Identification of Three Key Residues in Substrate Recognition Site 5 of Human Cytochrome P450 3A4 by Cassette and Site-Directed Mutagenesis[†]

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ABSTRACT: Cassette mutagenesis and site-directed mutagenesis were used to investigate the importance of individual amino acid residues at positions 364−377 of cytochrome P450 3A4 in determining steroid hydroxylation or stimulation by α-naphthoflavone. The mutants were expressed in an *Escherichia coli* system, and solubilized membranes were prepared. All mutants except R365G and R365K exhibited anti-3A immunoreactivity on Western blotting, although R372S and R375K were not detected as the Fe^{2+}−CO complex. Replacement of Arg-372 by Lys yielded a typical P450 spectrum. The results indicate that the highly conserved Arg residues at positions 365 and 375 may play a role in stabilizing the tertiary structure or in heme binding. Catalytic activities of 12 mutants were examined using progesterone and testosterone as substrates, and residues 369, 370, and 373 were found to play an important role in determining substrate specificity. Although the three mutants hydroxylated progesterone and testosterone primarily at the 6*β*-position like the wild-type, replacement of Ile-369 by Val suppressed progesterone 16α-hydroxylase activity, whereas substitution of Ala-370 with Val enhanced progesterone 16α-hydroxylation. Interestingly, substitution of Leu-373 with His resulted in production of a new metabolite from both steroids. Moreover, the mutants at positions 369 and 373 were more and less responsive, respectively, than the wild-type to α-naphthoflavone stimulation. Alterations in activities or expression of several mutants were interpreted using a three-dimensional model of P450 3A4. The results suggest that analogy with mammalian family 2 and bacterial cytochromes P450 can be used to predict P450 3A residues that contribute to regiospecific steroid hydroxylation.

Cytochrome P450[1] 3A4 is the most highly expressed P450 in the liver of most humans (Guengerich, 1990) and biotransforms a vast array of clinically, physiologically, and toxicologically important compounds. Representative agents include drugs [reviewed in Guengerich (1995)], steroids (Waxman et al., 1988), and carcinogens (Shimada et al., 1989; Shimada & Guengerich, 1989). Although P450 3A4 can accommodate a wide variety of structurally diverse substrates, it still exhibits remarkable regio- and stereoselectivity with many compounds. For example, the enzyme catalyzes the 6*β*- and 16α-hydroxylation of progesterone and the 2*β*-, 6*β*-, and 15*β*-hydroxylation of testosterone (Waxman et al., 1991). Like other P450 3A enzymes, 3A4 also exhibits differential stimulation of certain catalytic activities by flavonoids such as α-naphthoflavone (α-NF) (Huang et al., 1981; Schwab et al., 1988; Shou et al., 1994; Ueng et al., 1995).

Despite the wealth of information on the importance, regulation, and substrate specificity of the cytochrome P450 3A subfamily, structure−function analysis of these enzymes has not been rigorously approached. Unlike cytochromes P450 of family 2, 3A enzymes within or across species exhibit few dramatic substrate specificity differences that could provide obvious leads for site-directed mutagenesis of particular residues. For example, human cytochromes P450 3A4 and 3A5 display 84% amino acid sequence identity and metabolize many of the same substrates. Only a few differences between 3A4 and 3A5, such as differences in the regiospecific metabolism of progesterone, testosterone, cyclosporin A (Aoyama et al., 1989), and midazolam (Wandel et al., 1994), have been identified. In addition, the location and identity of residues responsible for flavonoid stimulation remain unknown. Based on the various hypotheses proposed for the mechanism of action of α-NF, the compound may interact with residues close to or part of the substrate binding site or with distal residues not involved in substrate binding (Shou et al., 1994; Schwab et al., 1988; Ueng et al., 1995, 1997).

On the basis of comparative sequence analysis and analogy with bacterial P450 101, the existence of six substrate recognition sites (SRSs) within family 2 has been proposed (Gotoh, 1992). In particular, extensive work from this laboratory has provided experimental evidence for the existence in P450 2B1 of five out of the six predicted SRSs, the only exception being SRS-3 (He et al., 1994, 1995). The importance of the SRSs for the substrate specificity of P450 2A and 2C enzymes is also well established [reviewed in Von Wachenfeldt and Johnson (1995)]. Experimental studies of P450 family 2 enzymes are in excellent agreement with more theoretical analysis of bacterial P450 crystal structures, which suggests that SRSs can be predicted (Hasemann et al., 1995).

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[1] Abbreviations: P450, cytochrome P450; α-NF, α-naphthoflavone; SRS, substrate recognition site; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DOPC, dioleoylphosphatidylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TLC, thin-layer chromatography; PCR, polymerase chain reaction; -OH, hydroxy; IgG, immunoglobulin.

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A sequence alignment of P450 3A4 with four bacterial P450s of known 3-D structure has indicated the putative location of the SRSs in 3A4 (Szklarz & Halpert, 1997). However, the identification of key residues within these regions requires further study. A very recent study in this laboratory of SRS-2 of P450 3A4 provided the first evidence for the location of residues that influence flavonoid stimulation in a region shown to constitute part of the active site of several bacterial and mammalian cytochromes P450 (Harlow & Halpert, 1997). In the present study, cassette mutagenesis, which allows for construction of multiple mutants simultaneously, was chosen to analyze SRS-5. This SRS in P450 3A4 was suggested to encompass amino acid residues 367−376. A degenerate oligonucleotide, including SRS-5 and an additional four codons at the N-terminus and three at the C-terminus, was designed to mutate residues 363−379. Mutants I369V,[2] A370V, and L373H showed altered patterns of progesterone hydroxylation, and L373H also exhibited altered testosterone hydroxylation. Substitutions at positions 369 and 373 correlated with alterations in α-NF activation. The results localize 3A4 residues involved in steroid hydroxylation in a region predicted on the basis of bacterial P450 structures and an alignment with mammalian family 2 enzymes.

EXPERIMENTAL PROCEDURES

Materials. All primers except the degenerate oligonucleotide used for cassette mutagenesis were made by the University of Arizona Macromolecular Structure Facility (Tucson, AZ). The pKK233-2 *E. coli* expression plasmid was purchased from Pharmacia (Alameda, CA). Topp3 cells were purchased from Stratagene (La Jolla, CA). Growth media for bacteria were obtained from Difco (Detroit, MI). Restriction endonucleases, DNA modification enzymes, Taq DNA polymerase, and reagents for PCR were purchased from GIBCO-BRL (Grand Island, NY). The TA Cloning Kit was obtained from Invitrogen (San Diego, CA). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), NADPH, dioleoylphosphatidylcholine (DOPC), and α-naphthoflavone (α-NF) were purchased from Sigma (St. Louis, MO). [^{14}C]Progesterone and [^{14}C]testosterone were purchased from DuPont−New England Nuclear (Boston, MA). HEPES was obtained from CalBiochem Corp. (La Jolla, CA). TLC plates [silica gel, 250 μm, Si 250 PA (19C)] were purchased from J. T. Baker (Phillipsburg, NJ). All other reagents and supplies not listed were obtained from standard sources.

