An A245T Mutation Conveys on Cytochrome P450\textsubscript{eryF} the Ability to Oxidize Alternative Substrates\textsuperscript{*}

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Cytochrome P450\textsubscript{eryF} (CYP107A1), which hydroxylates deoxyerythronolide B in erythromycin biosynthesis, lacks the otherwise highly conserved threonine that is thought to promote O–O bond scission. The role of this threonine is satisfied in P450\textsubscript{eryF} by a substrate hydroxyl group, making deoxyerythronolide B the only acceptable substrate. As shown here, replacement of Ala\textsuperscript{245} by a threonine enables the oxidation of alternative substrates using either H\textsubscript{2}O\textsubscript{2} or O\textsubscript{2} /spinach ferredoxin/ferredoxin reductase as the source of oxidizing equivalents. Testosterone is oxidized to 1-, 11α-, 12-, and 16α-hydroxytestosterone. A kinetic solvent isotope effect of 2.2 indicates that the A245T mutation facilitates dioxygen bond cleavage. This gain-of-function evidence confirms the role of the conserved threonine in P450 catalysis. Furthermore, a Hill coefficient of 1.3 and dependence of the product distribution on the testosterone concentration suggest that two testosterone molecules bind in the active site, in accord with a published structure of the P450\textsubscript{eryF}-androstenedione complex. P450\textsubscript{eryF} is thus a structurally defined model for the catalytic turnover of multiple bound substrates proposed to occur with CYP3A4. In view of its large active site and defined structure, catalytically active P450\textsubscript{eryF} mutants are also attractive templates for the engineering of novel P450 activities.

P450\textsubscript{eryF} (CYP107A1) catalyzes the stereospecific 6(S)-hydroxylation of deoxyerythronolide B (6-DEB) in the biosynthesis of erythromycin by Saccharopolyspora erythraea (Fig. 1) (1–3). The genetic manipulation of macrocyclic antibiotic biosynthetic pathways, including that of erythromycin, is currently under investigation as a route for the production of novel antibiotics (4). Hydroxylation catalyzed by P450 enzymes play key roles in these biosynthetic pathways, and modification of the substrate and regiospecificity of appropriate P450 enzymes is therefore of considerable interest. In an early example, targeted disruption of the gene encoding P450\textsubscript{eryF} in S. erythraea yielded a strain that was unable to hydroxylate 6-DEB and which therefore produced 6-deoxyerythromycin (3).

P450\textsubscript{eryF}, a soluble 45-kDa protein, has been crystallized, and its structure has been determined in complexes with both the natural substrate 6-DEB and alternative ligands (5, 6). Two endogenous proteins able to provide electrons for turnover of P450\textsubscript{eryF} have been cloned and expressed (7, 8), although spinach ferredoxin and FNR function as acceptable surrogate electron donors (9). The structure of P450\textsubscript{eryF} reveals two particularly interesting features of the enzyme. One is that the active site is much larger than the active sites of the other structurally defined bacterial P450 enzymes, as expected from the size of its macrocyclic substrate. The second is that the highly conserved threonine, Thr\textsuperscript{252} in P450\textsubscript{cam} (CYP101), is replaced in P450\textsubscript{eryF} by Ala\textsuperscript{245} (5, 6). The conserved threonine is thought to be required for dioxygen bond cleavage in the activation of molecular oxygen (10, 11). The crystal structure of P450\textsubscript{eryF} shows that the hydrogen bonding interactions normally satisfied by the threonine residue are replaced by hydrogen bonding interactions with the 5-hydroxyl group of the substrate (10). The resulting requirement for substrate-assisted catalysis in the 6-hydroxylation of 6-DEB makes P450\textsubscript{eryF} an ineffective catalyst for the oxidation of alternative substrates, even of closely related 6-DEB analogues (2). Replacement of Ala\textsuperscript{245} by a serine or threonine reportedly decreases the rate of 6-DEB hydroxylation to 10% and 1%, respectively, of the wild-type activity (10). This decrease in activity is thought to reflect disruption of the hydrogen bonding interactions involved in substrate-assisted catalysis by the new side-chain hydroxy group.

In P450\textsubscript{cam}, the most thoroughly characterized P450 enzyme (11, 12), the conserved threonine (Thr\textsuperscript{252}) clearly plays a critical catalytic role. Replacement of Thr\textsuperscript{252} by amino acids with non-hydrogen bonding sidechains virtually suppresses camphor hydroxylation in favor of the uncoupled reduction of O\textsubscript{2} to H\textsubscript{2}O\textsubscript{2} and H\textsubscript{2}O (13–15). In a key experiment, a threonine with a methylated hydroxyl group was introduced and the modified protein was shown to retain high catalytic activity (14). This experiment showed that the threonine may function by hydrogen bonding to a water molecule rather than by directly serving as a proton donor to the distal oxygen atom of the ferrous dioxy complex. This inference is supported by the crystal structure of the P450\textsubscript{cam} ferrous dioxygen complex (11). In accord with the proposed role for a hydrogen bond in oxygen activation, solvent isotope effect studies of the catalytic cycle have demonstrated that a KSIE of 1.8 is expressed during the second electron transfer step associated with O–O bond cleavage (15). No other step in the catalytic cycle exhibited a significant solvent isotope effect.

