Bioorganic Chemistry **28,** 306–314 (2000)
doi:10.1006/bioo.2000.1187, available online at http://www.idealibrary.com on IDEAL®

The Role of Serine-246 in Cytochrome P450eryF-Catalyzed Hydroxylation of 6-Deoxyerythronolide B

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Received July 6, 2000

A strongly conserved threonine residue in the I-helix of cytochrome P450 enzymes participates in a proton delivery system for binding and cleavage of dioxygen molecules. 6-Deoxyerythronolide B hydroxylase (P450eryF) is unusual in that the conserved threonine residue is replaced by alanine in this enzyme. On the basis of crystal structures of substrate-bound P450eryF, it has been proposed that the C-5 hydroxyl group of the substrate and serine-246 of the enzyme form hydrogen bonds with water molecules 519 and 564, respectively. This hydrogen bonding network constitutes the proton delivery system whereby P450eryF maintains its catalytic activity in the absence of a threonine hydroxyl group in the conserved position. To further assess the role in the proton delivery system of hydroxyl groups around the active site, three mutant forms of P450eryF (A245S, S246A, and A245S/S246A) were constructed and characterized. In each case, decreased catalytic activity and increased uncoupling could be correlated with changes in the hydrogen bonding environment. These results suggest that Ser-246 does indeed participate in the proton shuttling pathway, and also support our previous hypothesis that the C-5 hydroxyl group of the substrate participates in the acid-catalyzed dioxygen bond cleavage reaction. © 2000 Academic Press

Key Words: cytochrome P450; 6-deoxyerythronolide B hydroxylase; site-directed mutagenesis; proton delivery system; uncoupling of cytochrome P450-catalyzed reaction.

The mechanism of cytochrome P450-catalyzed monooxygenation of various substrates is well characterized, and a complete reaction cycle common to all P450 enzymes has been proposed *(1).* The most crucial step in this reaction cycle is the cleavage of a dioxygen molecule to produce an unusually highly oxidized ferryl oxygen species which is responsible for the ultimate oxidation of the substrate. Many studies have focused on the role of active site amino acid residues in binding and cleavage of dioxygen molecules *(2,3).* In particular, the role of a strongly conserved threonine residue in the I-helix has been well documented *(1,2).* For example, mutation of the conserved threonine residue of P450cam resulted in reduced monooxygenation

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0045-2068/00 $35.00

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and increased uncoupling, leading to a net transfer of electrons to molecular oxygen and production of hydrogen peroxide *(4)*. This conserved threonine residue is thought to participate in a proton delivery system which converts bound, iron-linked dioxygen molecules to highly oxidized ferryl oxygen species *(5)*.

However, not all P450 enzymes contain threonine at the conserved position in the I-helix. For example, allene oxide synthase in the jasmonic acid biosynthetic pathway and 6-deoxyerythronolide B hydroxylase (P450eryF) in the erythromycin biosynthetic pathway both lack the conserved threonine residue *(6,7)*. The conserved position is occupied by alanine (Ala-245) in P450eryF. Since P450eryF is one of a very few cytochrome P450s lacking the crucial threonine in the I-helix among hundreds of known P450 enzymes, its active site structure, particularly around the I-helix, has attracted numerous crystallographers. The crystal structure of P450eryF bound to substrate indicated that the distal I-helix still maintains a complex hydrogen bonding network involving two amino acid side chains, three water molecules, and the substrate *(6)*. Of the three water molecules, Wat564 hydrogen-bonded to the hydroxyl group of Ser-246 takes the place of the missing threonine hydroxyl group, and Wat519 forms a hydrogen bond with the C-5 hydroxyl group of 6-deoxyerythronolide B (6-DEB).

Following the solution of the crystal structure of P450eryF, mutant forms of P450eryF (A245T and A245S) were constructed and their crystal structures were compared with the wild-type enzyme *(8)*. The A245S mutant retained the monooxygenation activity and showed a hydrogen bonding network around Wat519 and the C-5 hydroxyl group of 6-DEB which was similar to that of the wild-type enzyme. In contrast, the A245T mutant showed disruption of the hydrogen bonding network around Wat564 and increased uncoupling. The hydrogen bonding network around Wat519 and Wat564, including the hydrogen bond involving the C-5 hydroxyl group of 6-DEB, thus appears to be essential for the catalytic activity of P450eryF. In an effort to further elucidate the role of hydroxyl groups around the active site in proton shuttling and cleavage of iron-linked dioxygen molecules, we prepared A245S, S246A, and A245S/S246A mutants of P450eryF and determined their kinetic constants, NADPH oxidation rates, and uncoupling rates.

