ionic IGEPEAL CA-630 (25, 29).

One complication in these experiments was that all three detergents were able to enter the CYP3A4 active site and cause type I spectral changes, which supports the notion that detergents can serve as substrates for CYP3A4 (30). Interestingly, despite the ability to cause the largest spin perturbations (Fig. 6A), CHAPS had virtually no effect on the BEC binding kinetics. Moreover, a low-to-high spin shift was complete when both CHAPS and BEC were present (Fig. 6C) as opposed to 90% conversion induced by BEC in a detergent-free buffer. We attribute this phenomenon to a cumulative effect of the two compounds, because it is unclear at the moment whether CHAPS can be displaced by BEC or the detergent stabilizes a conformer disfavoring coordination of the water ligand.

Unlike CHAPS, Emulgen 913 and IGEPEAL CA-630 strongly inhibited the CYP3A4-BEC interaction. This agrees with a previous investigation showing that non-ionic detergents inhibit CYP3A4 catalysis by interfering with substrate binding (30). The fact that neither studied detergent could completely eliminate the biphasicity of BEC association undermines the possibility that protein heterogeneity and/or hydrophobic interactions between the truncated CYP3A4 molecules are major factors complicating the BEC binding reaction. On the other hand, a 30:70% distribution between the slow and fast kinetic phases observed at subequimolar concentrations of BEC and its sharp change to ~35:65% when the BEC:CYP3A4 ratio exceeded unity (Fig. 5G) favor the hypothesis on CYP3A4 conformers with different reactivity toward BEC (14). Our data suggest that the relative content of such conformers might be affected by both substrate and detergent binding. One cause for protein heterogeneity could be variations in the Arg<sup>212</sup> conformation. In the two crystal structures of substrate-free CYP3A4 available to date, the Arg<sup>212</sup> side chain faces either the solvent or the active site (25, 29). As follows from our study, even this minor structural deviation could significantly affect the rate of BEC binding.

Finally, resonance Raman spectroscopy studies on nanodisc-incorporated CYP3A4 detected small changes in the modes associated with disposition of the heme peripheral groups and out-of-plane macrocycle distortion caused by BEC binding (31). A comparison of the ligand-free and BEC-bound structures showed that, indeed, the BEC-dependent change in the heme coordination state distorts the heme plane and vinyl group conformation (Fig. 7B). If the energy barrier between the 6- and 5-coordinated states is high, it could modulate the dynamics of BEC binding and, hence, contribute to the complexity of the reaction.

In conclusion, the crystallographic complex between CYP3A4 and BEC provides the first insights into the productive binding mode of a type I ligand. It also suggests the mechanism of BEC association, helps better understand previously accumulated data, and most importantly, may be useful for developing new and safer drugs.

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REFERENCES

1. Sono, M., Roach, M. P., Coulter, E. D., and Dawson, J. H. (1996) Heme-containing oxygenases. *Chem. Rev.* 96, 2841–2887

2. Guengerich, F. P., Hosea, N. A., Parikh, A., Bell-Parikh, L. C., Johnson, W. W., Gillam, E. M., and Shimada, T. (1998) Twenty years of biochemistry of human P450s: purification, expression, mechanism, and relevance to drugs. *Drug Metab. Dispos.* 26, 1175–1178

3. Wrighton, S. A., Szczepanski, E. G., Thummel, K. E., Shen, D. D., Korzewska, K. R., and Watkins, P. B. (2000) The human CYP3A subfamily: practical considerations. *Drug Metab. Rev.* 32, 339–361

4. Ekroos, M., and Sjögren, T. (2006) Structural basis for ligand promiscuity in cytochrome P450 3A4. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13682–13687

5. Scott, E. E., and Halpert, J. R. (2005) Structures of cytochrome P450 3A4. *Trends Biochem. Sci.* 30, 5–7

6. Niwa, T., Murayama, N., and Yamazaki, H. (2008) Heterotropic cooperativity in oxidation mediated by cytochrome p450. *Curr. Drug Metab.* 9, 453–462

7. Szkolka, G. D., and Halpert, J. R. (1998) Molecular basis of P450 inhibition and activation: implications for drug development and drug therapy. *Drug Metab. Dispos.* 26, 1179–1184

8. Ueng, Y. F., Kuwabara, T., Chun, Y. J., and Guengerich, F. P. (1997) Cooperativity in oxidations catalyzed by cytochrome P450 3A4. *Biochemistry* 36, 370–381

9. Denisov, I. G., Baas, B. J., Grinkova, Y. V., and Sligar, S. G. (2007) Cooperativity in cytochrome P450 3A4. Linkages in substrate binding, spin state, uncoupling, and product formation. *J. Biol. Chem.* 282, 7066–7076

10. Davydov, D. R., Baas, B. J., Sligar, S. G., and Halpert, J. R. (2007) AllostERIC mechanisms in cytochrome P450 3A4 studied by high-pressure spectroscopy: pivotal role of substrate-induced changes in the accessibility and degree of hydration of the heme pocket. *Biochemistry* 46, 7852–7864

11. Fernandez, H., Halpert, J. R., and Davydov, D. R. (2006) Resolution of multiple substrate binding sites in cytochrome P450 3A4: the stoichiometry of the enzyme-substrate complexes probed by FRET and Job's titration. *Biochemistry* 45, 4199–4209