Studies of the role of the conserved threonine in P450 enzymes have generally provided loss-of-function evidence for its catalytic role, with the exception of the P450\textsubscript{cam} mutants in which the threonine was replaced by a methyl ether derivative or a serine (14). Here, we carry out a gain-of-function study in which the missing threonine is introduced into P450\textsubscript{eryF}. This

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\textsuperscript{†} The abbreviations used are: P450, cytochrome P450; heme, iron protoporphyrin IX independent of the iron ligation and oxidation state; 6-DEB, 6-deoxyerythronolide B; FNR, spinach ferredoxin reductase; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography; KSIE, kinetic solvent isotope effect.
change of an alanine to a threonine makes it possible to oxidize substrates that, unlike DEB, are unable to contribute to substrate-assisted catalysis. The results refine our understanding of the role of the threonine in catalysis. Furthermore, the P450 eryF A245T mutant is a good model for the multiple-ligand binding observed with enzymes like CYP3A4, and is a potentially important framework for the design of tailored P450 catalysts.

**MATERIALS AND METHODS**

**General Methods**—Restriction enzymes were purchased from Promega. The primers were either purchased from Life Sciences (Manassas, VA) or the Biomolecular Resource Center (University of California, San Francisco, CA). All reagents, testosterone, and 2α-hydroxysteroidase were purchased from Sigma unless stated otherwise. The 2β-, 6α-, 6β-, 7α-, 7β-, 11α-, 11β-, 14α-, 16α-, 18-, and 19-hydroxysteroidase standards were gifts from the Steroid Reference Collection (Medical Research Council, London, United Kingdom). 15α- and 16β-Hydroxysteroidase were purchased from Steraloids (Newport, RI). DEAE (DE-52) cellulose was obtained from Whatman (Clifton, NJ) and Red Sepharose CL-6B from Amersham Pharmacia Biotec.

**Enzymes**—Spinach ferredoxin was purchased from Sigma and was used without further purification. The plasmid pMBPFNR containing the ferredoxin NADP+ reductase (FNR) gene linked to the C terminus of the Escherichia coli maltose-binding protein was provided by Dr. A. Alberti (16). The plasmid was transformed into E. coli XL-1 Blue competent cells (Stratagene, La Jolla, CA) and was expressed as described (17). FNR was purified as described by Apley et al. (18) with the modification that only the DEAE and Red Sepharose CL-6B column chromatographies were used. The protein was purified to homogeneity, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and was used without cleavage of the fusion maltose-binding protein.

**Subcloning, Expression, and Purification of P450 eryF**—The plasmid pPGS180–129, containing the P450 eryF gene, was a gift from Kosan Bioscience (Hayward, CA). NdeI and XbaI sites were introduced by overhang polymerase chain reaction at the N and C termini, respectively. The forward primer (start codon in boldface and NdeI site underlined) and reverse primer (stop codon in boldface and XbaI site underlined) were: 5′-GG AAG TTT CAG TGA CGA CGG TCC-3′ and 5′-CAG TGT TAG ATT ATC CGT CGA GCC GGC-3′. The polymerase chain reaction product was digested with NdeI and XbaI restriction enzymes and ligated into the pCWori + (His), vector (19). The resulting clone was transformed into E. coli XL-1 Blue cells. Insertion of the correct gene was verified by DNA sequencing (Biomolecular Resource Center, University of California, San Francisco, CA) and restriction analysis.

The E. coli XL-1 Blue cells containing the pCWori plasmid and expressing P450 eryF were grown overnight in 2× YT medium with 100 μg/ml ampicillin. One liter of 2× YT with 100 μg/ml ampicillin was inoculated with 5 ml of the saturated overnight culture and grown in a 2.8-liter flask at 37 °C to an A<sub>600</sub> of 1.0 (5–6 h). 3-α-Androstanediol was added to a final concentration of 80 μg/ml, and the cells were grown at 37 °C for another 1.5 h. The temperature was then lowered to 30 °C, and isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. The cells were grown for an additional 18 h.

