Crystal Structures of Epothilone D-bound, Epothilone B-bound, and Substrate-free Forms of Cytochrome P450epoK*

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Epothilones are potential anticancer drugs that stabilize microtubules by binding to tubulin in a manner similar to paclitaxel. Cytochrome P450epoK (P450epoK), a heme containing monoxygenase involved in epothilone biosynthesis in the myxobacterium Sorangium cellosolus, catalyzes the epoxidation of epothilones C and D into epothilones A and B, respectively. The 2.10-, 1.93-, and 2.65-Å crystal structures reported here for the epothilone D-bound, epothilone B-bound, and substrate-free forms, respectively, are the first crystal structures of an epothilone-binding protein. Although the substrate for P450epoK is the largest of a P450 whose x-ray structure is known, the structural changes along with substrate binding or product release are very minor and the overall fold is similar to other P450s. The epothilones are positioned with the macroside ring roughly perpendicular to the heme plane and I helix, and the thiazole moiety provides key interactions that very likely are critical in determining substrate specificity. Interestingly, there are strong parallels between the epothilone/P450epoK and paclitaxel/tubulin interactions. Based on structural similarities, a plausible epothilone tubulin-binding mode is proposed.

Since the discovery of paclitaxel (an active ingredient of Taxol®) from Taxus brevifolia (1) and its clinical success as an anti-cancer drug, there have been extensive efforts to find compounds with similar action. Those efforts resulted in the identification of three other classes of compounds from natural sources: epothilones, produced by the cellulose-degrading myxobacterium Sorangium cellosolus (2), the marine sponge-derived discodermolide (3, 4), and the coral-derived eleuthobins (5)/sarcodeitixins (6) (Fig. 1). All three classes, like paclitaxel, bind to and stabilize microtubules leading to mitotic arrest of the cycle at G2-M phase and subsequently induction of cell death in several cell lines (7, 8). Epothilones, however, offer some advantages, because they are effective against P-glycoprotein-expressing multidrug-resistant cell lines, and they offer some advantages, because they are effective against P-glycoprotein-expressing multidrug-resistant cell lines, and the water-solubility of epothilones is significantly greater than that of paclitaxel. Another advantage is that epothilones can be produced in large quantities using a heterologous expression system (9).

One step in the biosynthesis of epothilones involves a C12-C13 epoxidation (Fig. 1a) by a cytochrome P450, P450epoK (9). Cytochrome P450s (P450s) are heme-containing monoxygenases best known for their role in drug detoxification (10). However, P450s also are involved in steroid hormone biosynthesis (11) as well as the biosynthesis of important macroside antibiotics like erythromycin (12) and rapamycin (13). To date, there is no known protein structure complexed with an epothilone. In addition, epothilone represents the largest substrate of a P450 where the crystal structure of the enzyme-substrate complex is known and thus provides important insights in understanding how P450 architecture adapts to the requirements of substrate binding. Here we present the crystal structures of the oxidized P450epoK in substrate-free, epothilone D-bound, and epothilone B-bound forms at 2.65-, 2.10-, and 1.93-Å resolution, respectively.

EXPERIMENTAL PROCEDURES

Protein Purification and Crystallization—P450epoK was overexpressed in Escherichia coli and purified as will be described elsewhere. Single crystals of all forms of P450epoK reported here were obtained by the vapor-diffusion method using the sitting-drop technique. P450epoK crystals were obtained within a few days after microseeding. Crystals of the imidazole-bound form for multiple wavelength anomalous diffraction (MAD) phasing were grown at 23 °C in 100 mM imidazole, pH 6.5-7.0, 25% polyethylene glycol 5000 monomethyl ether/0.1 M Li2SO4/0.1 M MES, pH 6.5. Initial protein concentration was 20 mg/ml. Crystals of substrate-free form were also obtained by vapor-diffusion method at 23 °C from 18% polyethylene glycol 5000 monomethyl ether/0.1 M Li2SO4/0.1 M MES, pH 6.5. Initial protein concentration was 20 mg/ml. Crystals of epothilone-bound P450epoK were grown at 23 °C from 11% polyethylene glycol 550 monomethyl ether/0.05 M glycine, pH 8.4, and in the presence of a saturated amount of epothilone B or D. The microseeding method was also used for these crystal growths. The single crystal was carefully transferred to cryobuffer containing glycerol with the glycerol concentration brought up to 20% in four steps.