Modification of P450 3A4 cDNA. A modified 3A4 cDNA in pKK233-2 was created by site-directed mutagenesis for subsequent use in subcloning the degenerate oligonucleotide cassettes. The Transformer Site-directed Mutagenesis Kit (Clontech, CA) was used to destroy an undesirable *Aat*II site at amino acid positions 174−175 in the first step of mutagenesis using a 3A4 cDNA modified at the N-terminus (Harlow & Halpert, 1997) as the template. The mutagenic primer with the mutations underlined was 5′-CTTGAAAGATGTGTTTGGGGCCTACAGC-3′. Then, an *Hpa*I and an *Aat*II site were created by PCR. Fifty picomoles each of the upstream primer and the mutagenic primer (5′-ATAG-

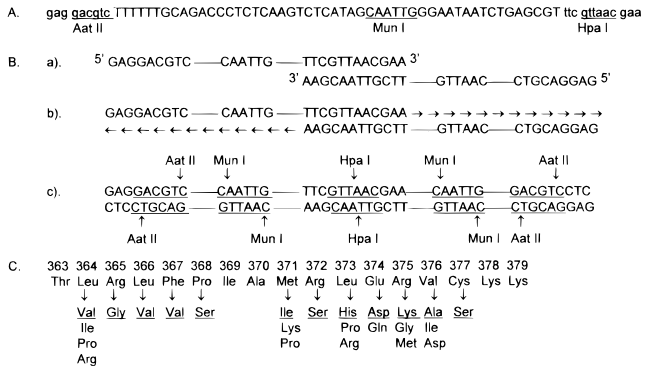

FIGURE 1: Cassette mutagenesis. (A) Degenerate oligonucleotide for mutagenesis of residues 363−379. The *Aat*II, *Mun*I, and *Hpa*I sites are underlined; the positions with capital letters were spiked with 0.9% of each of the other three nucleoside phosphoramidites (2.7% total) during synthesis. (B) Procedure for cassette mutagenesis. (a) The self-complementary region at the 3′ end was allowed to anneal to itself. (b) The second strand was synthesized by PCR. (c) The double-stranded product was digested with *Mun*I and *Hpa*I/*Aat*II. The fragments were inserted into the P450 3A4 cDNA in pKK233-2. (C) Single mutants created by cassette mutagenesis. The mutants studied in this report are underlined.

CAATTGGGAATAATCTGAGCGTTTCGTTAACCACC-ATGTC-3′), which included a unique *Mun*I site (in boldface), was used to amplify a 677 bp fragment containing codons 137−371. Reaction conditions were 1 cycle of 94 °C for 3 min, 50 °C for 3 min, and extension at 72 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min. The reaction product was cloned into the pCR vector (Invitrogen). Then the *Pst*I−*Mun*I fragment of P450 3A4 in pKK233-2 encoding residues 331−368 was replaced with the corresponding fragment from the PCR reaction in the pCR vector to create an *Hpa*I site at codons 360−361. Fifty picomoles each of the downstream and the mutagenic primer (5′-CC**CAATTG**CTATGAGACTTGAG-AGGGTCTGCAAAAAAGACGTCGAGATCAATGGGA-TG-3′), which had a *Mun*I site (in boldface), was used to amplify a 144 bp fragment including codons 368−414. The conditions were 1 cycle of 94 °C for 3 min, 55 °C for 3 min, and 72 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. The PCR product was initially cloned into the pCR vector. Then the *Mun*I−*Xcm*I fragment of P450 3A4 encoding residues 368−392 was replaced with the corresponding fragment from the PCR reaction in the pCR vector to create an *Aat*II site at codons 380−381.

Formation of the Double-Stranded Degenerate Cassette for Mutagenesis of Residues 363−379. A 72-base oligonucleotide, the complementary DNA of residues 360−381, was synthesized by Genemed Biotechnologies (San Francisco, CA) in order to mutate residues 363−379 of 3A4 by cassette mutagenesis (Figure 1A). During synthesis of positions 10 through 60, each nucleotide mixture contained 97.3% of the wild-type and 0.9% of each of the three other nucleotides. This composition of the mixture theoretically maximized the number of single mutants. The oligonucleotide also contained three extra nucleotides at the 5′ end, followed by an *Aat*II site. At the 3′ end, 12 nucleotides including an *Hpa*I site were self-complementary.

The degenerate oligonucleotide was converted to double-stranded form by PCR, using 25 pmol of degenerate oligonucleotide (Figure 1B). Reaction conditions were 10 cycles of 94 °C for 1 min, 37 °C for 1 min, a 10 min ramp to the extension temperature, and then 72 °C for 3 min. The

[2] Mutants are indicated using the single-letter code for the amino acid residue replaced, the position in the sequence, and the designation of the new residue, in that order (Johnson, 1992). For example, L373H refers to replacement of Leu at position 373 with His.

double-stranded degenerate cassette was initially cloned into the pNoTA/T7 plasmid (5 Prime → 3 Primer Inc.; Boulder, CO). The clones were sequenced by double-stranded sequencing using a Sequenase 2.0 Kit from U.S. Biochemical Corp. (Cleveland, OH).

Site-Directed Mutagenesis of Residues 365, 366, 369, 370, and 372. Mutagenesis of 3A4 residues 365, 366, 369, 370, and 372 was performed by PCR using the modified 3A4 cDNA, described under "Modification of P450 3A4 cDNA", as a template. All the oligonucleotides contained the unique restriction enzyme sites (in boldface) *Mun*I (CAATTG) or *Hpa*I (GTTAAC). The oligonucleotides with the mutated bases underlined, 5′-ATAG**CAATTG**GGAATAAC<u></u>TTGAGCGTTTCG-3′, 5′-CAC**GTTAAC**GAAACGCTCAGATTATTCCCAGTTGCTATGAC-3′, and 5′-TTCC**CAATTG**CTATGAAGCTTGAGAGGGT-3′, were made to mutate residues Arg-365, Ile-369, and Arg-372 to Lys, Val, and Lys, respectively. The oligonucleotide 5′-CATAG**CAATTG**GGAA*AGATCT*GAGCG-3′ was made to mutate Leu-366 to Ser and to create a unique *Bgl*II site (in italics), and the oligonucleotide 5′-C**CAATTG**TTATGCG*TCTAGA*GAGGGTCTG-3′ was synthesized to change Ala to Val at position 370. The silent mutations AGA → CGT and CTT → CTA at positions 372−373 introduced a unique *Xba*I site (in italics). The PCR reaction conditions for L366S, I369V, and A370V were 1 cycle of 94 °C for 3 min, 37 °C for 3 min, and 72 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 2 min. The reaction conditions for R365K and R372K were 1 cycle of 94 °C for 3 min, 50 °C for 3 min, and 72 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. The fragments from the PCR reaction were ligated into the pCR vector. R365K and L366S were constructed in pKK233-2 by replacing a 108 bp *Pst*I−*Mun*I fragment including codons 332−368 from the modified P450 3A4 cDNA with the corresponding fragment from R365K or L366S. A 497 bp *Hpa*I−*Kpn*I fragment containing codons 361−503 and a 468 bp *Mun*I−*Kpn*I fragment containing codons 368−503 from the modified 3A4 cDNA were substituted with the corresponding fragments from I369V and A370V to construct the mutated plasmids, respectively. Construction of R372K was performed by replacing a 39 bp *Mun*I−*Aat*II fragment including codons 368−381 from A370V in pKK233-2 with the corresponding fragment from R372K. The entire mutated fragments generated by PCR were sequenced to ensure that only the desired mutations were present.