12. Peyronneau, M. A., Delaforge, M., Riviere, R., Renaud, J. P., and Mansuy, D. (1994) High affinity of ergopeptides for cytochromes P450 3A. Importance of their peptide moiety for P450 recognition and hydroxylation of bromocriptine. *Eur. J. Biochem.* 223, 947–956

13. Renaud, J. P., Davydov, D. R., Heinwegh, K. P., Mansuy, D., and Hui Bon Hoa, G. H. (1996) Thermodynamic studies of substrate binding and spin transitions in human cytochrome P450 3A4 expressed in yeast microsomes. *Biochem. J.* 319, 675–681

14. Davydov, D. R., Halpert, J. R., Renaud, J. P., and Hui Bon Hoa, G. (2003) Conformational heterogeneity of cytochrome P450 3A4 revealed by high pressure spectroscopy. *Biochem. Biophys. Res. Commun.* 312, 121–130

15. Insi, E. M., and Guengerich, F. P. (2006) Kinetics and thermodynamics of ligand binding by cytochrome P450 3A4. *J. Biol. Chem.* 281, 9127–9136

16. Neth, A., Grinkova, Y. V., Sligar, S. G., and Atkins, W. M. (2007) Ligand binding to cytochrome P450 3A4 in phospholipid bilayer nanodiscs. The effect of model membranes. *J. Biol. Chem.* 282, 28309–28320

17. Das, A., Grinkova, Y. V., and Sligar, S. G. (2007) Redox potential control by drug binding to cytochrome P450 3A4. *J. Am. Chem. Soc.* 129, 13778–13779

18. Maurer, G., Schreier, E., Delabarde, S., Loosli, H. R., Nufer, R., and Shukla, A. P. (1982) Fate and disposition of bromocriptine in animals and man. I: Structure elucidation of the metabolites. *Eur. J. Drug Metab. Pharmacokinet.* 7, 281–292

19. Maurer, G., Schreier, E., Delabarde, S., Nufer, R., and Shukla, A. P. (1983) Fate and disposition of bromocriptine in animals and man. II: Absorption, elimination, and metabolism. *Eur. J. Drug Metab. Pharmacokinet.* 8, 51–62

20. He, Y. A., Zientek, M., Parge, H. E., Burke, B. J., Lee, C. A., and Wester, M. R. (2009) Crystal structures of CYP3A4 in complex with bromocriptine and clotrimazole: evidence of structural plasticity in the active site. In *Book of Abstracts for the 16th International Conference on Cytochrome P450*, p. 91, Okinawa, Japan

21. Sevioukov, I. F., and Poulos, T. L. (2010) Structure and mechanism of the complex between cytochrome P450 3A4 and ritonavir. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18422–18427

22. Collaborative Computational Project, Number 4 (1994) The CCP4 suite: programs for protein crystallography. *Acta Crystallogr. D Biol. Crystallogr.* 50, 760–763

23. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 60, 2126–2132

24. Viner, R. I., Novikov, K. N., Ritov, V. B., Kagan, V. E., and Alterman, M. A. (1995) Effect of different solubilizing agents on the aggregation state and catalytic activity of two purified rabbit cytochrome P450 isozymes, CYP1A2 (LM4) and CYP2B4 (LM2). *Bioclin. Biochem. Res. Commun.* 217, 886–891

25. Williams, P. A., Cosme, J., Vinkovic, D. M., Ward, A., Angove, H. C., Day, P. J., Vonrhein, C., Tickle, I. J., and Jhoti, H. (2004) Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. *Science* 305, 683–686

26. Harlow, G. R., and Halpert, J. R. (1997) Alanine-scanning mutagenesis of a putative substrate recognition site in human cytochrome P450 3A4. Role of residues 210 and 211 in flavonoid activation and substrate specificity. *J. Biol. Chem.* 272, 5396–5402

27. Harlow, G. R., and Halpert, J. R. (1998) Analysis of human cytochrome P450 3A4 cooperativity: construction and characterization of a site-directed mutant that displays hyperbolic steroid hydroxylation kinetics. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6636–6641

28. Fishelovitch, D., Hazan, C., Shaik, S., Wolfson, H. J., and Nussinov, R. (2007) Structural dynamics of the cooperative binding of organic molecules in the human cytochrome P450 3A4. *J. Am. Chem. Soc.* 129, 1602–1611

29. Yano, J. K., Wester, M. R., Schoch, G. A., Griffin, K. J., Stout, C. D., and Johnson, E. F. (2004) The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05 Å resolution. *J. Biol. Chem.* 279, 38091–38094

30. Hosea, N. A., and Guengerich, F. P. (1998) Oxidation of nonionic detergents by cytochrome P450 enzymes. *Arch. Biochem. Biophys.* 353, 365–373

31. Mak, P. J., Denisov, I. G., Grinkova, Y. V., Sligar, S. G., and Kincaid, J. R. (2011) Defining CYP3A4 structural responses to substrate binding. *J. Biol. Chem.* 286, 1357–1366

32. Hendlich, M., Rippmann, F., and Barnickel, G. (1997) LIGSITE: automatic and efficient detection of potential small molecule-binding sites in proteins. *J. Mol. Graph. Model.* 15, 359–363