Alanine-scanning mutagenesis was performed on amino acid residues 210–216 of cytochrome P450 3A4, the major drug-metabolizing enzyme of human liver. Mutagenesis of this region, which has been proposed to align with the C-terminal ends of F-helices from cytochromes P450BM-3, P450terp, and P450cam, served as a test of the applicability of the substrate recognition site model of Gotth (Gotth, O. (1992) J. Biol. Chem. 267, 83–90) to P450 3A4. The results, using two steroid substrates, indicated that substitution of Ala for Leu210 altered the responsiveness to the effector α-naphthoflavone and the regioselectivity of testosterone hydroxylation. Replacement of Leu211 by Ala also decreased the stimulation by α-naphthoflavone, whereas mutations at residues 212–216 had little effect. The diminished flavonoid responses of the 210 and 211 mutants were observed over a wide range of pregnenolone and α-naphthoflavone concentrations. Further characterization was performed with the additional effectors β-naphthoflavone, flavone, and 4-chromanone. The finding that P450 3A4 with one altered residue, Leu210 → Ala, can have both an altered testosterone hydroxylation profile and response to flavonoid stimulation provides evidence that the substrate binding and effector sites are at least partially overlapping.

Human cytochrome P450 3A4 (P450 3A4) is capable of catalyzing the oxidative metabolism of a wide range of chemical compounds of very different structures (1). Of particular interest is the large number of drugs known to be metabolized by P450 3A4 (2). This enzyme may account for as much as 60% of the P450 in a human liver sample (3), making it one of the single most important human xenobiotic-metabolizing enzymes. Although 3A4 can accommodate a wide variety of structurally diverse substrates, it still exhibits remarkable regions and stereoselectivities with many compounds. For example, the enzyme catalyzes the β-, 6β-, and 15β-hydroxylation of testosterone, the 6β- and 16α-hydroxylation of progesterone (4), the 1- and 4-hydroxylation of midazolam (5), and the M1-, M17-, and M21-oxidation of cyclosporin A (6). In addition to these properties, metabolic activities of enzymes of the P450 3A subfamily are modulated by naturally occurring phenolic compounds known as flavonoids, resulting in either the stimulation or inhibition of enzyme activity (7–11). Several sites for effector binding have been postulated based on the proposed mechanisms of action. Hypotheses include (a) α-NF increases the affinity of P450 for the substrate (12), (b) α-NF increases the affinity of P450 for reductase (10), (c) α-NF binds in the same pocket as the substrate and increases coupling efficiency (13), or (d) α-NF is an allosteric effector, binding at a distinct site and causing a conformational change in the substrate binding pocket (12–14).

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. Based on comparative sequence alignments and analogy with P450 101, the existence of six substrate recognition sites (SRSs) within family 2 has been proposed (15). Extensive analysis of P450 2B enzymes by this laboratory (16–23) and of enzymes in the P450 2A (24–26) and 2C (27–32) subfamilies have so far confirmed that virtually all amino acid residues and chimeric fragments identified as critical for the substrate specificity of P450 2 forms fall within or near the putative SRSs. To assess the applicability of the SRS model to the P450 subfamily 3A we have targeted P450 3A4 residues that are predicted (33) by a structure-based alignment to correspond to active site residues from P450BM-3, P450cam, and P450terp with the goal of identifying residues that form or influence the enzyme active site and/or effector binding site. We have chosen to start our analysis of P450 3A structure-function at a region that has been aligned by Hasemann et al. (33) with SRS-2. The SRS-2 residues 209 of P450s 2A4 and 2A5 (24–26, 34) and 206 of P450 B1 (17) have been identified as substrate contact residues. The precise equivalents of these family 2 residues in P450BM-3, P450cam, or P450terp are uncertain due to a high degree of structural variability among the bacterial enzymes in these regions. We reasoned that verification of an equivalent SRS-like region in P450 3A4 in this difficult to align region would be an excellent indicator of the accuracy of the alignment and of whether other SRSs predicted by the alignment are likely to exist. We report here the use of alanine-scanning mutagenesis to examine the structure-activity relationships of seven residues from the P450 3A4 region analogous to SRS-2. Based on our analysis, a Leu → Ala substitution at residue 210 or 211 diminishes responsiveness to α-NF. This report details the first site-directed mutagenesis study of any P450 3A enzyme and
The P450 3A4 cDNA was cloned directly into the pCRII vector (underlined) to facilitate cloning rather than an NcoI site followed by religation of the entire modified 3A4 insert was sequenced and was indicated by a *.