The cells were harvested by centrifugation at 10,000 × g for 5 min, resuspended, and then lysed by stirring for 1 h at 4 °C in 50 mM Tris (pH 7.5) buffer containing 1 mM EDTA and 2 mM/ml lysozyme. The cells were sonicated on ice for 5 min (1 min on, 1 min off, 50% power), and the cell debris was removed by centrifugation at 45,000 × g for 20 min. The cell-free extract was loaded onto a 10-ml Ni<sup>2+</sup>-NTA-agarose (Qiagen) 2.5-cm-diameter column that had been equilibrated with 50 mM Tris (pH 7.5) buffer containing 0.5 mM NaCl and 10 mM imidazole (binding buffer). The column was then washed with 10 volumes of binding buffer followed by 50 mM Tris (pH 7.5) buffer containing 0.5 mM NaCl and 20 mM imidazole (wash buffer). The protein was eluted by washing the column with 10 volumes of 50 mM Tris (pH 7.5) buffer containing 0.5 mM NaCl and 0.5 mM imidazole (eluant buffer). The protein was >90% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Coomassie Blue staining and was dialyzed against storage buffer (50 mM Tris, 1 mM EDTA, and 10% glycerol) overnight. The enzyme was divided into aliquots and was stored at 70 °C. The yield was 80 mg/liter culture.

**Site-directed Mutagenesis**—The P450<sub>eryF</sub> A245T, A245S, and A245V mutants were constructed using the QuickChange site-directed mutagenesis kit (Stratagene) with the wild-type P450<sub>eryF</sub>-(His)<sub>6</sub> gene as the template. The specific base substitutions of GCG were ACC for the A245T, TCT for the A245S, and GTT for the A245V mutant. The sequence of the mutant was confirmed by DNA sequencing, and the plasmid was transformed into competent E. coli XL-1 Blue cells. The protein was expressed and purified under the same condition as the wild-type protein. The yields were 38, 30, and 12 mg/liter of culture for the A245T, A245S, and A245V mutants, respectively.

**Steady-state Kinetic Measurements**—The steady-state kinetic constants were determined by the HPLC method (20). The reaction mixture contained 10 μM A245T P450<sub>eryF</sub> and 5 μM H<sub>2</sub>O<sub>2</sub>, and testosterone (55–700 μM). At selected times, aliquots were quenched with 340 units of catalase, the reaction mixture was extracted with CH<sub>2</sub>Cl<sub>2</sub>, and the organic layer was evaporated to dryness at room temperature under a stream of argon. The dried sample was dissolved in the mobile phase before loading onto the HPLC column. The HPLC analysis was performed on a Hewlett-Packard HP 1090 liquid chromatograph equipped with a C-18 reverse-phase column (Beckman Ultrasphere, 5 μm, 4.6 × 250 mm). The column was eluted isocratically with 70% methanol containing 0.1% triethanolamine (pH adjusted to 7.0). The flow rate was 1.0 ml/min, and the UV detector was set at 254 nm. The retention time for testosterone under these conditions was 10.2 min.

Catalysis by P450<sub>eryF</sub> and its A245T mutant in the presence of electron transport proteins was monitored using an HPLC-based fixed-time assay. The conditions were as follows: 10 μM P450, 20 μM ferredoxin, 5 μM NADP<sup>+</sup>, 200 μM NADH, 1.7 mM glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, 170 units of catalase, and 100 units of superoxide dismutase in a total volume of 200 μl. The mixtures were incubated at 25 °C for 2 h before they were quenched and subjected to HPLC analysis.

**Spectroscopic Measurement of Ligand Binding Constants**—The binding constants of wild-type P450<sub>eryF</sub> and its A245T mutant toward testosterone were determined using difference UV-visible spectroscopy at 25 °C in 50 mM Tris buffer (pH 7.5) containing 10 mM EDTA. The enzyme concentration was 2 μM for wild-type P450<sub>eryF</sub> and 0.85 μM for the A245T mutant. The reference spectrum was subtracted from the spectrum measured after the addition of testosterone concentrations ranging from 166 to 990 μM. The difference between the peak at 390 nm and the trough at 416 nm was used to calculate the spectroscopic dissociation constant K<sub>D</sub> (see below).

**Identification of Testosterone Metabolites**—HPLC, TLC, mass spectrometry, and chemical oxidation were used to identify the testosterone metabolites. For mass spectrometric analysis, the testosterone metabolites were separated by HPLC on a preparative C-18 reverse-phase column, the products were collected and dried, and the samples submitted for mass spectrometric analysis by the UCSF Biomolecular Mass Spectrometry Facility. For HPLC analysis, the hydroxylated testosterone standards were profiled by C-18 reverse phase column HPLC with the following gradient: 50% solvent B to 100% solvent B in 30 min, followed by 100% solvent B for 10 min, where solvent A is H<sub>2</sub>O and solvent B is 50% (v/v) 2-propanol in water. The flow rate was 1.0 ml/min, and the UV detector was set at 254 nm. Authentic hydroxylated testosterone standards were compared with the testosterone metabolites by co-elution on HPLC using the same elution conditions. Finally, in the case of TLC, the authentic standards with Jones reagent (chromic acid in 70% H<sub>2</sub>O) were used to identify the metabolites and the authentic standards with Jones reagent (chromic acid in 70% H<sub>2</sub>O) were used to identify the metabolites.
Testosterone Oxidation by P450<sub>eryF</sub>

sulfuric acid and water), which oxidizes dissubstituted alcohols to ketones (20).