Insertion of the Cassette Mutants. The single mutants at positions 364−368 were constructed in the pKK233-2 vector by replacing a 23 bp *Hpa*I−*Mun*I fragment including codons 361−368 from L366S with the corresponding fragment from the mixed cassette mutants in the pNoTA/T7 vector. Construction of the mutants at positions 371−377 was performed by replacing a 39 bp *Mun*I−*Aat*II fragment containing codons 369−380 from A370V with the corresponding fragments from the mixed mutated cDNA in the pNoTA/T7 vector. The cassette regions were confirmed by sequencing.

Heterologous Expression. *E. coli* Topp3 cells were used for P450 expression. Methods for expression and solubilization of membranes were as described previously (John et al., 1994). Two independent solubilized membrane preparations were made for each mutant.

Steroid Hydroxylase Assays. The catalytic activities of cytochrome P450 3A4 wild type and mutants were examined in a reconstituted system. *E. coli* solubilized membrane fractions were preincubated at 25 °C for 10 min with 2 equiv of rat liver NADPH−cytochrome P450 reductase and DOPC (0.1 mg/mL). Aliquots were then incubated with 25 μM [^{14}C]progesterone or testosterone in 0.1 mg/mL DOPC, 0.05% CHAPS, 2 equiv of rat liver cytochrome b_5, and 50 mM HEPES buffer (15 mM $MgCl_2$, 0.1 mM EDTA, pH 7.6) with or without 25 μM α-NF. NADPH (1 mM) was added, and samples were incubated for 10 min at 37 °C. Metabolites were resolved on TLC plates by three cycles of chromatography in benzene/ethyl acetate/acetone (10:1:1,v/v/v) for progesterone metabolites or two cycles of chromatography in dichloromethane/acetone (4:1, v/v) for testosterone metabolites.

Immunochemical Methods. SDS−polyacrylamide gels (7.5%) were run as described (Laemmli 1970). Transfer of proteins and immunodetection using anti-3A12 IgG were performed as described (Kedzie et al., 1991; Ciaccio & Halpert, 1989).

Computer Modeling. A 3-D molecular model of cytochrome P450 3A4 constructed previously (Szklarz & Halpert, 1997) was used to dock progesterone in the active site. The structures were displayed on a Silicon Graphics workstation using InsightII software (Biosym/MSI, San Diego, CA). Energy minimization and molecular dynamics simulations were carried out with the Discover program (Biosym/MSI) using the consistent valence force field. The parameters for heme and ferryl oxygen were those described by Paulsen and Ornstein (1991, 1992). Progesterone was placed in the active site of the P450 3A4 model in a 16α binding orientation with the oxidation site fixed at about 5.6 Å from the heme iron, and the $C_{16}-H_{16\alpha}$ bond aligned with ferryl oxygen, heme iron, and sulfur of Cys-442, as described earlier (Szklarz et al., 1995). This results in a hydrogen-bonding distance between the ferryl oxygen and the hydrogen atom to be abstracted from the substrate. Docking of progesterone was performed using molecular dynamics, as described previously (Szklarz & Halpert, 1997). For these simulations, C_{16} and $H_{16\alpha}$ atoms of the substrate were fixed, while the rest of the molecule, along with the side chains of protein residues within 5 Å from the substrate, was allowed to move. Initially, the system was minimized using the steepest descent method and harmonic potential, with a nonbond cutoff of 10 Å, to a maximum gradient of 5 kcal mol^{-1} $Å^{-1}$. For the subsequent molecular dynamics simulations, the leap-frog algorithm was used, the system was equilibrated for 0.1 ps, and the simulations were continued for 1 ps at 300 K using 1 fs time steps. The system was then minimized again using conjugate gradients to a maximum gradient of 1 kcal mol^{-1} $Å^{-1}$, first the substrate molecule only, and then the side chains of protein residues neighboring docked progesterone. The nonbond interaction energy, both electrostatic and van der Waals forces, between the substrate and the enzyme was evaluated with the Docking module of the InsightII package.

RESULTS

Mutagenesis Strategies. As described under Experimental Procedures, a 72-base oligonucleotide containing random mutations at positions 10 through 60 was synthesized in order to mutate the P450 3A4 cDNA in the region corresponding to residues 363−379 (Figure 1A). The 5′ end of the oligonucleotide contained an *Aat*II site. The 12-base 3′ end,

containing an *Hpa*I site, was designed for self-annealing. The annealed region served as the primer and the rest of the oligonucleotide as the template for second-strand synthesis, as shown in Figure 1B (Hill et al., 1987). The double-stranded DNA, including two copies of a degenerate nucleotide sequence, was inserted into the pCR vector. At each degenerate position, the nucleotide mixture used contained 97.3% wild-type and 0.9% of each of the other nucleotides, which should have produced 33.5% wild-type and 37.9% single, 20.2% double, 6.6% triple, and 1.3% quadruple mutants. Based on DNA sequencing of 28 colonies, the wild-type, single, double, triple, and quadruple mutants constituted 18%, 39%, 25%, 11%, and 7% of the colonies characterized (disregarding deletions and frameshifts), respectively, close to the theoretical prediction. Twenty-three single mutants were recovered with at least one mutation in each position in the region corresponding to residues 364−377 except positions 369 and 370 (Figure 1C). Some of the single mutants were derived from double, triple, and deletion cassettes cut with *Mun*I and *Hpa*I/*Aat*II. Site-directed mutagenesis was used to mutate Ile-369 or Ala-370 to Val and to create a unique *Xba*I site at positions 372−373. An additional mutant was made to change Leu-366 to Ser and create a unique *Bgl*II site at this position. P450 3A4 L366S and A370V in the *E. coli* expression vector served as the parental plasmids for insertion of the cassettes using *Hpa*I−*Mun*I or *Mun*I−*Aat*II fragments. The plasmids with cassettes remained circular upon treatment with *Bgl*II or *Xba*I, while the undigested parental plasmids, which retained a *Bgl*II or *Xba*I site, were linearized. Since circular DNA transforms bacteria 10−1000-fold more efficiently than linear DNA (Conley & Saunder, 1984; Cohen et al., 1972), the plasmids with cassettes were efficiently recovered when subsequently transformed into *E. coli*, and no parental plasmid was obtained during the subcloning.