**Results**

**Alanine-scanning Mutagenesis of P450 3A4**

**Materials**—Primers for PCR amplification were obtained from the University of Arizona Macromolecular Structure Facility (Tucson, AZ) and National Bioscience, Inc. (Plymouth, MN). Restriction enzyme and bacterial growth media were purchased from Life Technologies, Inc. CHAPS, progesterone, testosterone, α-NF, NANDPH, and DOPC were purchased from Sigma. [4-14C]Progesterone was obtained before adding 1.0 mM isopropyl-1-thio-

**Primers used for site-directed mutagenesis.**

**Materials**—Primers for PCR amplification were obtained from the University of Arizona Macromolecular Structure Facility (Tucson, AZ) and National Bioscience, Inc. (Plymouth, MN). Restriction enzymes and bacterial growth media were purchased from Life Technologies, Inc. CHAPS, progesterone, testosterone, α-NF, NANDPH, and DOPC were purchased from Sigma. [4-14C]Progesterone was obtained from DuPont NEN. [4-14C]Testosterone was obtained from Amersham Life Science, Inc. HEPES was purchased from Calbiochem. Thin layer chromatography plates (silica gel, 250 mm, Si 250 PA (19C)) were purchased from Baker (Phillipburg, NJ). All other reagents and supplies not listed were obtained from standard sources.

**Cloning and Expression of P450 3A4**—The P450 3A4 cDNA was isolated from human liver by PCR amplification of a first strand cDNA library created using the 3′-rapid amplification of cDNA ends kit (Life Technologies, Inc.) and total human liver RNA (Clontech; Palo Alto, CA). The N-terminal (5′-GTTACAGGCTTCATACGTCATCCATGGTACCTAC-3′) and C-terminal (5′-GAAAAATTTACGCTAATTTGCTGCTACTCCCATTGACTACCT-3′) primers were designed to amplify the entire 1.5-kilobase pair coding sequence (35). Reaction conditions were as follows: one cycle of 94°C for 5 min, 45°C for 5 min, and 72°C for 5 min, followed by 29 cycles of 94°C for 2 min, 45°C for 2 min, and 72°C for 2 min. To increase expression of the cDNA in Escherichia coli, N-terminal modifications were made by using the primer 5′-GGCCCATGATGCCTGTGTTATGAGGCATTTTCTGGTGCTCTC-3′ as described previously (36) except for the incorporation of an NcoI site (underlined) to facilitate cloning rather than an NdeI site. Following modification, the 3A4 cDNA was cloned directly into the pCRII vector using the TA Cloning Kit (Invitrogen, San Diego, CA) and subsequently subcloned as an NcoI-KpnI fragment into the E. coli expression plasmid pSE380 (Pharmacia Biotech Inc.), creating pSE3A4. A BamHI site that originated from the pCRII multiple cloning site was deleted from pSE3A4 by cutting at the closely spaced SpeI sites and removing the SpeI site that contains the region of the three BamHI site followed by religation of the SpeI ends. The entire modified 3A4 insert was sequenced and was determined to be most similar to the 3A4 variant hPCN1 (37), differing only by a silent mutation (CTC → CTG) at the position that encodes Leu49.

