INVESTIGATION OF THE PROTON TRANSFER SYSTEM IN P450eryF*

Cytochrome P450eryF (CYP107A) from Saccaropolyspora ertherea catalyzes the hydroxylation of 6-deoxyerythronolide B, one of the early steps in the biosynthesis of erythromycin. P450eryF has an alanine rather than the conserved threonine that participates in the activation of dioxygen (O₂) in most other P450s. The initial structure of P450eryF (Cupp-Vickery, J. R., Han, O., Hutchinson, C. R., and Poulos, T. L. (1996) Nat. Struct. Biol. 3, 632–637) suggests that the substrate 5-OH replaces the missing threonine OH group and holds a key active site water molecule in position to donate protons to the iron-linked dioxygen, a critical step for the mono-oxygenase reaction. To probe the proton delivery system in P450eryF, we have solved crystal structures of ferrous wild-type and mutant (Fe²⁺) dioxygen-bound complexes. The catalytic water molecule that was postulated to provide protons to dioxygen is absent, although the substrate 5-OH group donates a hydrogen bond to the iron-linked dioxygen. The hydrogen bond network observed in the wild-type ferrous dioxygen complex, water 63-Glu360-Ser246-water 53-Ala 241 carbonyl in the 1-helix cleft, is proposed as the proton transfer pathway. Consistent with this view, the hydrogen bond network in the O₂-A245S and O₂-A245T mutants, which have decreased or no enzyme activity, was perturbed or disrupted, respectively. The mutant Thr245 side chain also perturbs the hydrogen bond between the substrate 5-OH and dioxygen ligand. Contrary to the previously proposed mechanism, these results support the direct involvement of the substrate in O₂ activation but raise questions on the role water plays as a direct proton donor to the iron-linked dioxygen.

Cytochromes P450 (P450)¹ (1), a family of heme enzymes, catalyze the monooxygenation of a wide variety of substrates by utilizing dioxygen (O₂) and electrons derived from NAD(P)H. P450s play an important role in steroid hormone biosynthesis, drug metabolism, xenobiotic detoxification, and steroid biosynthesis (2). Some bacterial P450s participate in the biosynthesis of pharmacologically important molecules. For example, cytochrome P450eryF (P450eryF, CYP107A) hydroxylates 6-deoxyerythronolide B (6-DEB) to erythronolide B at the C-6 atom as one step in erythromycin A production in Saccaropolyspora ertherea (3, 4). Electrons provided from NAD(P)H via cytochrome P450 reductase or an iron-sulfur protein are required to reduce the iron-linked O₂ molecule to the peroxide level, whereas protons are needed to protonate the distal O₂ oxygen atom to promote heterolysis of the O–O bond. Much of what we know regarding the proton shuttle system in P450s derives from studies with P450cam (5, 6). Thr252 and Asp251 are critical residues in an H-bonded network that enables water protons to be delivered to the iron-linked O₂ molecule (6–9). P450eryF is unusual in that alanine (Ala²⁴⁵) replaces the threonine found in most other P450s. However, the substrate 5-OH group is located close to the oxygen binding site, suggesting that the substrate 5-OH replaces the missing threonine OH group (10). Another unique feature of P450eryF is that a water molecule is located between the substrate and Ala²⁴⁵ and is in position to donate a proton to an iron-linked O₂ molecule. This proton supply mechanism is consistent with mutagenic (11) and computational studies (12–14). Nevertheless, an important lesson from P450cam is that the H-bonded network and water structure changes significantly in the O₂ complex (15). Therefore, developing detailed mechanistic schemes based on P450 structures in the Fe³⁺ state may be of limited value. In addition to the possibility of similar structural changes in P450eryF, the “catalytic” water in P450eryF is only 3.6 Å from the ferric iron so this water must reposition in the O₂ complex. To further probe the potentially unique features of the P450eryF proton relay system, we solved the structure of the WT O₂-P₄₅₀eryF at 1.7 Å. We also solved the structure of oxy-complexes of two mutants, A245T and A245S, that have significantly reduced activity (11). The crystal structures of the O₂-P₄₅₀eryF complexes, together with the O₂-P₄₅₀cam structure, provide important insights on the differences and similarities in P450 O₂ activation mechanisms.