Expression of Cytochrome P450 3A4 Wild-Type and Mutant Enzymes in E. coli. P450 3A4 wild-type[3] and one mutant at each position (Figure 1C, underlined) were transformed into bacteria. Optimal expression of P450 3A4 wild-type was obtained after 50 h incubation at 30 °C in cultures shaken at 190 rpm. Solubilized membrane preparations yielded 160−230 nmol of P450 per liter of culture detected as the reduced CO complex. Most of the mutants exhibited the same expression ranges as the wild-type. However, the substitution of Pro-368 with Ser led to a 30-fold lower expression level than that of wild-type, while R365G, R372S, and R375K could not be detected from Fe^{2+}−CO vs Fe^{2+} difference spectra. To preserve the positive charge at positions 365 and 372, Arg was replaced by Lys using site-directed mutagenesis. Solubilized membrane preparations of R372K contained 30−40 nmol of P450 per liter of culture, whereas no hemoprotein was detected in the preparations of the R365K mutant. Immunoblotting using anti-P450 3A12 IgG revealed immunoreactive protein in R375K, but not R365K (data not shown). To increase R365K expression, different incubation temperatures and times were tested. After IPTG induction, the bacteria were shifted to 25, 30, or 37 °C, and harvested after 4, 21, 24, 28, 44, 50, 72, 78, 95, or 109 h. Expression levels of P450 for the above conditions were determined by whole-cell immunoblotting. Only a trace of R365K protein was observed using anti-3A12 IgG after 4 h growth at 25, 30, or 37 °C. At all later time points, no immunoreactive 3A4 was detected (data not shown).

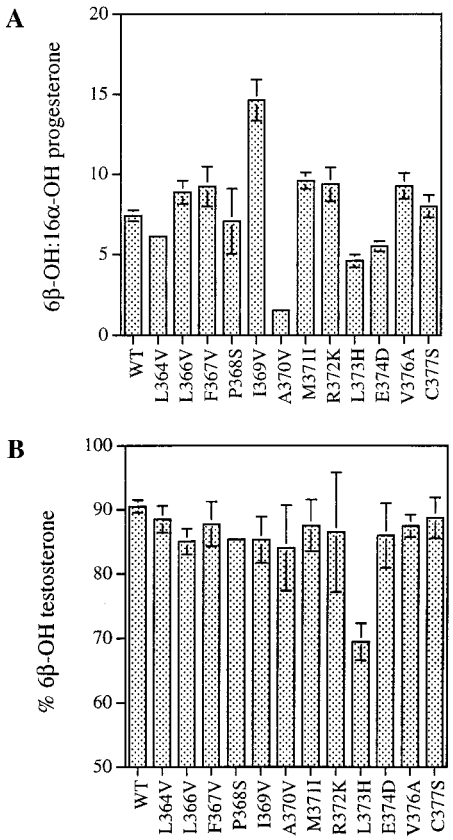

FIGURE 2: Progesterone and testosterone metabolite profiles of single mutants at residues 364, 366−374, and 376−377. Assay conditions are as described under Experimental Procedures. The wild-type amino acids, the positions, and the substituted amino acids in that order are shown at the bottom of the bar graph. (A) 6β-OH:16α-OH progesterone ratio. The values represent the mean ± SD of six analyses. (B) Formation of 6β-hydroxytestosterone as a percent of total metabolites. The values represent the mean ± SD of four analyses.

Steroid Hydroxylase Activities of P450 3A4 Wild-Type and Mutants. Cytochrome P450 3A4 hydroxylated progesterone primarily at the 6β-position and to a lesser extent the 16α-position. The progesterone metabolite profile of 3A4 wild-type from *E. coli* in this study (6β-OH:16α-OH ratio = 7.4) was the same as that exhibited by 3A4 in microsomes from vaccinia-infected Hep G2 cells (6β-OH:16α-OH ratio = 7.5) (Aoyama et al., 1989). None of the mutants at positions 364, 366−368, 371−372, 374, or 376−377 exhibited significant alterations in the regio- or stereospecificity of progesterone hydroxylation. For these mutants, the 6β-OH:16α-OH ratio ranged from 5.5 to 9.6 (Figure 2A), and no novel metabolites were formed. In addition, these mutants retained at least two-thirds of the progesterone 6β-hydroxylase activity of wild-type 3A4 except for F367V, P368S, and R372K, which exhibited one-tenth to one-fifth of wild-type activity. Substitutions at residues 369, 370, or 373 caused major changes in the progesterone metabolite profile (Figure 2A and Figure 3). Replacement of Ile-369 by Val suppressed 16α-hydroxylase activity, resulting in a progesterone 6β-OH:16α-OH ratio (=14.6) that was 2-fold higher than the parental P450 3A4. The Ala-370 → Val substitution enhanced 16α-hydroxylase activity. As a consequence, the

[3] The 3A4 cDNA referred to as wild-type has been modified at the N-terminus to maximize expression in *E. coli* (Gillam et al., 1993; Harlow & Halpert, 1997). The modifications result in deletion of codons 3−12 and conversion of Ser to Phe at position 18.

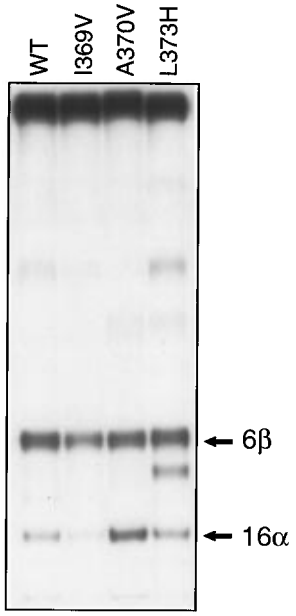

FIGURE 3: Autoradiogram of progesterone metabolites produced by P450 3A4 wild-type and single mutants. P450 (5 pmol) was incubated with 25 μM [^{14}C]progesterone for 10 min at 37 °C in 100 μL of buffer containing 10 pmol of NADPH−cytochrome P450 reductase and 10 pmol of cytochrome b_5. Samples were quenched by the addition of 50 μL of tetrahydrofuran, and 50 μL aliquots were applied to a TLC plate. Cytochrome P450 3A4 wild-type and the three mutants that exhibited major changes in the progesterone metabolite profiles are shown. The 16α- and 6β-hydroxylase activities, in nmol min^{-1} (nmol of P450)$^{-1}$, were as follows: WT (0.84, 6.08), I369V (0.21, 3.09), A370V (3.49, 5.52) and L373H (1.49, 6.67). The unknown metabolite of L373H was produced at a rate of 2.27 nmol min^{-1} (nmol of P450)$^{-1}$.

progesterone 6β-OH:16α-OH ratio (=1.6) decreased almost 5-fold. Interestingly, substitution of Leu-373 with His conferred a new hydroxylase activity with retained hydroxylation at the 6β- and 16α-positions (Figure 3). Overall, the results of all the amino acid substitutions investigated are consistent with our previous suggestion that the inherent chemical reactivity of the allylic 6-position is a major contributor to the regioselectivity of steroid hydroxylation by P450 3A4 (Harlow & Halpert, 1997).