**Alanine-scanning Mutagenesis of Residues 210–216**—Primers used to modify codons 210–216 were designed in the reverse orientation and are shown in Fig. 1. The forward primer used for PCR annealed to a C-terminal primer used for PCR amplification of the first strand cDNA library created using the 3′-rapid amplification of cDNA ends kit (Life Technologies, Inc.) and total human liver RNA (Clontech; Palo Alto, CA). The N-terminal (5′-GTTACAGGCTTCATACGTCATCCATGGTACCTAC-3′) and C-terminal (5′-GAAAAATTTACGCTAATTTGCTGCTACTCCCATTGACTACCT-3′) primers were designed to amplify the entire 1.5-kilobase pair coding sequence (35). Reaction conditions were as follows: one cycle of 94°C for 5 min, 45°C for 5 min, and 72°C for 5 min, followed by 29 cycles of 94°C for 2 min, 45°C for 2 min, and 72°C for 2 min. To increase expression of the cDNA in Escherichia coli, N-terminal modifications were made by using the primer 5′-GGCCCATGATGCCTGTGTTATGAGGCATTTTCTGGTGCTCTC-3′ as described previously (36) except for the incorporation of an NcoI site (underlined) to facilitate cloning rather than an NdeI site. Following modification, the 3A4 cDNA was cloned directly into the pCRII vector using the TA Cloning Kit (Invitrogen, San Diego, CA) and subsequently subcloned as an NcoI-KpnI fragment into the E. coli expression plasmid pSE380 (Pharmacia Biotech Inc.), creating pSE3A4. A BamHI site that originated from the pCRII multiple cloning site was deleted from pSE3A4 by cutting at the closely spaced SpeI sites and removing the SpeI site that contains the region of the three BamHI site followed by religation of the SpeI ends. The entire modified 3A4 insert was sequenced and was determined to be most similar to the 3A4 variant hPCN1 (37), differing only by a silent mutation (CTC → CTG) at the position that encodes Leu49.

**Preparation of Solubilized E. coli Membranes**—CHAPS-solubilized membrane preparations were made essentially as previously described (33). Briefly, pSE3A4 containing E. coli DH5α cells were grown at 37°C with 240-rpm shaking in 250 ml of liquid TB media (12 g of Bacto-tryptone, 24 g of Bacto-yeast extract, 4 ml/liter glycerol) to midlog phase before adding 1.0 mM isopropyl-1-thio-β-galactopyranoside and 80 mg/ml b-amino levulinic acid. Cells were harvested after an additional 48-h incubation at 30°C with 190-rpm shaking. Membranes were solubilized in MOPS buffer (100 mM MOPS (pH 7.3), 10% glycerol, 0.2 mM dithiothreitol, 1 mM EDTA) containing 0.5% CHAPS. Recovery yields of 120–180 mmol of P450 3A4/liter of culture were routine as determined from reduced carbon monoxide difference spectra. P420 contamination was <5%.

**Rat NANDPH-P450 Reductase Expression and Purification**—The cDNA encoding rat NANDPH-P450 reductase was kindly provided by Dr. Todd Porter (College of Pharmacy, University of Kentucky, Lexington, KY). Reductase was expressed from a T7 expression plasmid, which was created by moving the XbaI-HindIII fragment from pOR262 (39) containing the N-terminally modified rat reductase with a fused ompa signal peptide and ribosome binding site into pET29a (+) (Novagen, Madison, WI). The resulting plasmid, pETOR262, was transformed into E. coli strain HMS174(DE3) (Novagen). Cells were grown at 37°C with 240 rpm in 1-liter cultures of TB medium with 30 μl of kanamycin to micro phase and then into micro phase with 0.1 mM isopropyl-1-thio-β-galactopyranoside and grown for an additional 20 h at 30°C with 190-rpm shaking. Reductase was solubilized from E. coli membranes and purified on a 2′,5′-ADP column as described previously (39).