MATERIALS AND METHODS

Chemicals and reagents. The Sculptor *in vitro* mutagenesis system and QIAEX DNA purification kit were obtained from Amersham (Buckinghamshire, UK) and Qiagen (Chatsworth, CA), respectively. Tri-Sil for GC analysis was purchased from Pierce (Rockford, IL). All other chemicals were from Sigma-Aldrich (St. Louis, MO) and were of the highest grade available.

Site-directed mutagenesis. Site-directed mutagenesis was carried out using the Sculptor *in vitro* mutagenesis system according to the manufacturer's instructions and the following oligonucleotides: 5′-TCA CCG ACG ACT CGA AAC C-3′ (A245S), 5′-TCA CCG CCG CCT CGA AAC C-3′ (S246A), and 5′-TCA CCG CCG ACT CGA AAC C-3′ (A245S/S246A) (mutated bases are underlined). A *Sac*I–*Sty*I fragment of the P450eryF gene *(9)* in pWHM808 was cloned into the M13mp18 vector and single-stranded DNA was used as a template for mutagenesis. Each mutated *Sac*I–*Sty*I

fragment in M13mp18 was inserted into pWHM808 and transformed into *Escherichia coli* DH5α MCR strain.

Expression and purification of P450eryF enzymes. Mutated P450eryF enzymes were expressed and purified according to the method reported previously *(10)* with slight modifications. Transformed *E. coli* were grown in 1 L of LB broth containing ampicillin (100 μg/ml) for 2.5 h at 37°C with shaking (150 rpm), and δ-aminolevulinic acid (79 mg) was added to the culture. Expression was induced by addition of 1 mM IPTG after a further 30 min and the cells were grown for an additional 18 h. Cells were harvested by centrifugation at 6000*g* for 20 min at 4°C, washed with 50 mM Tris–Cl (pH 7.5) containing 1 mM EDTA, mixed with lysozyme (2 mg/ml) and Triton X-100 (0.1%), and sonicated for 3 min at 6-s intervals in 50 ml of buffer A (50 mM Tris–Cl (pH 7.5), 1 mM EDTA, and 0.2 mM DTT) containing 0.2 mM PMSF. Cellular debris was removed by centrifugation at 39,000*g* for 1 h at 4°C and the remaining proteins were precipitated with ammonium sulfate (30–80% saturation). The pellets were dissolved in buffer A and dialyzed against two changes of buffer A for 8 h at 4°C. The dialyzed crude extract was applied to a Q-Sepharose anion exchange column. The column was washed twice with buffer A containing first 0.1 M KCl and then 0.2 M KCl, and bound proteins were eluted with a linear gradient from 0.2 M to 1 M KCl in buffer A. P450eryF fractions were collected by monitoring the absorption ratio at 418/280 nm, pooled, concentrated by Centricon, and applied to a Mono-Q anion exchange FPLC column. P450eryF enzyme was eluted with a linear gradient from 0.1 M to 0.3 M KCl in buffer A.

Spectroscopy. Optical absorption spectra (700 to 360 nm) of ferric, sodium dithionite-reduced ferrous, and CO-complexed ferrous P450eryF were obtained with a Lambda 18 UV-Vis spectrophotometer (Perkin–Elmer). Appropriate amounts of ferric P450eryF were dissolved in 0.1 M KH_2PO_4, pH 7.5, and reduced with 200 μl of saturated sodium dithionite. CO complexes were prepared by vigorously bubbling carbon monoxide through the reduced P450eryF solution. Circular dichroism (CD) spectra of ferric P450eryF enzymes were obtained with a JASCO 715 polarimeter (Shimadzu).