Previous studies have established that >90% of cytochrome P450 3A4-mediated testosterone hydroxylation occurs at the 6β-position with small amounts of 2β-OH and 15β-OH products also formed (Aoyama et al., 1989; Harlow & Halpert, 1997). All the mutants except L373H displayed the same metabolite profile as the wild-type enzyme, and the percentage of 6β-hydroxylation ranged from 84 to 89% (Figure 2B). However, the replacement of Leu-373 by His produced an unknown metabolite that migrated slightly slower than 15β-hydroxytestosterone and constituted 18% of total metabolites. As a result, the percentage of 6β-hydroxylation of L373H decreased to 70% (Figure 2B).

Effect of α-NF on Progesterone Hydroxylation by Cytochrome P450 3A4 Wild-Type and the Mutants at Positions 369, 370, and 373. A number of biochemical approaches have been used to study the molecular mechanism of P450 3A4 activation by the flavonoid α-NF. These analyses have resulted in several different hypotheses, one of which is that the compound interacts with residues close to or part of the substrate binding site (Shou et al., 1994). Therefore, the P450 3A4 wild-type and three mutants at positions 369, 370, and 373, which exhibited major changes in progesterone metabolite profiles, were chosen to test the effect of α-NF

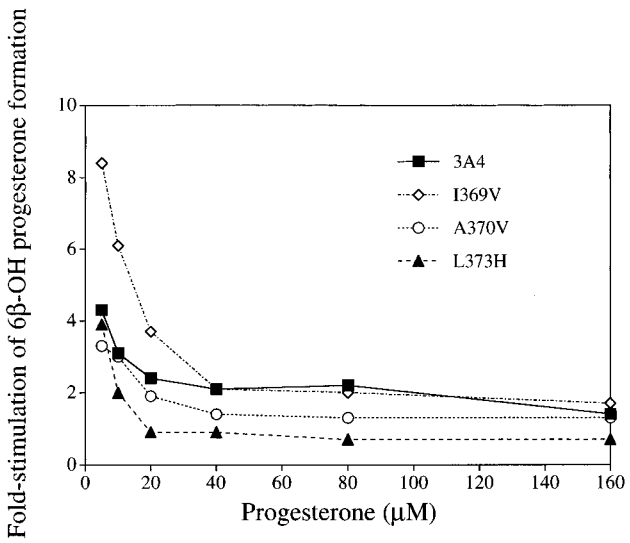

FIGURE 4: Relationship between fold stimulation by α-NF and progesterone concentration. Values represent the average of duplicate determinations.

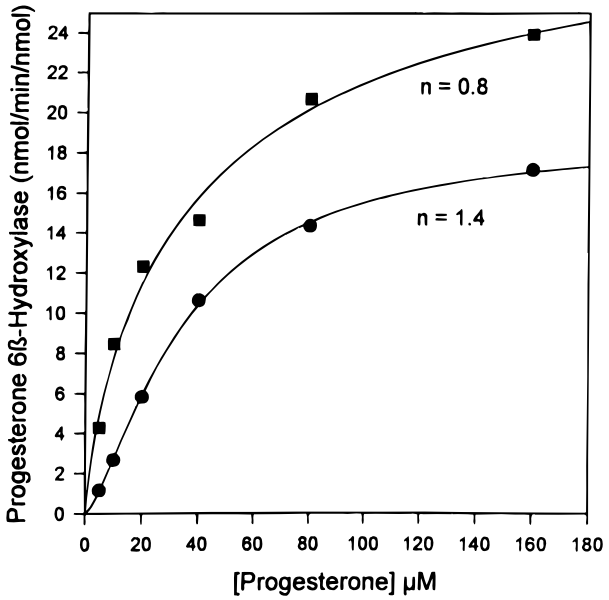

FIGURE 5: Steady-state kinetics of progesterone 6β-hydroxylation catalyzed by wild-type P450 3A4 in the absence (●) or presence (■) of 25 μM α-naphthoflavone. Data were analyzed and lines were drawn as described by Ueng et al. (1997) with Sigma Plot from Jandel Scientific (San Rafael, CA) using the equation $v = V_{max}S^n/(S_{50}{}^n + S^n)$. The n value was 1.4 ± 0.1 in the absence of α-NF and 0.8 ± 0.2 in the presence of the compound.

on progesterone hydroxylation. Figure 4 shows the fold stimulation of 6β-hydroxylation by 25 μM α-NF at different progesterone concentrations. This α-NF concentration was shown previously to be optimal for stimulation of wild-type 3A4 (Harlow & Halpert, 1997). As the progesterone concentration was increased, the responsiveness of the 3A4 wild-type to α-NF decreased (Harlow & Halpert, 1997). This is also evident from Figure 5, in which the steady-state kinetics of 6β-hydroxylation by the enzyme were analyzed using a sigmoidal V_{max} model. Clear evidence for cooperativity was obtained in the absence of α-NF, whereas this cooperativity was abolished by the addition of α-NF. The diminished response to α-NF at high substrate concentrations can be readily explained by occupation of the effector site by progesterone (Schwab et al., 1988). In terms of the mutants, I369V was more responsive and L373H less