**Steroid Hydroxylase Assays—**CHAPS-solubilized E. coli membrane preparations were used directly by reconstituting 10 pmol of P450 with 40 pmol of E. coli-expressed rat NANDPH-P450 reductase, 10 pmol of rat cytochrome b5, and 0.1 mg/ml DOPC in a minimal volume; assays were performed for 5–15 min at 37°C in 15 mM MgCl2, 50 mM HEPES buffer (pH 7.6), 0.1 mg/ml DOPC, 0.06% CHAPS, and 1 mM NANDPH. L-α-NF stock solutions were made in 100% methanol. Care was taken so that methanol concentrations in all reactions were equivalent and did not exceed 1% of the total reaction volume. Progesterone and L-α-NF concentrations varied according to the experiment and are described in the individual figure legends. Assays involving testosterone were performed using 25 μM testosterone with or without 25 μM L-α-NF. Identification of metabolites of progesterone (6β-OH- and 16alpha-OH-progesterone) and testosterone (2β-OH-, 6β-OH-, and 16α-OH-testosterone) was by co-chromatography using authentic standards (Steraloids, Wilton, NH). A commercial source of 16β-OH-testosterone was available; identification of this metabolite was by relative mobility in TLC and comparison with the published values for TLC analysis of testosterone metabolites (40).

**RESULTS**

**Alanine-scanning Mutagenesis of P450 3A4 Amino Acid Residues 210–216**—The SRS mutant (15) for P450 family 2 enzymes has proven to be useful for predicting residues that may influence the geometry of the active site and thus affect enzymatic activity. In a search for residues that determine substrate specificity and flavonoid stimulation of human P450 3A4, a P450 family 3 enzyme, we made site-directed mutations over a short region in P450 3A4 that is predicted to align with the C-terminal end of helix F. This region contains the active site residues Thr488, Phe490, and Met492 from P450, 3A4 and P450BM-3, respectively (Fig. 2), and corresponds to SRS-2 in P450 family 2. Alanine-scanning mutagenesis (41) was used to individually change P450 3A4 amino acid residues 210–216 to alanine. All of the resulting alanine substitution mutants were sufficiently expressed in an E. coli heterologous expression system at levels comparable with the N-terminally modified wild type construct (data not shown).

**Progesterone Hydroxylase Activities of P450 3A4 Wild Type and Alanine Substitution Mutants 210–216**—Solubilized membrane fractions from E. coli expressing P450 3A4 wild type or individual alanine substitution mutants were examined in reconstituted systems containing HEPES buffer with MgCl2.

**Fig. 2. A portion of the multiple sequence alignment by Hase-**

He et al. (33). Underlined residues indicate amino acids that help form the active site. P450 3A4 residues that were individually changed to alanine in this study are indicated by a dot. The single-letter amino acid code is used with gaps in individual sequences indicated by a dot.
cytochrome b<sub>5</sub>, DOPC, and 0.06% CHAPS as described under "Experimental Procedures" for progesterone hydroxylase activity. Table I displays the results of these analyses. All mutants retained progesterone hydroxylase activity, forming two predominant metabolites, 6α- and 16α-hydroxyprogesterone. In the absence of α-NF, the ratios of 6β-hydroxyprogesterone to 16α-hydroxyprogesterone (β/α ratios) of most of the mutants were similar to the wild type enzyme, the exception being mutants L210A and L211A, which had slightly elevated β/α ratios.