MATERIALS AND METHODS

Crystallization—WT and mutants P450eryF were expressed and purified as reported earlier (16). The natural substrate 6-DEB was from KOSAN Bioscience (Hayward, CA). Crystallization of P450eryF followed published procedures (16). 1 μl of mother liquor consisting of 0.1 M Tris–HCl, pH 8.5, 0.3 M sodium acetate, and 30–50% polyethylene glycol 4000 was overlaid over 1 μl of a WT protein drop without 6-DEB in the sitting drop vapor diffusion crystallization tray (Hampton Research, Aliso Viejo, CA). Fine needle clusters that formed in a few days were crushed into microcrystals and used for microseeding. Microseeding...
Calculation of reflections were randomly picked for the calculation of consisting of 0.1M Tris-HCl, pH 8.5, 0.28M sodium acetate, 22% poly-
ing was carried out 24 h after setting up crystallization sitting drops

Preparation of Ferrous (Fe2+): Dioxegen-bound Single Crystals—The following manipulations with the exception of dioxegen-soaking were carried out at room temperature. Oxidized crystals were transferred into cryobuffer (mother liquor with 20% glycerol) and then reduced with 10 mM sodium dithionite in a glove box (COY, Grass Lake, MI) with the dioxogen concentration kept <15 ppm. A clear color change from dark brown to bright red was apparent within 2 min. The crystals were kept in the dithionite solution for another 20 min. After reduction, crystals were washed thoroughly in deoxgenated cryobuffer for 10 min in the glove box. Reduced crystals then were soaked in the oxygen-saturated cryobuffer under atmospheric condition at −10 °C. The crystal was quickly scooped up in a nylon loop and flash-cooled in liquid nitrogen in preparation for data collection.

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RESULTS AND DISCUSSION

Active Site Structure of WT O2-P450eryF—The simulated-annealing omit maps for the O2-WT complex clearly show dioxegen coordinated to the heme iron (Fig. 1). The Fe–O bond was retrained to 1.9 Å using a force constant of 4.0 kcal/mol/Å2, whereas the Fe–O–O bond angle was retrained to 130° with a force constant of 35 kcal/mol/rad2. Water molecules were added by using the water-pick program of CNS with the threshold of 5.0 σ. After another cycle of refinement, several water molecules were added manually around the substrate. The graphic program O (19) was used for the model building. The same procedure was used for the mutant-oxy structures. Cavity volume calculations were done using VOIDOO (20).

Active Site structure and electron density maps of the WT O2-P450eryF. Active site residues, substrate, and heme are shown as a stick model (green, carbon; blue, nitrogen; red, oxygen; orange, sulfur; purple, iron). Water molecules 53 and 63 are shown as red spheres. Yellow broken lines show the presumed proton transfer pathway. The substrate C-5 and C-6 atoms are labeled. The simulated-annealing omit maps of Fc−F, contoured at 3 σ (A) and of the 2Fc−F, contoured at 1.5 σ (B). The dioxygen ligand and waters 53 and 63 are omitted from the map calculation.
carbonyl O atom is 3.3 Å from the distal O atom. The C-6 atom of the substrate, which is the hydroxylation site, is 3.2 Å from the proximal oxygen atom. The substrate C-5 OH forms a 2.7-Å H-bond with the distal oxygen atom. These steric and H-bond interactions force the dioxygen ligand to point toward the I-helix cleft between Ala241 and Ala245.

Fig. 2 shows a superposition of O2/P450eryF and oxidized P450eryF. Although the substrate slightly rotates about C-13, O2 binding does not change the substrate-heme distances significantly, probably because of restrictions imposed by the H-bond between 5-OH and O2. This is different than in P450cam where diatomic ligand binding, CO and O2, displaces the substrate by 1 Å further from the heme (15, 22, 23).