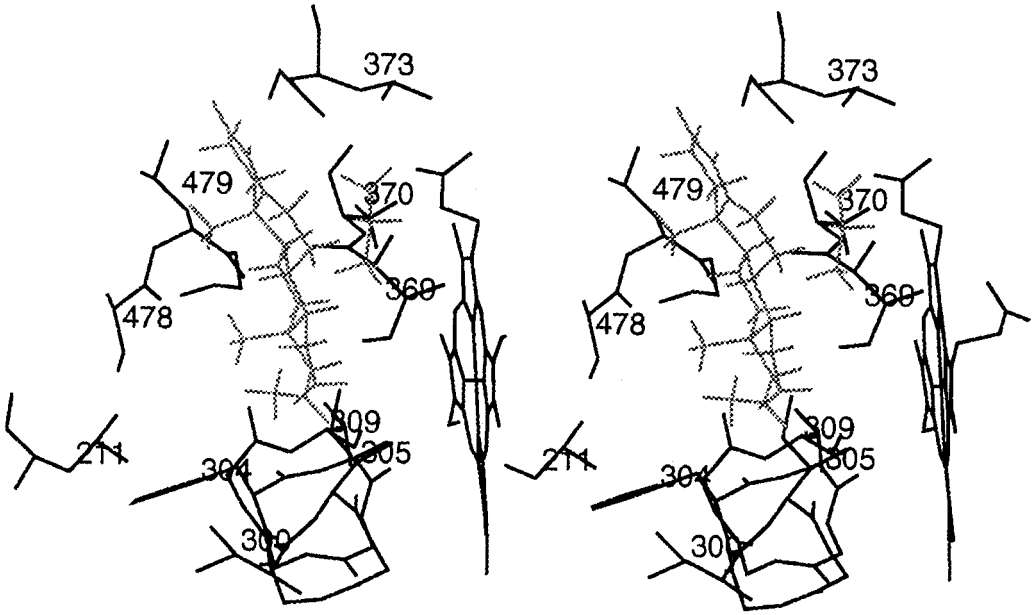

FIGURE 6: Progesterone docked into the P450 3A4 model in a 16α binding orientation. The substrate is shown in gray, with all hydrogens displayed. Upon the replacement of Ala-370 with Val (gray), the increased van der Waals contacts with the substrate may stabilize the 16α binding orientation.

responsive than the parental enzyme to stimulation by 25 μM α-NF (Figure 4). For example, at 5 and 10 μM progesterone, the 6β-hydroxylase activity of I369V was stimulated 8.5- and 6.1-fold as compared with 4.3- and 3.1-fold for the wild-type and 3.0- and 2.0-fold for L373H. At substrate concentrations $\geq$20 μM, essentially no stimulation of 6β-hydroxylase activity by α-NF was seen for L373H. All three mutants exhibited sigmoidal kinetics in the absence of α-NF (n values from 1.3 to 1.5) but hyperbolic kinetics in the presence of α-NF ($n \leq 1.0$) (data not shown). Qualitatively, the relationship between fold stimulation of progesterone 16α-hydroxylation and progesterone concentration was similar as for 6β-hydroxylation for the wild-type and three mutants (data not shown).

Docking of Progesterone into the Active Site of the P450 3A4 Model. A very recent homology model of P450 3A4 has suggested that the enzyme is characterized by a relatively large active site, which can accommodate progesterone or the much larger erythromycin (Szklarz & Halpert, 1997). Progesterone docked in a 6β binding orientation interacted with residues 370 and 373, which suggested that they may be important for enzyme function (Szklarz & Halpert, 1997). In the present study, a major role for residues 369, 370, and 373 has been confirmed, especially in 16α-hydroxylation of this substrate. To explain some of the changes in activity observed upon 3A4 mutagenesis, progesterone was docked into the enzyme model in a 16α binding orientation (Figure 6). Residues 369 and 370 are found within 4 Å and residue 373 within 5 Å from the substrate and can interact with progesterone through hydrophobic interactions. In the A370V mutant, the number of van der Waals contacts increases, which leads to an increase in the nonbond interaction energy. Thus, it is likely that the presence of Val instead of Ala at position 370 decreases the mobility of the substrate in the active site and stabilizes the 16α binding orientation of progesterone.

DISCUSSION

By extensively mutagenizing cytochrome P450 3A4 in the region that contains the putative substrate recognition site 5, we have identified specific residues that play important roles in either protein structure or substrate specificity. The Arg residues at positions 365 and 375 may contribute to the folding and stability of the protein, including heme binding. The hydrophobic residues at positions 369, 370, and 373 contribute to steroid hydroxylase specificity, and substitutions at positions 369 and 373 also affect α-NF stimulation.

The mutation of Arg residues at positions 365, 372, and 375 had a profound effect on P450 expression and/or stability. Solubilized membranes, except those from P450 3A4 mutants at position 365, yielded immunoreactive protein on Western blotting, but R372S and R375K mutant proteins were not detected as the $Fe^{2+}-CO$ complex. However, the replacement of Arg-372 by Lys yielded a typical P450 spectrum. According to a recent P450 3A4 model based on P450 BM-3 (Lewis et al., 1996), an ion pair between Asp-61 and Arg-372, which may stabilize protein conformation, is apparent in the 3A4 structure. Charge−charge interactions between residues 61 and 372 are also present in our 3A4 model (Szklarz & Halpert, 1997). Thus, it is likely that the substitution of Arg-372 with Ser leads to misfolding or unfolding of the protein accompanied by the loss of heme, whereas the positively charged Lys can successfully replace Arg. Interestingly, Asp-61 and Arg-372 are conserved within the P450 3A subfamily.

Arg-375 of P450 3A4 seems to play an important role in heme binding. In an alignment between P450 3A4 and four bacterial P450s of known 3-D structures (Szklarz & Halpert, 1997), this residue aligns with an Arg at the corresponding positions in P450cam, P450eryF, and P450terp. The only exception is P450 BM-3, which has a Leu instead of an Arg (Figure 7). This Arg residue interacts with the heme propionate group either directly through hydrogen bonds as in P450cam (Poulos et al., 1987) and P450eryF (Cupp-Vickery & Poulos, 1995) or indirectly through water molecules as in P450terp (Hasemann et al., 1994). Similar interactions are also possible in our P450 3A4 model. In the 3A4 R375K mutant, hydrogen bonds with the heme propionate group may be disrupted because of the shorter

BM3:	320-	E	A	L	R	L	W	P	T	A	P	-	A	F	S	L	Y	A-	335
TERP:	306-	E	A	V	R	W	T	A	P	V	K	-	S	F	M	R	T	A-	321
ERYF:	280-	E	I	L	R	Y	I	A	P	P	E	-	T	T	T	R	F	A-	295
CAM:	287-	E	L	L	R	R	F	S	L	V	-	-	A	D	G	R	I	L-	301
3A4:	362-	E	T	L	**R**	L	F	P	**I**	**A**	M	-	R	**L**	E	**R**	V	C-	377
3A5:									V		I						T		
2A4:	358-	E	I	Q	R	F	A	D	L	I	P	M	G	L	A	R	R	V-	374
2B1:	355-	E	I	Q	R	F	S	D	L	V	P	I	G	V	P	H	R	V-	371

FIGURE 7: Sequence alignment among bacterial P450s, P450 3A4 and 3A5, and P450 2A4 and 2B1. The residues involved with protein structure or substrate specificity in P450 3A4 are bolded. Only the residues that differ between 3A4 and 3A5 are shown for P450 3A5.

side chain of Lys compared with Arg. This may explain the inability of the mutant to bind heme.