Previous studies have shown that, depending on the substrate and α-NF concentrations employed, individual metabolites from a single substrate can be differentially stimulated/inhibited by α-NF (13, 42). Table I demonstrates that the 16α-hydroxylase activity of wild type P450 3A4 is stimulated to a slightly greater extent by α-NF than the 6β-hydroxylase activity of the enzyme. At a substrate concentration of 25 μM progesterone, wild type P450 3A4 had a β/α ratio equal to 6.0 in the presence of the methanol control and 4.5 in the presence of 25 μM α-NF. One of the mutants, L210A, showed the opposite pattern of stimulation; i.e. 6β-hydroxylase activity was stimulated more by α-NF than the 16α-hydroxylase activity. Because production of the 16α-OH-progesterone metabolite was only stimulated 1.3-fold (compared with 6.0-fold for wild type enzyme) in the presence of α-NF, the β/α ratio was 19.2 for L210A as compared with 4.5 for wild type P450 3A4. Another interesting mutant is L211A, which showed less than 2-fold stimulation of both the 6β- and 16α-hydroxylase activities by α-NF (1.2- and 1.8-fold, respectively). Alanine substitution mutants 212–216 maintained β/α ratios more closely resembling the wild type enzyme in either the absence or presence of α-NF; for these mutants, α-NF always caused at least 2.2-fold stimulation and increased the 16α- over the 6β-hydroxylase activity.

The effect of α-NF on the observed progesterone hydroxylase activity was tested over a range of α-NF concentrations to determine whether the low levels of stimulation of the activities of the L210A and L211A mutants could be overcome by using higher concentrations of α-NF. When the progesterone concentration was kept at 25 μM and the α-NF concentration was increased to 200 μM, no further increase in progesterone hydroxylation was seen for P450 3A4 wild type, L210A, or L211A as compared with the stimulation by 25 μM α-NF (data not shown).

**Kinetic Analysis of the 6β-Hydroxylation of Progesterone in the Absence and Presence of α-NF**—It was previously noted (12) that in the absence of α-NF the rates of progesterone 6β-hydroxylation by rabbit and human liver microsomes gave nonlinear kinetics in a double reciprocal plot. Upon the addition of α-NF, however, the double reciprocal plot became linear. To determine whether the kinetics for L210A and L211A, the two mutants least responsive to α-NF stimulation, were similar to P450 3A4 wild type, the dependence of progesterone 6β-hydroxylation on substrate concentration in the presence or absence of 25 μM α-NF was determined (Fig. 3). Double reciprocal plots showed that wild type P450 3A4, L210A, and L211A displayed nonlinear kinetics in the absence of α-NF. When 25 μM α-NF was included, the double reciprocal plot became linear.

The kinetic analyses revealed that at high substrate concentrations the progesterone hydroxylase velocities obtained in the absence of α-NF approached those obtained in its presence. To better illustrate this phenomenon, the -fold stimulation by 25 μM α-NF was plotted as a function of progesterone concentration (Fig. 4). At high substrate concentrations (320 μM progesterone), essentially no stimulation of 6β-hydroxylase activity by α-NF was seen for wild type P450 3A4, L210A, or L211A (Fig. 4A). As the progesterone concentration was lowered, wild type 3A4 became more responsive to stimulation by α-NF. P450 3A4 mutants L210A and L211A were less responsive than wild type to α-NF stimulation, especially at the lower concentrations of progesterone. For example, at 10 μM progesterone and 25 μM α-NF, the 6β-hydroxylase activity of L210A was stimulated 4.3-fold and L211A was stimulated 2.6-fold as compared with the 10-fold stimulation of the wild type enzyme. Fig. 4B demonstrates a similar relationship between stimulation of progesterone 16α-hydroxylation and progesterone concentration. At 320 μM progesterone, no α-NF stimulation of 16α-hydroxylase activity was observed. As the progesterone concentration was decreased, the responsiveness of the wild type enzyme to α-NF stimulation increased. Thus, at 10 μM progesterone, the 16α-hydroxylase activity was stimulated 30-fold by α-NF. P450 L211A was stimulated only 6-fold at 10 μM progesterone, and L210A was stimulated just 2-fold.