Data Collection and Processing—With the exception of MAD data, x-ray diffraction data were collected at the Advanced Light Source, Lawrence Berkeley Laboratory, beam line 8.2.2 with an Area Detector System Corporation Quantum 315 charge-coupled device detector at cryogenic temperature. MAD data sets of imidazole-bound P450epoK were collected at the Stanford Synchrotron Radiation Laboratory beam...
line 1–5 with an Area Detector System Corporation Quantum-4R charge-coupled device detector. Three wavelengths around the iron edge, 1.738 Å (peak), 1.741 Å (inflection), and 1.653 Å (remote), were chosen. The inverse-beam data collection procedure was used to ensure good completeness and redundancy for Bijvoet pairs. Data were processed and scaled using HKL2000 (14). Crystallographic statistics are given in Table I.

MAD Phasing—The iron position was located, and initial phases were refined with three-wavelength MAD data sets at 2.9 Å using SOLVE (15) enabling the location of several a-helices and bulky side chains. Solvent flattening and density modification using RESOLVE (16) resulted in a more clearly interpretable electron density map. Statistics for the MAD data sets are given in Table II. Crystallographic statistics are given in Table I.

RESULTS AND DISCUSSION

Comparison with Other P450s—To accommodate the bulky substrate, epothilone, P450epoK has a large substrate-binding cavity of 1060 Å³ in volume. Despite the presence of the very large substrate binding site, the overall structure of P450epoK and its outer dimensions are similar to that of P450cam (23) whose substrate binding cavity is only 240 Å³ in volume. Like other P450s, P450epoK exhibits the typical triangular prism-shaped P450 fold (Fig. 2a) with a side 60 Å long and 30 Å thick and the heme prosthetic group embedded between the I and L forms.

Table I

| Data set         | Substrate-free | Epothilone D-bound | Epothilone B-bound |
|------------------|----------------|--------------------|--------------------|
| Unit cell (Å)    | a = b = 61.52; c = 256.73 | a = b = 60.43; c = 252.77 | a = b = 61.52; c = 252.84 |
| Space group      | P4,22          | P4,22              | P4,22              |
| Resolution range (Å) | 50–2.65        | 50–2.10            | 50–1.93            |
| Reflections (observed/unique) | 72,065/14,049  | 167,058/27,331     | 203,413/34,489     |
| Completeness (overall/outer shell; %) | 91.7/94.9     | 96.2/95.4          | 91.0/72.4          |
| Rmerge (overall/outer shell; %) | 5.7/9.3        | 6.3/9.3            | 7.7/9.3            |
| r.m.s.d. bond length (Å) | 0.016          | 0.015              | 0.016              |
| r.m.s.d. bond angle (degree) | 1.56           | 1.50               | 1.25               |
| Number of water molecules | 103            | 200                | 266                |

Fig. 1. Tubulin-binding anti-cancer drugs. a, structure of epothilones and epoxidation reaction catalyzed by P450epoK. Epothilones D and B have higher tubulin polymerization activity and cytotoxicity than epothilones C and A, respectively (8, 45). b, paclitaxel. Taxane skeleton is composed of rings A–D. c, eleutherobin; d, sarcodictyin; e, discodermolide.
helices. In addition, the substrate binding cavity is surrounded mainly by the heme and the I and F helices (Fig. 2b). Although the folds are the same, P450epoK and P450cam differ in the location of several helices (Fig. 3). The significant differences, which are important for substrate binding (24, 25), include the B’, F, and G helices (Figs. 3 and 4a). The B’ helix of P450cam is important for substrate binding, especially via an H-bond between Tyr^{386} and the camphor carbonyl oxygen atom. In P450epoK the B’ helix region is composed of two helices, B’1 and B’2. Both helices are farther from the active site than the B’ helix in P450cam to accommodate the larger substrate. The B’2 helix is especially important, because residues from this helix help to form part of the substrate binding pocket. On the other hand, the F helix, which forms the roof over the substrate, is even closer to the heme than that of P450cam. The closer approach of the F helix to the active site is possible, because the C-terminal end of the F helix has smaller side chains on the side facing the heme: Gly^{178} and Ala^{180}, which correspond to Thr^{381} and Thr^{383} of P450cam, respectively. This close approach of the F helix to the substrate or product enables the carbonyl O atom of Ala^{180} in the F helix to accept an H-bond from the substrate/product C3 OH group, which will be considered in more detail below.

As in other P450s, the I helix experiences a kink near a conserved Thr residue, Thr^{385} in P450epoK, that is important to the oxygen activation machinery (26, 27). Another conserved structural element in P450s is the Cys thiolate heme ligand and its immediate surroundings. P450epoK is no exception with Cys^{385} near the N-terminal end of the I helix coordinating the heme iron (Fig. 2b). Also similar to other P450s are the H-bond and ionic interactions between the heme propionates and His and Arg residues (Arg^{107}, His^{103}, and Arg^{107} in P450epoK).