The third important Arg residue in SRS-5 of P450 3A4 is Arg-365. As shown in Figure 8, the side chain of this amino acid participates in a complex network of hydrogen bonding, which may stabilize the region of helices K and the meander proximal to heme. Thus, the guanidino group of Arg-365 is hydrogen-bonded to the backbone carbonyl oxygen atoms of His-402 and Pro-411, the side-chain oxygen atom of Glu-362, and the side chain of Trp-408. Moreover, the side-chain oxygen atom of Glu-362 is also hydrogen-bonded to the side chain of Arg-418. Glu-362, Arg-365, and Arg-418 of 3A4 are absolutely conserved in known bacterial enzymes, while other residues participating in the network are less conserved [Figure 7; see also the alignment in Szklarz and Halpert (1997)]. Furthermore, hydrogen bonds equivalent to those found in 3A4, except the bond between Arg-365 and Trp-408, are also conserved within the crystal structures of the four bacterial P450s. These hydrogen bonds are lost upon mutation of Arg-365 to Lys in P450 3A4. The immunoblotting results indicate that the mutant protein is expressed only transiently. This may be due to the decreased stability of the protein in the region of helices K and the meander, which promotes the unfolding of the whole structure. Thus, Arg-365 and its hydrogen bond network may contribute to the maintenance of the tertiary structure of cytochrome P450 3A4.

Alignment of P450 sequences with that of P450cam has been useful in predicting which regions of mammalian P450s contain amino acids that may contact the substrate (Poulos, 1991; Gotoh, 1992). The sequence alignment between cytochrome P450 3A4 and P450cam (Szklarz & Halpert, 1997) locates a region containing residues 364−377 of 3A4 in SRS-5. In P450cam, this region contains two substrate contact residues, Val-295 and Asp-297 (Poulos et al., 1987). Substitution of Ile-369, Ala-370, or Leu-373 in P450 3A4 had a profound effect on progesterone hydroxylase profiles. The L373H mutant in particular exhibits novel progesterone and testosterone hydroxylase activity. The corresponding residues in cytochrome P450 family 2 enzymes (Figure 7) have also been reported to alter catalytic activities. A Leu-365 → Met substitution in P450 2A4, corresponding to position 369 in 3A4, confers the coumarin hydroxylase activity of P450 2A5 (Lindberg & Negishi, 1989). Replacement of Val-363 or Val-367 in P450 2B1 with Ala, corresponding to positions 370 and 373 in 3A4, confers androgen 15α-hydroxylase and 6β-hydroxylase activities, respectively (He et al., 1994). Other key residues identified in SRS-5 of family 2 include 359, 364, 368, and 369 in P450 2C enzymes (Kaminsky et al., 1992; Hsu et al., 1993; Ramarao et al., 1995), and 380 in P450 2D1 (Matsunaga et al., 1990). Replacement of the corresponding residues (367, 372, 375, or 376) in P450 3A4 did not significantly change progesterone or testosterone metabolite profiles. In general, the results indicate agreement among the studies with cytochrome P450 family 2 and family 3 enzymes and indicate that P450cam is an appropriate model for these mammalian enzymes in the region surrounding Val-295, as suggested (Poulos, 1991).

Enzyme−substrate interactions and the possible mechanism responsible for changes in specificity in some of the P450 3A4 mutants were investigated using computer modeling. Due to the large size of the 3A4 active site (Szklarz & Halpert, 1997), the steroid substrate can fit in virtually any orientation, and changes in hydroxylation at a given site are likely to be related to changes in the mobility of the compound. Thus, in the A370V mutant, Val stabilizes a 16α binding orientation of progesterone through an increase in van der Waals contacts with the substrate (Figure 6), leading to an increase in the nonbond interaction energy. Likewise, the loss of 16α-hydroxylase activity in the 3A4 I369V mutant may be related to increased mobility of progesterone in the enlarged active site of the mutant.

P450 3A4 and 3A5 exhibit 84% amino acid sequence identity and metabolize many of the same substrates. However, each enzyme produces a distinct pattern of

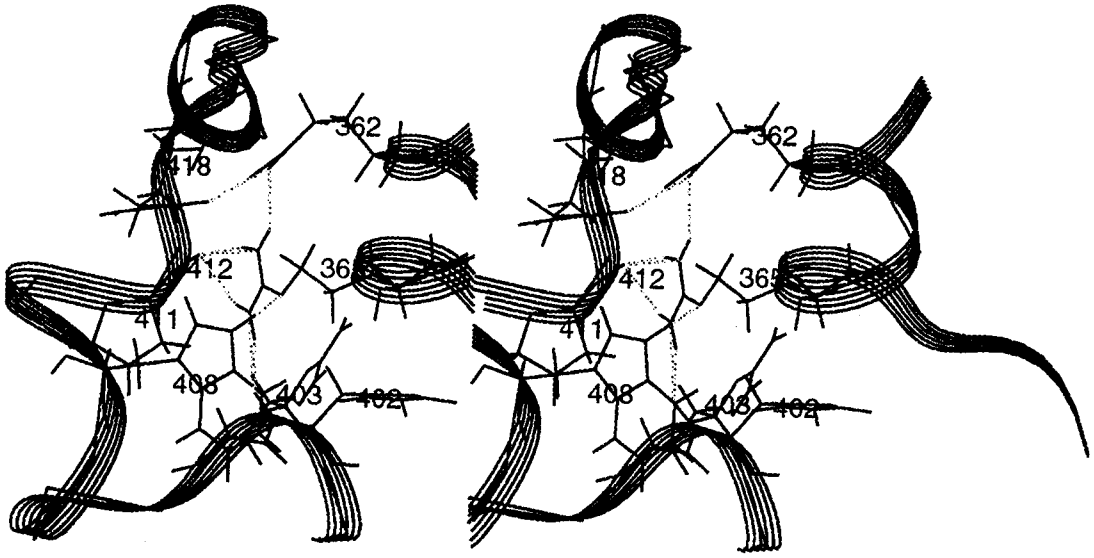

FIGURE 8: Topology of the P450 3A4 model in the neighborhood of residue 365. Hydrogen bonds are shown as dotted gray lines.