**Stereoselectivity of Testosterone Hydroxylation by Alinate Substitution Mutants**—Table II summarizes the hydroxylase activities for wild type P450 3A4 and all of the mutants. As previously reported (4, 42), wild type P450 3A4 produces primarily three metabolites, 2β-, 6β-, and 15β-OH-testosterone, with 6β-hydroxylation accounting for 91% of the activity. All mutants retained testosterone hydroxylase activity, remaining primarily testosterone 6β-hydroxylases. In the absence of α-NF, the metabolite percentages were approximately the same between the wild type enzyme and alinate substitution mutants 212–216. L210A had lower testosterone 2β- and 15β-hydroxylase activities than the wild type enzyme both in the absence and presence of α-NF. L210A produced an additional metabolite, 16α-OH-testosterone at a rate (0.08 nmol/min/mg) much higher than the wild type enzyme (<0.01 nmol/min/mg). L211A, in the absence of α-NF, had a higher percentage of 2β- and 15β-hydroxylase activities than the wild type enzyme.

As with the unequal α-NF stimulation of wild type P450 3A4 progesterone 16α- and 6β-hydroxylase activities, the testosterone 2β- and 15β-hydroxylase activities were preferentially
stimulated 12.6- and 9.6-fold, respectively, as compared with the 2.9-fold stimulation of the testosterone 6β-hydroxylase activity. In the presence of α-NF the testosterone metabolite profiles of mutants at positions 212–216 remained remarkably similar to the wild type enzyme. However, altered α-NF responsiveness of P450 3A4 mutants L210A and L211A was observed with testosterone, as illustrated in Fig. 5. L210A, because of decreased stimulation of 2β- and 15β-hydroxylase activities, maintained a high percentage of 6β-OH-testosterone activity (94% versus the wild type 74%). In contrast, the P450 3A4 mutant L211A, because of decreased stimulation of 6β-OH-testosterone activity, displayed a lowered percentage of 6β-OH-testosterone formation (53% versus the wild type 74%). P213A showed α-NF stimulation of 2β- and 15β-hydroxylase activities 2-fold greater than wild type enzyme, although the metabolite percentages in the presence of α-NF closely resembled the wild type profile. The remaining mutants maintained testosterone metabolite ratios and responsiveness to α-NF very similar to the wild type enzyme (Table II).

Response of P450 3A4 Wild Type, L210A, and L211A to Partial or Modified Analogs of α-NF—A previous study (13) tested a number of partial or modified analogs of α-NF for their ability to modulate P450 3A4 activity. Using polycyclic aromatic hydrocarbons as test substrates, it was found that α-NF, flavone, and 4-chromanone could stimulate P450 3A4 as measured by progesterone 6β- and 16α-hydroxylase activities. However, rather than being an inhibitor, β-NF was an effective stimulator of P450 3A4 as measured by progesterone 6β- and 16α-hydroxylase activities. However, rather than being an inhibitor, β-NF was a mild stimulator of P450 3A4 progesterone hydroxylase activity (1.3- and 1.9-fold stimulation of the 6β-OH and 16α-OH progesterone activities, respectively).
TABLE II

| 3A4 sample | MeOH | α-NF | α-Fold stimulation by α-NF |
|------------|------|------|---------------------------|
|            | 2β-OH | 6β-OH | 15β-OH | 2β-OH | 6β-OH | 15β-OH |
| Wild type  | 0.07 (5) | 1.3 (91) | 0.05 (4) | 0.88 (17) | 3.8 (74) | 0.48 (9) |
| L210A      | 0.02 (1) | 1.9 (97) | 0.03 (2) | 0.17 (4) | 3.8 (94) | 0.09 (2) |
| L211A      | 0.21 (9) | 2.0 (84) | 0.17 (7) | 1.6 (34) | 2.5 (53) | 0.61 (13) |
| R212A      | 0.06 (4) | 1.4 (93) | 0.05 (3) | 0.77 (15) | 3.8 (77) | 0.38 (8) |
| F213A      | 0.03 (2) | 0.8 (96) | 0.03 (2) | 0.72 (17) | 3.0 (72) | 0.48 (11) |
| D214A      | 0.07 (5) | 1.2 (91) | 0.05 (4) | 0.65 (18) | 2.7 (73) | 0.35 (9) |
| F215A      | 0.08 (5) | 1.4 (92) | 0.05 (3) | 0.70 (13) | 4.4 (80) | 0.40 (7) |
| L216A      | 0.12 (5) | 2.1 (91) | 0.10 (4) | 1.27 (15) | 6.1 (74) | 0.91 (11) |

Values are nmol of product formed/min/nmol of P450 and are the mean of quadruplicate determinations. Numbers in parentheses represent the amount of metabolite as a percentage of the total (2β-OH-, 6β-OH-, and 15β-OH-testosterone).