Spectra of Reduced P450<sub>eryF</sub> and Its A245T Mutant—Complexed with Testosterone and 6-DEB in the Presence of CO—The UV-visible spectra of reduced wild-type P450<sub>eryF</sub> and its reduced A245T mutant complexed with 6-DEB were measured in the presence of CO. The incubations contained 2 μM P450, 40 μM 6-DEB for the wild-type and 10 μM 6-DEB for the mutants, 8 μM ferredoxin, 2 μM FNR, and 250 μM NADPH. The reduced CO spectra of the enzyme complexed with testosterone (392 μm) were similarly measured.

Isotope Effects on the Oxidation of Testosterone by the P450<sub>eryF</sub>, A245T Mutant—The turnover rate for testosterone hydroxylation catalyzed by the A245T mutant was measured in parallel in H<sub>2</sub>O and D<sub>2</sub>O buffers. The reaction mixture contained 10 μM A245T, 10 mM H<sub>2</sub>O<sub>2</sub>, and 30–540 μM testosterone in 50 mM Tris buffer, pH 7.5 (pD 7.1), and the reaction was carried out at 25 °C (21). Aliquots were taken at 2, 4, 6, and 8 min, and the reaction was quenched with 340 units of catalase. The unreacted substrate and products were extracted into CH<sub>2</sub>Cl<sub>2</sub>, and were analyzed on a C-18 reversed phase column as described earlier.

Data Analysis—For steady state kinetic analyses, initial velocities were calculated from the slopes of the plots of product or substrate concentration versus reaction time. The kinetic constants were calculated using Equation 1 and the Enzyme Kinetics suite of programs (22).

\[ v = V_{max}A/[K_m + A] \]  
\[ (\text{Eq. 1}) \]

[<i>A</i>] is the substrate concentration, \( V_{max} \) is the initial velocity, \( K_m \) is the maximum velocity, and \( K_a \) is the Michaelis constant. The \( k_{cat} \) was calculated from the \( V_{max} \) and the enzyme concentration was calculated from the difference absorbance of the reduced P450-CO complex at 450 nm versus 490 nm (\( \Delta A = 91 \text{ nm}^{-1} \text{ cm}^{-1} \), values for P450<sub>cam</sub> (23)).

The Hill coefficient was determined both by nonlinear regression fit (KaleidaGraph, Abelbeck Software) of the data to the Hill equation (Equation 3) and by a linear fit of the data to the logarithmic form of the Hill equation (Equation 4).

\[ \log[v/(V_{max} - v)] = n \log S - \log K' \]  
\[ (\text{Eq. 2}) \]

\[ n \] is the number of substrate binding sites per enzyme active site, and \( K' \) is a constant comprising interaction factors and intrinsic dissociation constants.

For spectral binding assays, the binding constant \( K_a \) was calculated using Equation 4.

\[ \Delta A = \Delta A_{max}[A]/(K_a + [A]) \]  
\[ (\text{Eq. 4}) \]

\[ [A] \] is the substrate concentration, \( \Delta A \) is the absorbance change upon substrate binding, \( \Delta A_{max} \) is the maximum absorbance change, and \( K_a \) is the dissociation constant.

For analysis of substrate inhibition, nonlinear regression (KaleidaGraph, Abelbeck Software) was used to fit the data to a modified two-substrate binding equation (Equation 5) (24).

\[ v/F_s = V_{max}/1 + K_{nu}S + [S]/K_{cat} \]  
\[ (\text{Eq. 5}) \]

\( K_{cat} \) and \( K_{nu} \) are the \( K_a \) values for the reactions of E and the ES complex, respectively, with the substrate \( S \).

RESULTS

Expression and Characterization of the P450<sub>eryF</sub> A245T, A245S, and A245V Mutants—P450<sub>eryF</sub> in the original pKOS180–125e vector was expressed in E. coli DH5α cells, and the protein was purified using Q-Sepharose, Mono Q, and Superose 6 chromatographies in a yield of 2.9 mg/1 liter of culture (2). In order to improve the expression level and to simplify the purification, we put a sequence coding for a 6-His tag at the N terminus of the gene and subcloned the gene into the pCWori<sup>+</sup> vector. By adding δ-aminovalinic acid before the isopropyl-1-thioline, the turnover rate of the engineered strain was improved greatly, so that we obtained 80 mg of protein/liter of culture. The activity of wild-type P450<sub>eryF</sub>-(His)<sub>6</sub> toward 6-DEB, measured as reported previously (9, 10, 25), showed that the turnover rate of the tagged protein was comparable to that reported earlier for the untagged protein (103 min⁻¹) (2). The expression levels of the A245T, A245S, and A245V mutants were somewhat lower than that of the wild-type protein. The purified A245T protein had an A<sub>118/280</sub> ratio of 1.63 and an absorption maximum at 449 nm when reduced with sodium dithionite under an atmosphere of CO (Fig. 2). The absorption maxima of the A245S and A245V Fe<sup>2+</sup>-CO complexes were also at 449 nm (data not shown). The protein concentration was estimated using the extinction coefficients reported for the P450<sub>cam</sub> reduced CO-bound form relative to the base-line absorbance at 490 nm (23). The absorption spectra of the ferric A245T, A245S, and A245V mutants had a maximum at 418 nm that shifted to a small extent to approximately 392 nm when 6-DEB was bound, a spectroscopic shift characteristic of a low to high spin shift similar, albeit smaller, than that observed when 6-DEB binds to wild-type P450<sub>eryF</sub> (10).