Enzyme assay. Stock solutions for kinetic analysis of P450eryF enzymes were prepared as follows: 6-DEB, 10 mM in absolute ethanol; erythronolide B (EB), 10 mM in absolute ethanol; spinach ferredoxin, 20 μM in 0.1 M KH_2PO_4, pH 7.5; spinach ferredoxin-$NADP^+$ oxidoreductase, 0.01 U/μl in 0.1 M KH_2PO_4, pH 7.5; NADPH, 100 mM in 0.1 M KH_2PO_4, pH 7.5. Assay mixtures contained O_2-saturated 0.1 M KH_2PO_4, pH 7.5, 0.53 μM ferredoxin, 1×10^{-4} U of ferredoxin-$NADP^+$ oxidoreductase, 66 μM 6-DEB, 4 mM NADPH, and 1.88 μM P450eryF enzyme in a total volume of 150 μl . The reaction was initiated by the addition of NADPH and terminated after 0, 2, 5, 10, 20, or 30 min by the addition of ethyl acetate. The reaction mixture was extracted twice with ethyl acetate and dried by evaporation under vacuum.

Derivatization of reaction products and GC analysis. Tri-sil solution (30 μl) was mixed with the enzymatic reaction products and heated to 85°C for 5 min, and the silinated derivatives were subjected to analysis by gas chromatography using a GC-17A instrument (Shimadzu) equipped with a 30 m $\times$ 0.25 mm Rtx-1 capillary column (Restek). The temperature program consisted of an initial incubation at 100°C for 1 min, a linear increase of 10°C/min to 260°C, and a final incubation of 20 min at

260°C. The temperatures of injector and detector were set at 275°C. Retention times of trimethysilinated 6-DEB and EB were 25.7 min and 30 min, respectively. K_m and k_{cat} values of P450eryF enzymes were obtained by reciprocal plot.

NADPH oxidation and formation of hydrogen peroxide. NADPH oxidation rates were measured by monitoring absorbance at 340 nm. Reaction mixtures consisted of O_2-saturated 0.1 M KH_2PO_4, pH 7.5, 8 μl of 20 μM ferredoxin, 20 μl of 0.01 U/μl ferredoxin-$NADP^+$ oxidoreductase, 100 μl of 100 mM NADPH, and 8 μg of P450eryF enzyme in a total volume of 2.5 ml. For quantitative analysis of H_2O_2 production, the enzymatic reaction was terminated after 2, 5, 10, 20, or 30 min by the addition of 5% trichloroacetic acid, and the absorption of red ferrithiocyanate complex formed in the presence of peroxides was monitored at 480 nm *(11)*. The amount of hydrogen peroxide produced was determined by comparison with a standard curve.

RESULTS AND DISCUSSION

Purification of Mutant P450eryF Enzymes

The DNA sequence of each mutant P450eryF cDNA was confirmed by dideoxy sequencing, and purified mutant P450eryF enzymes were analyzed by SDS–PAGE. The purified proteins were homogeneous and the mobility of each mutant protein exactly matched that of wild-type P450eryF (Fig. 1).

Spectral Properties of Mutant P450eryF Enzymes

Absorption spectra of P450eryF enzymes are shown in Fig. 2. All of the enzymes showed a characteristic absorption at 418 nm in the ferric state, and the CO-complexed reduced ferrous forms showed strong absorption at 450 nm. The difference spectra between reduced and CO-complexed P450eryF enzymes also indicated that all of the mutant P450eryF enzymes were functional cytochrome P450 proteins (Fig. 3). These results indicated that all of the mutant P450eryF enzymes retained the characteristic spectral properties of P450 enzymes. Possible alteration of the wild-type three dimensional structure was further checked by analyzing CD spectra of the mutant enzymes. No significant difference was observed by CD spectroscopy between any of the mutants and the wild-type enzyme (data not shown). We therefore concluded that mutation at A245 or S246 did not cause any significant three-dimensional structural change, and that overall structure was maintained by all of the mutant enzymes. Spectral changes induced by substrate binding are well characterized in all P450 proteins *(8)*. Difference spectra of P450EeryF enzymes bound to substrate are shown

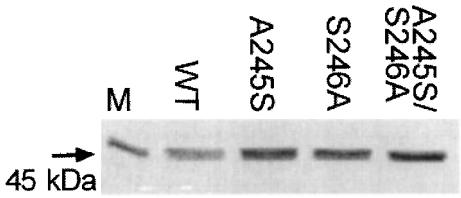

FIG. 1. SDS-PAGE of purified wild-type and mutant P450eryF proteins. Ten micrograms of protein was loaded in each lane of a 12% SDS–polyacrylamide gel. M indicates ovalbumin (MW = 45,000).