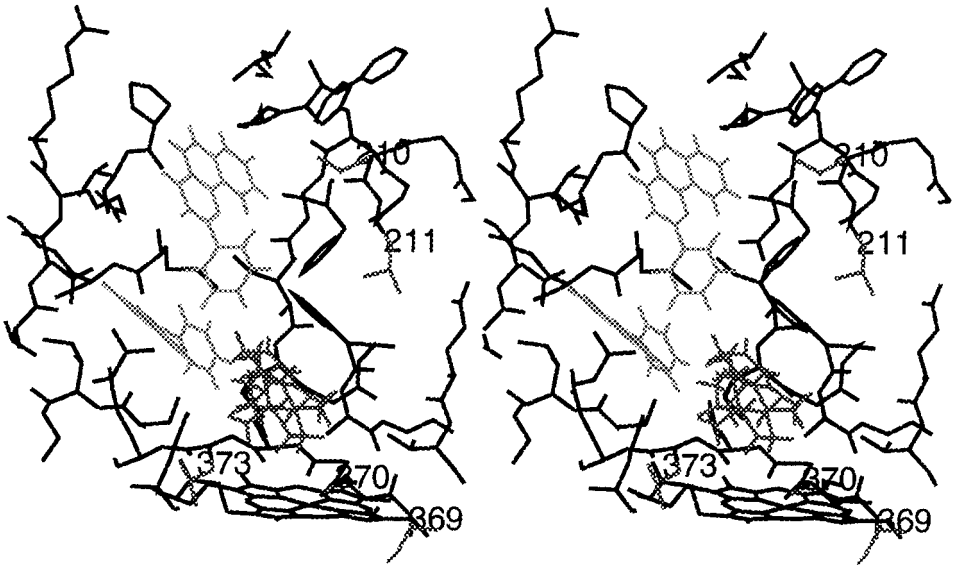

FIGURE 9: α-Naphthoflavone and progesterone docked into the P450 3A4 model. Side chains of residues 210 and 211 (Harlow & Halpert, 1997) and 369, 370, and 373 are shown as dotted gray lines. Progesterone in a 6β binding orientation with all hydrogens displayed is in darker gray close to heme. α-NF shown in lighter gray can bind in the vicinity of the substrate, near residue 373, or further from the substrate but close to residues 210 and 211. α-NF was docked into the active site with progesterone fixed. The orientation of the activator was optimized using molecular dynamics and minimization, essentially as described for progesterone under Experimental Procedures, except that no atom of α-NF was fixed.

metabolites of certain compounds such as progesterone, testosterone, cyclosporin A, and midazolam. Comparison of the amino acid sequences of SRS-5 reveals only three residues that are different between 3A4 and 3A5 (Figure 7). Two of the residues have been investigated for their effect on progesterone and testosterone metabolism in this study. Previous studies have established that both enzymes catalyze mainly 6β-hydroxylation of these steroid hormones. Several minor oxidation products of these steroids (e.g., 2β- and 15β-hydroxytestosterone and 16α-hydroxyprogesterone), comprising up to ~20% of the total metabolites, are formed by P450 3A4 but not 3A5 (Aoyama et al., 1989). Replacement of Met-371 by Ile in 3A4 did not cause major changes in either testosterone or progesterone metabolite profiles. However, when Ile-369 in P450 3A4 was mutated to the corresponding Val in 3A5, progesterone 16α-hydroxylase activity was suppressed. As a consequence, the progesterone 6β-OH:16α-OH ratio increased almost 2-fold. The result indicates that the residue at position 369 may play an important role in partial interconversion of progesterone hydroxylase specificity of human cytochromes P450 3A4 and 3A5. Interestingly, the mutant enzyme does not exhibit significant alterations in the regio- or stereospecificity of testosterone hydroxylation. Moreover, the substitution of Ala-370 with Val affects progesterone but not testosterone hydroxylation. This is consistent with findings from a number of studies that the precise identity of substrate contact residues varies with the substrate tested (Hasler et al., 1994; Szklarz et al., 1995; He et al., 1996; Kaminsky et al., 1992; Straub et al, 1994; Matsunaga et al., 1990). Progesterone differs from testosterone only by virtue of a 17β-acetyl as opposed to 17β-hydroxy group, suggesting that the larger side chain of progesterone interacts with additional amino acid side chains not involved in binding testosterone.

In recent years, substantial progress has been made in elucidating the mechanism of P450 3A4 activation by flavonoids. An early study of 3A4-mediated progesterone hydroxylation in human liver microsomes provided evidence for activation by the substrate as well as by α-NF (Schwab et al., 1988). Kinetic analysis indicated that activation by α-NF was largely due to a decreased K_m for progesterone with little change in V_{max}. The results were interpreted in terms of an allosteric model, although the authors could not determine whether the interaction with effectors occurred at the catalytic site or at a separate site. Subsequent studies of the related rabbit P450 3A6 demonstrated enhanced binding of a substrate analog in the presence of α-NF or progesterone (Johnson et al., 1988). Very recent results with P450 3A4 heterologously expressed in *E. coli* are also consistent with an allosteric model to explain autoactivation by substrate and stimulation by α-NF (Ueng et al., 1997; Harlow & Halpert, 1997). However, it has also been proposed using the enzyme expressed in HepG2 cells that stimulation of 3A4-catalyzed phenanthrene metabolism reflects the simultaneous presence of substrate and activator in the active site as opposed to an allosteric mechanism (Shou et al., 1994). The major evidence for this was that α-NF served as a substrate for 3A4 and mainly changed the V_{max} rather than K_m for phenanthrene. As pointed out by Ueng et al. (1997), the two models are not mutually exclusive and differ primarily in terms of the proximity between the catalytic site and putative effector site.

Our approach has been to utilize site-directed mutagenesis in conjunction with molecular modeling to map the substrate binding and effector sites in P450 3A4. A very recent study of SRS-2 in P450 3A4 identified two mutants, at positions 210 and 211, that displayed decreased flavonoid stimulation but only minor changes in steroid metabolite profiles in the absence of activator (Harlow & Halpert, 1997). In the present study of SRS-5, three mutants at positions 369, 370, and 373 were identified that exhibited major changes in progesterone hydroxylation. Two of these mutants, I369V and L373H, also displayed an altered response to α-NF. In order to rationalize the mutagenesis results in terms of the proposed mechanisms of 3A4 activation, substrate and effector were docked in the active site of our 3A4 model

(Figure 9). Although the precise location of effector is uncertain, three important observations are apparent. First, the substrate binding cavity is sufficiently large to accommodate two molecules of activator in addition to the substrate. Progesterone is shown as the substrate and α-NF as the activator in Figure 9, but there is sufficient space for three molecules of any combination of the two compounds. In other words, progesterone can be readily accommodated as an effector and α-NF as a substrate. Second, residues 210 and 211, on the one hand, and 369, 370, and 373, on the other hand, are too far from each other to interact with the same substrate or effector molecule simultaneously. Third, in one docking orientation of α-NF, residue 373 can be involved in hydrophobic interactions with both the effector and the substrate. Therefore, the mutagenesis data and model lend themselves to two interpretations of the effects of the SRS-5 substitutions. One interpretation is that residues 369, 370, and 373 are mainly involved in substrate interactions. However, since occupation of the effector site presumably changes the shape of the substrate binding pocket (Ueng et al., 1997; Harlow & Halpert, 1997), the altered α-NF response of the SRS-5 mutants I369V and L373H could reflect an indirect effect of the modified active site. A second interpretation is that the altered behavior of L373H toward α-NF also reflects perturbation of the interaction with the effector. Additional side-chain substitutions at this position will be required to elucidate its precise role in P450 3A4 activation.

In conclusion, this study demonstrates a useful approach, by a combination of cassette and site-directed mutagenesis, to identify key residues involved in substrate specificity as well as in protein stability and heme binding. Moreover, the utilization of molecular models for interpretation of enzyme−substrate and hydrogen-bond interactions provides an additional insight into the possible function of individual residues. The further study of mutant enzymes that influence α-NF stimulation should be invaluable in elucidating the mechanism of flavonoid activation of P450 3A4 enzymes.

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