Alternative Substrates: Oxidation of Testosterone—The binding of testosterone to P450<sub>eryF</sub> and its A245T, A245S, and A245V mutants was determined by difference spectroscopy, a method that reflects the spin state change that accompanies the binding of most ligands within the P450 active site (data not shown). The spectroscopic dissociation constants for the binding of testosterone to P450<sub>eryF</sub> and its A245T and A245S mutants were nominally 410, 560, and 1500 μM (Table I). No binding of testosterone to the A245V mutant was spectroscopically detected. Due to the limited water solubility of testosterone, \( K_a \) values greater than 200 μM, such as these, are approximate. These values are to be compared with the binding of 6-DEB, the natural substrate, which exhibits a \( K_a \) value of 2 μM (2).

The oxidation of testosterone by P450<sub>eryF</sub> has been evaluated using two catalytic systems, one supported by spinach ferredoxin and FNR, and the other by H<sub>2</sub>O<sub>2</sub>. Spinach ferredoxin and FNR have been used as surrogates for the endogenous electron donor partners of P450<sub>eryF</sub> (9). The catalytic rate for the oxidation of 6-DEB by wild-type P450<sub>eryF</sub> using the spinach ferredoxin system is reportedly 103 min⁻¹ (2), but the enzyme has no detectable ferredoxin-dependent testosterone oxidation activity (Table I). The wild-type enzyme also does not support the H<sub>2</sub>O<sub>2</sub>-dependent oxidation of testosterone, as only a negligible turnover of < 0.01 min⁻¹ was observed. In contrast, testosterone is oxidized by the P450<sub>eryF</sub> A245T and A245S mutants using either O<sub>2</sub>/ferredoxin/FNR or H<sub>2</sub>O<sub>2</sub> as the source of oxidizing equivalents (Fig. 3). The rates of these reactions were estimated by integrating the HPLC areas of the substrate and the four principal products (see below) as a function of time (Table I). For the A245T mutant, these rates were 0.48 and 0.0027 min⁻¹, respectively, for the H<sub>2</sub>O<sub>2</sub> and ferredoxin-dependent reactions, and for the A245S mutant they were 0.30...
Identification of Testosterone Metabolites—HPLC analysis shows that four major products are formed in the H$_2$O$_2$-dependent oxidation of testosterone by the A245T mutant. The identities of the four metabolites were established by comparison with authentic standards by both reverse-phase (RP-18) and normal-phase silica gel TLC. In contrast, similar analysis by reverse- and normal-phase silica gel TLC ruled out identification of both compound A as either 6- or 19-hydroxytestosterone and compound D as 2α-hydroxytestosterone. Oxidation of the hydroxyl groups of compounds A and D to keto groups with Jones reagent ruled out the identification of either one of them as 15β-hydroxytestosterone because the chemical oxidation products of these compounds did not coelute on TLC with that from similar oxidation of 15α-hydroxytestosterone. Furthermore, the products from the Jones oxidation of A and D had different $R_f$ values on thin layer chromatography, indicating that they were hydroxylated on different carbons. As standards are available for all the other ring and methyl monohydroxylated testosterone, none of which co-eluted with metabolite A or D, one of these two compounds must be an isomer of 1-hydroxytestosterone and the other an isomer of 12-hydroxytestosterone, the only positions for which standards were not available. The sites of hydroxylation of testosterone are indicated in Fig. 4.