Of the known P450 structures, the closest homologue to P450epoK at 30% identity and 48% similarity is P450eryF (28), which also utilizes a polyketide, 6-deoxyerythronolide B (6-DEB), as a substrate. As seen for the substrate bound to P450eryF (28), epothilones D and B are oriented roughly perpendicular to the heme plane and I helix (Figs. 2b and 5). P450eryF has a long loop preceding the B’ helix, which might provide flexibility required to accommodate the large 14-member ring of 6-DEB. However, this loop is much shorter in P450epoK despite the larger size of epothilone. The F helix of P450epoK (Cys^{185}-Leu^{183}), which forms the roof of the substrate binding site, exhibits a 4.7-Å r.m.s. difference in backbone compared with P450eryF (Glu^{166}-Val^{176}), and is positioned closer to the heme for protein-substrate interactions. The G helix (Glu^{194}-Asn^{219}) exhibits a 4.8-Å r.m.s. difference in backbone, and its N-terminal position is located farther from the heme than in P450eryF (Arg^{181}-Glu^{206}). The relocation of these helices results in a P450epoK F/G loop that is 5 residues longer than the corresponding loop in P450eryF. The longer F/G loop could provide additional flexibility for substrate entry.

In addition to the size of the macrolide ring, another important difference between P450epoK and P450eryF is that in P450epoK the macrolide ring of the substrate is rotated about 90° relative to 6-DEB (Fig. 4b). This is necessary to accommodate the thiazole ring. The only other orientation of epothilone that would position the C12-C13 double bond for proper epoxidation would have the thiazole ring pointing toward the I helix. Because the I helix is one of the most conserved regions in P450 and is critical for oxygen activation (26, 27, 29), large variations in the I helix cannot be tolerated. Therefore, the thiazole ring must point toward the B’2 helix thus requiring a reorientation of the macrolide ring relative to P450eryF. In P450epoK this region is farther from the active site and provides side chains such as Phe^{396} that specifically interact with the thiazole ring.

**Heme Coordination**—Normally substrate-free oxidized P450s are low spin with a water molecule coordinated to the sixth coordination position. Upon substrate binding, this ligand is displaced giving a pentacoordinate high spin heme (30, 31). P450epoK is somewhat different, because the epothilone B and D complexes give a low spin species (spectra not shown) like substrate-bound P450BS, which has a water molecule at the sixth coordination site (32). Consistent with a low spin heme, the structure reveals that a water molecule, which is H-bonded to the epothilone B epoxide O atom (Fig. 5b), is coordinated to the heme iron. The substrate-free and epothilone D-bound structures are low spin and expected to be hexacoordinate. Although there is some electron density at the sixth coordination site, the limited resolution of the substrate-free data, 2.65 Å, precludes a clear identification of a single water molecule coordinated to the heme iron.

**Conformational Changes upon Epothilone Binding**—Until now P450cam (23, 33), P450BM-3 (34, 35), and P450 2C5 (36, 37) were the only P450s where both the substrate-free and
-bound crystal structures are known, whereas P450cam was the only structure with product bound (38). P450epoK represents the second example where all three structures are known. In P450cam there is very little difference in structures between the three forms, whereas with P450BM-3, there are large changes between the substrate-free and -bound structures primarily due to motions of the F and G helices and the F/G loop (34), whereas with P450 2C5 similar but smaller changes are observed (37). Large changes in these regions also have been observed upon ligand binding for a thermophilic P450, CYP119 (39). Given that epothilone is the largest substrate of a P450 with known structure, we anticipated some significant changes in structure upon substrate binding. However, the differences between the substrate-bound and -free P450epoK structures are small and are confined to the F/G loop and B’1 helix regions (Fig. 6, a and b) generally thought to provide the entry point for substrates in P450s (24). The B’1 helix moves closer toward and the F/G loop moves away from the active site to optimize interactions with the substrate. These differences are similar to what was observed in the

**Fig. 3. Stereo diagram of superimposed P450s.** Top panel, P450epoK (red) on P450cam (green). Bottom panel, P450epoK (red) on P450eryF (green). The models were superimposed by overlaying the heme groups. Key helical regions and the F/G loop are labeled.

**Fig. 4. Superimposed structures of F, G, and B’1 helices and connecting loops.** a, P450epoK (green) and P450cam (cyan). b, P450epoK (green) and P450eryF (red). The substrates (epothilone D for P450epoK; d-camphor for P450cam; 6-DEB for P450eryF) and heme are shown as stick models.
P450BM-3 substrate-bound (34) and -free (35) forms except the changes are much smaller in P450epoK. It appears that the plausible substrate access channel, indicated in Fig. 2a, is similar to what has been proposed for P450BM-3 and P450cam. However, P450epoK must be able to adopt an even more open conformation to allow substrate entry and product release. That the substrate-free P450epoK crystallized in the nearly closed form is very likely due to crystal packing forces favoring the substrate-bound closed conformation as opposed to the postulated open conformation. With the substrate-free P450BM-3 just the opposite occurred: crystal packing favored the open form (35).