DISCUSSION

The results presented here describe for the first time the identification of certain key amino acid residues that play a role in substrate specificity and flavonoid stimulation of P450 3A4. The region selected for mutagenesis was predicted to be structurally similar to a portion of the F-helices of several bacterial enzymes of known crystal structure (33). However, even the alignment of proteins of known structure is subject to interpretation in this and several other key regions (for review, see Ref. 43). For example, a previous study of SRS-1 of P450 2C2 used a degenerated cassette to introduce random amino acid substitutions into a portion of the enzyme; this was helpful in confirming an alignment with P450 cam (32). Targeting contiguous residues that are predicted to form SRSs has the potential of identifying both the residues that comprise the active site and, just as importantly, adjacent residues that do not contribute to the active site. For mutagenesis of the proposed SRS-2 region of P450 3A4, Ala was used as the substituting residue because of its small side chain, low tendency to distort the C-α backbone, and minimal electrostatic effects. Of the seven P450 3A4 residues targeted by Ala in this report, substitutions at positions 212 and 214–216 did not affect activity, suggesting that these residues do not effectively influence the substrate or effector binding sites. F213A displayed greater α-NF stimulation of testosterone hydroxylase activities than wild type P450 3A4. The remaining two mutants, L210A and L211A, exhibited altered basal activity and/or flavonoid responsiveness, suggesting that the substrate binding and effector sites are at least partially overlapping.

The presence of highly conserved residues can be an indication of the importance of a position to enzyme function. The two Leu residues at positions 210 and 211 of P450 3A4 are highly conserved, being present in P450s 3A1 and 3A2 from rat, 3A3 and 3A7 from human, 3A6 from rabbit, 3A8 from monkey, and 3A12 from dog. The exception is human P450 3A5, which instead has Phe at position 210. Substitution of Ala at Leu residues 210 and 211 did not cause P450 3A4 to lose its preference for steroid 6β-hydroxylation. This observation is in contrast to mutations at SRS-2 position 209 of 2A4 and 2A5 (24, 26, 44) and the corresponding position 206 of 2B1 (17), which conferred a radical shift in steroid hydroxylation specificities. Hydroxylation of steroids by P450 3A4, which is likely to have a very large active site, may be controlled more by the inherent chemical reactivity of the allylic 6-position than by steric constraints exerted by the enzyme, similar to P450 2B4-mediated hydroxylation of camphor. With P450 2B4, because of considerable movement of the substrate molecule in the active site, camphor hydroxylation occurred preferentially at sites predicted by chemical reactivities (45). Subtle changes on testosterone hydroxylation could be seen for the P450 3A4 mutants L210A and L211A at positions other than the 6-position of the substrate. For example, in the absence of stimulator, L210A had a lower percentage of testosterone 2β- and 15β-hydroxylation and increased testosterone 16α-hydroxylation compared with the wild type enzyme. Interestingly, substitution of Ala at the adjacent position, Leu211, had an opposite effect on the percentage of testosterone 2β- and 15β-hydroxylation activities, increasing them above the wild type levels. In the proposed alignment (Fig. 2), Leu210 aligns with active site residues Thr185 of P450 cam and Phe188 of P450 sar. In the alignment of
results with α-NF stimulation of P450 3A4 testosterone and progesterone hydroxylation follow this general rule; namely, the progesterone 16α-hydroxylase activity and the testosterone 2β- and 15β-hydroxylase activities (positions that are located at the narrow ends of both steroids) are stimulated more than hydroxylation at the 6β (middle of the molecule) position. The two substitutions Leu$^{210}$ → Ala and Leu$^{211}$ → Ala seem to have opposite effects on differential stimulation of hydroxylation at the end versus the middle of the steroid; L210A shows less α-NF stimulation of end position hydroxylation, while L211A shows less stimulation of 6β-hydroxylation. Previously, the importance of the exact residue identity at position 209 of P450 2A5 was studied by engineering nine individual amino acid substitutions (24). A more detailed analysis of positions 210 and 211 of P450 3A4 will require additional substitutions, additional substrates, and computer modeling (46).