Product Profiles—The product profile of the reaction supported by ferredoxin/FNR differs from that supported by H$_2$O$_2$. The ratio of the four metabolites produced by the A245T mutant with H$_2$O$_2$ is A:B:C:D = 1:0.48:0.20:0.13 (Fig. 3), and with ferredoxin/FNR it is 1:0.48:0.20:0.13 (Table I), and for the ferredoxin/FNR reaction is 1:1:0:0. The zero in these ratios indicates not detectable. These ratios show that differences exist in the regiochemistry of testosterone oxidation supported by the two mutants and the two oxidizing systems. Differences in the ratios due to a threonine or serine substitution are not unexpected, given that the two amino acid side chains differ in size and therefore occlude different active site volumes, although the differences could also reflect differences in the mechanism of hydroxylation by the two catalytic systems. The more interesting differences in the product ratios are those between the reactions supported by H$_2$O$_2$ and ferredoxin/FNR, as the latter give exclusively metabolites A and B. One possible explanation for these differences is that P450$_{eryF}$ undergoes a conformational change when it binds ferredoxin that favors one testosterone orientation over another. To examine this possibility, the testosterone metabolite profile produced by the A245T mutant in the presence of H$_2$O$_2$, ferredoxin, and FNR (but not NADPH) was determined and compared with the profile formed with H$_2$O$_2$ alone. The two product profiles were essentially identical, indicating that the binding of oxidized ferredoxin is not responsible for the differences in product profiles. The catalytic complexes formed by reduction of O$_2$ versus reaction with H$_2$O$_2$ thus differ in ways that influence the substrate hydroxylation regiochemistry.
The experimental data suggest the existence of more than one substrate-binding mode for the P450<sub>eryF</sub> A245T mutant. In the H<sub>2</sub>O<sub>2</sub>-dependent testosterone hydroxylation reaction catalyzed by this enzyme, the rate of formation of metabolite D is faster than that of metabolite A at low testosterone concentrations, but at high testosterone concentrations (E:S molar ratio > 1:30), compound A is formed faster than compound D (Fig. 5). This is consistent with a large active site that, for example, can accommodate two substrate molecules and favors the two-versus one-substrate-bound form as the substrate concentration is increased. We have searched for independent spectroscopic evidence for the existence of two substrate-bound states of the P450<sub>eryF</sub> A245T mutant, the first containing one and the second two substrate molecules. However, even at a low enzyme:substrate ratio of 1:1, no spectroscopic break indicative of two distinct substrate binding events has been detected. This is true despite the low spectroscopically determined affinity (K<sub>d</sub> = 560 μM) of the P450<sub>eryF</sub> A245T mutant for testosterone. It is to be noted, however, that, despite a similarly large spectroscopic binding constant for the binding of androstenedione to wild-type P450<sub>eryF</sub> (K<sub>d</sub> = 356 μM), two molecules of androstenedione are bound in the active site at the low 1:2 molar ratio of enzyme to steroid used for the crystal structure (6).

Analysis of the spectroscopic titration curve for the binding of testosterone to the A245T mutant by the Hill equation yielded a Hill coefficient of 1.5 indicative of positive cooperativity (Fig. 6D). The cooperativity determined from the H<sub>2</sub>O<sub>2</sub>-dependent testosterone hydroxylation kinetics is more complicated. (a) The formation of metabolite A displays no cooperativity (Hill coefficient = 1.1 ± 0.1) (Fig. 6A); (b) at low substrate concentration, the formation of compound D displays positive cooperativity (Hill coefficient = 1.4 ± 0.1) (Fig. 6B), but (c) at high substrate concentration (>500 μM) substrate inhibition is observed for the formation of metabolite D (K<sub>i</sub> = 390 ± 160 μM). Overall, the substrate consumption rate displays positive cooperativity, with a Hill coefficient of 1.3 ± 0.1 (Fig. 6C), in agreement with the cooperativity indicated by the spectroscopic binding plot. The data suggest that the binding of one sterol facilitates the binding of a second, so that single occupancy of the site may be disfavored.

**Reduction CO Spectra of P450 Complexed with 6-DEB and Testosterone**—To determine whether electrons are transferred from reduced ferredoxin to the P450 enzyme, P450<sub>eryF</sub> and its A245T and A245S mutants were incubated with ferredoxin, FNR, NADPH, and testosterone under an atmosphere of CO. The 450 nm peaks obtained with all three proteins are weak, indicating that electron transfer to the proteins in the presence of testosterone is inefficient (Fig. 7). As a control, the CD spectrum was obtained in a similar experiment with the P450<sub>eryF</sub> A245T mutant in which testosterone was displaced after the start of the experiment by the addition of 6-DEB (Fig. 8). The 450-nm peak appeared instantly and gradually reached a higher maximum. 6-DEB thus has a higher ability to promote electron transfer from ferredoxin/FNR to the heme iron atom. This is consistent with the observation from the substrate binding studies that the binding of 6-DEB causes a much greater shift from low to high spin than does that of testosterone. This finding also supports the possibility that the multiplicity and orientation of testosterone binding may influence the efficiency of the electron transfer, and therefore product distribution.