In addition to a small adjustment in the backbone, various side chains move to accommodate the substrate. Most notable is Arg71 in the loop between B and B’1 helix, the guanidium group of which moves about 4 Å closer to the active site (Fig. 6c). This movement enables Arg71 to hold two water molecules close to the substrate, especially the thiazole ring. In addition, the movement of Arg71 enables the thiazole ring to form a π-π stacking interaction with Phe96, whose side chain provides a key substrate contact.

Epothilone/P450epoK Interactions—As found for P450eryF (28), there are a number of water molecules around epothilone that form H-bonding bridges between epothilone and protein atoms (Fig. 7). The H-bond network involves 6 residues for the epothilone D-bound form and 7 residues for the epothilone B-bound form, respectively. Most of the residues involved in these backbone H-bonds are small, such as Gly and Ala, which provide the required room for the large substrate. Arg71, which, as already mentioned, moves into the active site upon epothi-
lone binding, is the only side chain that forms part of the H-bond network with the substrate. Phe$_{96}$, which forms $\pi-\pi$ stacking interactions with the thiazole ring and the guanidium group of Arg$_{71}$, is very likely a critical residue for epothilone specificity. In addition to H-bonding and aromatic interactions, Leu$_{183}$ in the F helix and Ala$_{250}$ in the I helix form non-bonded contacts with the substrate/product thiazole ring (Fig. 7).

Similar to other P450s, the carbons to be oxidized are close to the heme iron (Figs. 5a and 7a). In this case, the C12-C13 double bond reacts with the iron-linked oxygen atom to give an epoxide. The distances of C12-Fe and C13-Fe are 4.7 and 5.0 Å, respectively, which are comparable to the distance between the iron and the carbon atom to be hydroxylated in P450cam and P450eryF (4.2 and 4.7 Å, respectively). The substrate orientation is suitable for synchronous oxygen transfer from an Fe(IV)-O species.

The conformation of P450epoK-bound epothilones is somewhat different from the crystal structure of free epothilone (2). The thiazole side chain is perpendicular to the macroside ring in P450epoK, whereas free epothilone has the thiazole moiety in-plane with the macroside ring. However, the conformation of the macroside ring predicted to bind to tubulin (31) is the same as that bound to P450epoK.

**Implication for Tubulin/Epothilone Interactions**—Both paclitaxel and epothilone bind to tubulin to stabilize microtubules, arresting cells in mitosis. Epothilones competitively inhibit the binding of paclitaxel to polymerized tubulin, suggesting that the two types of antitumor compounds share an overlapping binding site in tubulin (7, 8). Therefore, it is of interest to see if the binding of epothilones to P450epoK mimic to any degree the interactions found between paclitaxel and tubulin (40). As shown in Fig. 8, the C2-phenyl ring of paclitaxel is the structural homologue to the epothilone thiazole ring. Both form $\pi-\pi$ stacking interactions with a neighboring aromatic ring (His$_{229}$ of $\beta$-tubulin and Phe$_{96}$ of P450epoK) in addition to other non-bonded contacts with aliphatic side chains (Leu$_{217}$ of $\beta$-tubulin and Ala$_{250}$ of P450epoK). The aromatic moiety of epothilone is critical, because its removal or direct attachment of the aromatic moiety to C15 results in the loss of tubulin binding and cytotoxic properties (41). Therefore, it is reasonable to expect that the thiazole ring of epothilone makes similar $\pi-\pi$ stacking interaction when it binds to tubulin. Structure-activity relationship studies on epothilones have also shown that the location of the N atom in the aromatic ring is important for tubulin binding activity, because the 2-pyridyl- and 2-thiazyl-containing compounds exhibit properties comparable to the natural

![Fig. 7. Stereo view showing substrate-binding site. Atom colors are the same as in Fig. 4. H-bonds, which bridge between epothilone and protein atoms, are shown as broken yellow lines. a, epothilone D-bound form; b, epothilone B-bound form.](image-url)
Although epothilone is the largest natural substrate for P450 with known structure, the overall fold of P450epoK is very similar to other P450s. Unexpectedly, the structures of the substrate/product-bound P450epoK are very similar to the substrate-free structure with no major changes in the substrate access channel. Because P450epoK must undergo a large open/close motion to allow substrate to enter, we attribute crystal packing forces as the reason substrate-free P450epoK crystalized in the closed form. The largest change observed is in Arg141, which moves into the active site to help critical water molecules maintain H-bonds to the substrate/product. Similarities of how the thiazole ring of epothilone interacts in the P450epoK active site to how paclitaxel interacts with tubulin together with the mutant data suggest a possible binding mode for epothilone to tubulin.

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