From the crystallographic analysis of the ferric enzyme, water molecule 519, which hydrogen bonds to the carbonyl of Ala241 and the substrate 5-OH group, was proposed to be the direct proton donor to the dioxygen ligand (10). This view was supported by the A245S and A245T mutant structures that show a much higher mobility of water 519 and exhibit 16 and 1% wild-type activities, respectively (11). As shown in Fig. 2, the distal oxygen atom of O2 is only 0.7 Å from water 519 in the ferric enzyme. Thus, it might be anticipated that water 519 moves into a position for direct H-bond donation to the distal oxygen atom, similar to what is observed in P450cam (Fig. 3) (15). However, there is no evidence for the presence of water 519. Although there is a small cavity near the O2 molecule, this cavity is much smaller than in P450cam (77 versus 206 Å3) and is too small to accommodate a water molecule. Therefore, the water molecule previously assumed to be the proton donor has been expelled from the active site, leaving no water directly H-bonded to the distal oxygen as in P450cam.

In P450cam, there is a substantial change in the conformation of the I-helix and the H-bonded water network that provides a continuous H-bonded link among the distal dioxygen O atom, water molecules, and key active site groups such as Thr252 (15). In sharp contrast, there is little change in the I-helix or local H-bonded network in P450eryF. The H-bond network found in ferric P450eryF involving Glu360, Ser246, Ala241 carbonyl, and waters 53 and 63 (Fig. 1) remains intact in the O2-complex with the exception that Wat519 has been expelled. We next probed the functional relevance of this hydrogen bond network by solving the structures of mutant-O2 complexes that have reduced enzyme activity.

**Active Site Structures of A245S and A245T Mutant O2/P450eryF**—The A245S and A245T mutants have substantially decreased enzyme activities, 16 and 1%, respectively (11). The O2-complex structures were solved to see whether the mutations resulted in perturbations in the local H-bonded network that could be related to the loss in activity. Even though Ala245 has been replaced by residues of quite a different size and polarity at a critical location in the active site, the overall ligand geometry remains unchanged (Figs. 4 and 5). The similar conformation suggests that the other groups interacting with the ligand, the substrate, and Ala241 are the key determinants for the dioxygen ligand conformation. As in the WT structure, the mutants maintain the same substrate 5-OH-O2 H-bond and the Cβ atom of Ala241 forms one wall of the O2 cavity, both of which force the ligand to point toward the I-helix.

Although the activity is decreased by 84% by the A245S mutation, the active site structure including water is essentially unchanged with the exception that the OH group of Ser245 points toward the I-helix cleft to make new H-bonds with water 63 and the carbonyl group of the Ala241 (Fig. 4). A possible source of the decrease in activity in the A245S mutant is a disruption in the strict H-bond donor/acceptor relationship in the proton transfer system because of the new H-bonds formed with the mutant Ser245 side chain.
In the O₂:A245T complex (Fig. 5), the larger threonine side chain results in larger and more functionally relevant structural changes. The 5-OH group of the substrate makes van der Waals contact with the Cγ atom of Thr₂⁴⁵, pushing Thr₂⁴⁵ toward the I-helix cleft (Fig. 5C). As a result, the γ-OH of Thr₂⁴⁵ occupies a similar position as water 63 in the ferric state and thus displaces water 63 from the active site. The Oγ atom of Thr₂⁴⁵ is too far (4 Å) from the Oγ atom of Ser₂⁴⁶ to make an H-bond. Therefore, the H-bond network is indeed disrupted at this site in this mutant (Fig. 5A). Thr₂⁴⁵ also contacts the distal O₂ O atom (Fig. 5C). These interactions and especially the disruption of the water H-bonded network by the displacement of water 63 probably disrupts efficient proton transfer to dioxygen, which explains the extremely low activity of the A245T mutant.

Also of interest is a comparison between the A245T mutant and P450cam (Fig. 6). In P450cam-oxy complex, Thr²⁵² moves closer to the O₂ ligand and donates an H-bond to the distal O₂ O atom. This movement of Thr²⁵² away from the I-helix and the associated change in the I-helix conformation allow additional waters to move into the I-helix groove and thus complete the H-bonded network to the dioxygen. However, Thr²⁴⁵ in P450eryF cannot adopt the Thr²⁵²-like conformation because of steric interactions with the 5-OH group of the substrate. Thus, Thr²⁴⁵ does not form an H-bond with the ligand to assist with dioxygen activation and instead inhibits the dioxygen activation process. To accommodate the larger substrate and to make the substrate 5-OH group available for the dioxygen activation, it appears that P450eryF evolved to replace the threonine with alanine.