**Isotope Effects on the Oxidation of Testosterone by the P450<sub>eryF</sub> A245T Mutant**—The turnover rate for testosterone hydroxylation catalyzed by the A245T mutant was measured in parallel in normal and deuterated buffers (Fig. 9). The kinetic constants averaged from two independent experiments using the Michaelis-Menten equation are shown in Table III. The kinetic solvent isotope effect is significant (KSIE = 2.2), indicating that a proton transfer step is at least partially rate-limiting. The magnitude of the KSIE is consistent with the model proposed for P450<sub>cam</sub> (KSIE = 1.5–2.1) involving a hydrogen bonding network with a threonine hydroxyl group and an active site water molecule (15, 26). In addition to the KSIE effect, the K<sub>m</sub> measured in deuterated buffer is 4–7-fold smaller than in normal buffer. This large difference in K<sub>m</sub> is unexpected and could, in principle, reflect a change in the protein conformation in going from H<sub>2</sub>O to D<sub>2</sub>O. However, the CD spectra of the A245T mutant in 5 mM KPi buffer (proton and deuterium solution, pH 7.5 and pD 7.1) were identical, indicating that no major conformational change was associated with the change from H<sub>2</sub>O to D<sub>2</sub>O (data not shown). Furthermore, the spectroscopically determined constants for the binding of testosterone to the A245T mutant in H<sub>2</sub>O and D<sub>2</sub>O buffer were similar (560 μM in H<sub>2</sub>O and 615 μM in D<sub>2</sub>O), which indicates that there is no significant structural difference in the active site in H<sub>2</sub>O and D<sub>2</sub>O. The reason for the apparent lower K<sub>m</sub> value in D<sub>2</sub>O is unclear.

**Discussion**

The general sequence of steps for the catalytic turnover of P450 enzymes involves: (a) substrate binding with concomitant low to high spin shift of the ferric iron, (b) electron transfer to give the ferrous protein, (c) binding of O<sub>2</sub> to form the ferric dioxy (Fe<sup>2+</sup>O<sub>2</sub>) complex, (d) transfer of a second electron to give a ferric peroxy (Fe<sup>3+</sup>OOH) complex that is converted by O-O bond scission to a ferryl (formally Fe<sup>V</sup>+O<sub>2</sub>) complex, (e) hydrogen atom abstraction from the substrate, followed by radical recombination to give the hydroxylated product, and (f) dissociation of the hydroxylated product with return of the enzyme to the resting ferric state (11, 27). This catalytic mechanism requires the uptake of two protons that are eventually incorporated into a molecule of water. Although it is supported by work with many P450 enzymes, this mechanism is largely based on studies of P450<sub>cam</sub> by the groups of Gunsalus, Sligar,
Ishimura, Poulos, and others. These P450 cam studies culminated recently in determination of the crystal structures of (a) the ferrous dioxy complex, (b) a less well defined species that may be the ferryl intermediate, and (c) the enzyme-product complex (12). Although the putative ferric peroxide intermediate was not detected, the crystal structures provide a partial cinematic view of the structural changes associated with catalysis.

Formation of the Fe$^{2+}$-CO complexes of the A245T, A245S, and A245V mutants occurs only to a limited extent when the proteins are incubated with testosterone and ferredoxin/FNR in the presence of CO (Fig. 7). In contrast, rapid formation of the ferrous-CO complex is observed when 6-DEB displaces testosterone from the A245T active site (Fig. 8). These results indicate that the binding of testosterone does not satisfy all the requirements for efficient electron transfer to the heme iron atom. The fact that testosterone only induces a modest low to the high spin shift of the iron provides a partial explanation for this inefficient electron transfer. Furthermore, differences in the water content of the 6-DEB- and testosterone-bound states are likely to also alter the reduction potential of the iron (28).

Due to conformational changes associated with oxygen binding, Thr$^{202}$ of P450 cam is hydrogen-bonded to both an ordered water molecule and the distal oxygen atom (12). This finding is
consistent with the observation that most mutations of Thr252 result in uncoupling of the enzyme and loss of catalytic activity (13–15). A decrease in substrate hydroxylation and increase in uncoupling are also observed when the conserved threonine is mutated in some but not all P450 enzymes (29–34). The catalytic role for the conserved threonine is supported by the crystallographic data on the P450cam ferrous dioxy complex, but, in the catalytic arena, the role of the threonine is more ambiguous and rests primarily on loss-of-function data in which its mutation provides strong support for the conclusion that the iso- 

Aikens and Sligar (15) demonstrated that the only step in the P450cam catalytic sequence that exhibits a significant KSIE is the second electron transfer associated with ferryl complex formation and substrate hydroxylation. Camphor hydroxylation was shown to exhibit single and multiple turnover KSIE of 1.8 and 1.5, respectively. The observation that the second electron transfer gives rise to a measurable KSIE suggests that this electron transfer is coupled to protonation of the iron-bound dioxygen. The calculated KSIE, allowing for the two protons shown by a proton inventory to be involved in catalysis, is 2.1 (15). However, these studies do not distinguish between an isotope effect on O–O bond breaking versus some other aspect of the reactions linked to the second electron transfer. We have determined the KSIE for the turnover of testosterone by the P450eryF A245T mutant using H2O2 as the oxidizing agent and have obtained a KSIE of 2.2 (Table III), a value in agreement with those for the O2/putidaredoxin/putidaredoxin reductase-dependent turnover of P450cam (15, 26). This agreement provides strong support for the conclusion that the isotope effect occurs during the O–O bond cleavage step. Furthermore, the similarity in KSIE values argues that the role of Thr245 in the P450eryF A245T mutant is similar to that of Thr252 in P450cam.