Ortiz de Montellano and co-workers (24) have reported that A245T has higher activity than WT in testosterone hydroxylation. A possible cause of higher activity is that testosterone does not clash with Thr²⁴⁵, placing the threonine side chain in a similar position as P450cam, and recovers water 63. These plausible structural changes would enable proton supply through the WT-like hydrogen bond network and Thr²⁴⁵-assisted O₂ activation.

**Dioxygen Activation by P450eryF**—The only direct H-bond donor to dioxygen in P450eryF is the substrate 5-OH.
group is critical, because conversion of the 5-OH group to a carbonyl eliminates enzyme activity (11). It has generally been assumed that the 5-OH group replaces the I-helix threonine found in most other P450s, and as the O2-P450cam structure shows (15), Thr252 is in position to donate an H-bond to the distal oxygen atom, similar to the substrate 5-OH in P450eryF. However, the similar roles of Thr252 and the P450eryF substrate 5-OH group came into question when Kimata et al. (25) reported that the substitution of the O\(^{-}\)H9253 proton with methyl group (Thr to O\(^{-}\)-Me Thr) retains significant hydroxylation activity, suggesting that the O\(^{-}\)H9253 proton is not directly involved in catalysis. Although both P450cam and P450eryF have similar solvent H-bonded networks involving the I-helix, P450cam but not P450eryF has a water directly H-bonded to the distal oxygen. Although water 519 appeared to be an ideal candidate for the direct proton donor to dioxygen based on the Fe\(^{III}\) structure, once dioxygen binds the cavity, housing water 519 is simply too small (\(-77 \text{ Å}^3\)), compared with \(-206 \text{ Å}^3\) in P450cam, to accommodate water. Hence, if water does bind here, there must be some rearrangement in the active site once the peroxy intermediate forms. In the absence of any information supporting such a change in structure, we propose the mechanism shown in Fig. 7. In this scheme, one proton is delivered from water 63 via the Ala245 carbonyl. The most probable other proton source is the substrate 5-OH, because the 5-OH is the only direct H-bond partner for O\(^2\) and the 5-OH proton is essential for the catalytic activity. The proton abstraction from 5-OH by O\(^2\) transiently forms a substrate alkoxide, which is stabilized by the H-bond with the leaving water molecule. The energetic feasibility of forming an alkoxide is supported by density functional calculations that show that the iron-linked peroxy dianion has a higher proton affinity than the substrate alkoxide (14). After O–O bond fission, a leaving water molecule completes an H-bonded network that provides a proton to the substrate alkoxide via Glu360. The Fe(V)–O intermediate or its electronic equivalent next hydroxylates the C-6 atom to complete the catalytic event. One final problem is the ultimate source of protons, which for both P450cam and P450eryF is assumed to be bulk solvent. In both structures,
there is no continuous H-bonded connection between the active site and bulk solvent. However, when the concerted motion of side chains and water is taken into account, the proton delivery pathway in P450cam does connect to the molecular surface (26). Thus, dynamics may well play a critical role in enabling bulk solvent protons to be delivered to the internal H-bonded network.

CONCLUSIONS

The ferric P450eryF structure suggested that a well-ordered active site water molecule H-bonded to a substrate OH group serves as a direct proton donor to the iron-linked dioxygen (10). Although ferric P450cam has no homologous water molecule, the O₂-P450cam structure does have a water molecule H-bonded to the Thr²⁵² OH group capable of donating protons to dioxygen (15). Therefore, the previous picture of O₂ activation mechanisms for these two P450s shares common features: a catalytic water molecule H-bonded to a substrate or side chain OH group capable of donating protons to dioxygen. In forming the O₂-P450eryF complex, however, the water molecule that makes an H-bond to the Thr²⁵² OH group serves as a direct proton donor to dioxygen. Instead of being expelled from the active site, leaving no water capable of donating protons to dioxygen (15). Therefore, the previous picture on the structural underpinnings of catalysis that can be derived from resting state structures while emphasizing the importance of structural studies on critical intermediates.

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Crystal Structures of the Ferrous Dioxygen Complex of Wild-type Cytochrome P450eryF and Its Mutants, A245S and A245T: INVESTIGATION OF THE PROTON TRANSFER SYSTEM IN P450eryF
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