The H2O2-dependent hydroxylation of testosterone by the P450eryF A245T and A245S mutants yields at least four products (Fig. 3). Metabolites A and D are 1- and 12-hydroxytestosterone, although which is which is not known, and metabolites B and C are 16α- and 11α-hydroxytestosterone, respectively (Fig. 4). The product profiles differed, however, depending on whether the A245T or A245S mutant was used, and whether the reaction was supported by H2O2, or ferredoxin/FNR. The possibility that binding of oxidized ferredoxin causes an active site conformational change is excluded by the finding that the product profile is the same with H2O2 alone as with H2O2 in the presence of ferredoxin/FNR but no NADPH. A conformational change could still be mediated by the binding of reduced ferredoxin, but this would probably not be relevant because the ferredoxin would be oxidized in the oxygen activation step that precedes substrate hydroxylation. The probable explanation for the differences in product profiles is that they are due to the greater ability of some testosterone complexes than others to initiate electron transfer and catalysis. Electron transfer to the iron imposes a distinct set of conditions on the catalytic turn- 

In the 2.1-Å x-ray structure of P450eryF complexed with androstenedione (6), two molecules of the steroid are found within the active site. The two androstenedione molecules are positioned in parallel, one above the other, with their 3-keto groups facing in the same direction, their β-face toward the heme, and their sterol A and B rings above the porphyrin ring. The more distant androstenedione molecule is ~5.5 Å farther from the heme than the closer one. The carbon on the androstenedione closest to the heme is C-1, which is 3.8 Å from the iron atom, and the distances of the C-11 and C-12 carbons from the iron are 4.93 and 6.10 Å, respectively. For comparison, the position of 6-DEB that is hydroxylated in the P450cam complex is 4.8 Å from the iron atom. Our data on the regiospecificity of testosterone hydroxylation by the A245T mutant are consistent with the crystal structure of the androstenedione complex, as C-1 is one of the primary testosterone hydroxylation sites. Testosterone must translate or rotate within the active site to form the four metabolites, but it is difficult to explain the
formation of 16α-hydroxytestosterone without invoking a completely different binding mode for the sterol. Thus, it is possible for the sterol to also bind in an orientation different from that in the crystal structure, for one rather than two sterols to bind (see below), and for rapid substrate motion to occur within the P450 active site (36).

The substrate dependence of the metabolite profiles (Fig. 5) obtained in the \( \text{H}_2\text{O}_2 \)-dependent oxidation of testosterone by the A245T mutant suggests, in fact, that two testosterone molecules are bound in the active site. This interpretation is supported by the observation of positive cooperativity, as indicated by a Hill coefficient of 1.5 in binding of the sterol to the protein and 1.3 in the kinetic consumption of the sterol (Fig. 6). Homotopic cooperativity could explain the dependence of the rate of formation of metabolites A and D on the testosterone concentration (Fig. 5). Korzekwa et al. (24) have proposed a two-substrate model for analyzing atypical kinetics in which both the ES and ESS complexes can form products. However, this two-substrate kinetic model does not give a good fit of the substrate saturation curve for the hydroxylation of testosterone, including the substrate inhibition, unless it is assumed that the rate of oxidation of the ESS complex is negligible. This suggests that at high substrate concentrations the probability of forming ineffective complexes with two or more substrate molecules within the active site increases, resulting in substrate inhibition.

CYP3A4 catalyzes the 2β-, 6β-, and 15β-hydroxylation of testosterone (37). The active site of a CYP3A4 homology-based model has been reported to resemble that of P450eryF (38). Interestingly, CYP3A4 and other mammalian P450 enzymes are subject to homo- and heterotropic cooperativity (39-42). This cooperativity is of interest due to its effect on drug pharmacokinetics. In the case of CYP3A4, Halpert and co-workers have identified residues that may be involved in the binding of effectors. Mutation of these residues to larger amino acids that decrease the size of the binding pocket mimics the influence of effector binding on catalysis, abolishing cooperativity. This result suggests the coexistence of two binding sites that give rise to cooperativity and non-hyperbolic oxidation kinetics. The Korzekwa kinetic model has been used to analyze (a) the sigmoidal kinetics observed for the metabolism of carbamazepine by CYP3A4, naphthalene by CYP2B6, CYP2C8, CYP2C9, and CYP3A5, and dapsone by CYP2C9; (b) the nonhyperbolic kinetics observed for the oxidation of naphthalene by CYP3A4 and naproxen by CYP2C9; and (c) the activation by 7,8-benzoflavone of phenanthrene metabolism by CYP3A4 (24). Domanski et al. (43) described sigmoidal kinetics for CYP3A4 toward progesterone in the absence of \( \alpha \)-naphthoflavone, using a modified two-site model equation where the rate of oxidation of the ES, as opposed to ESS, complex is zero. The availability of a crystallographically defined system in which the binding of two substrates can be clearly demonstrated and analyzed should help to elucidate the properties expected of P450 enzymes, such as CYP3A4, in which such binding is thought to occur.

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