The mutagenesis results, which identify residues that are able to influence stereospecificity of testosterone hydroxylation and effector activity, do not yet point to an obvious mechanism for α-NF stimulation. An early study with rabbit and human liver microsomes demonstrated that α-NF stimulation of progesterone 16α-hydroxylation and 17β-estradiol 2-hydroxylation was largely due to a decreased $K_m$ with little change in $V_{max}$ (12). From their kinetic data the authors were not able to distinguish whether the interaction between enzyme and effector occurred at the active site or at a separate effector binding site. A more recent study (13) using P450 3A4 expressed in HepG2 cells presented evidence that phenanthrene and α-NF are present simultaneously in the active site and that the stimulation of phenanthrene metabolism by α-NF is a result of increased $V_{max}$ and not decreased $K_m$. Again, the authors were unable to pinpoint an exact mechanism for α-NF action. Our kinetic results (Fig. 3), using the data points from the highest progesterone concentrations, are most consistent with α-NF stimulating P450 3A4 progesterone hydroxylation rates by lowering the $K_m$ and not by increasing the $V_{max}$. The mechanism of α-NF stimulation of P450 3A4 remains to be elucidated, but we do now have evidence that single residue substitutions in an active site region of P450 3A4 can influence the ability to respond to effector, suggesting that the active site and the effector site are at least partially overlapping.

The finding that the structure-based alignment of Hasemann et al. (33) was accurate enough to predict the existence of key P450 3A4 residues in one of the most difficult to align regions suggests that the SRS model may be highly appropriate for the 3A subfamily. We anticipate that mutagenesis of the more conserved SRS regions will be useful for elucidating the key residues that determine structure-activity relationships of the important P450 3A subfamily. Additional mutants with altered substrate specificities and/or altered responsiveness to flavonoids should help to determine whether substrate binding and effector sites are partially or completely overlapping.

The use of mutant enzymes that exhibit enhanced as well as diminished stimulation by α-NF may prove to be valuable in elucidating the mechanism of action of flavonoids on P450 3A enzymes. In order to understand the mechanistic basis for flavonoid action, wild type and mutant enzymes will need to be studied in terms of some of the key individual steps in the P450 cycle. For example, the stoichiometry of hydrogen peroxide or hydroxylated product formed/NADPH or oxygen consumed can give a measure of the degree of uncoupling observed in the presence or absence of effector. Recent studies with site-directed mutants of bacterial cytochromes P450$_{cam}$ and P450$_{BM-3}$ provide an excellent illustration of the usefulness of these types of experiments in understanding the mechanistic basis of al-

![Compounds tested and the fold stimulation observed of progesterone hydroxylase activities of P450 3A4 wild type, L210A, and L211A. A, chemical structures of the stimulators/inhibitors used in this study. ANF, α-naphthoflavone; BNF, β-naphthoflavone. Shown are the effects of 25 μM test compound on progesterone 6β-hydroxylation (B) or progesterone 16α-hydroxylation (C) using 25 μM progesterone as test substrate. Incubations were performed for 10 min as described under “Experimental Procedures.” Values are an average of duplicate determinations.](image-url)

Hasemann et al. (33) Leu$^{210}$ of P450 3A4 is also aligned with position 209 of P450 2A4 and position 206 of P450 2B1. These results suggest that the alignment of P450 3A4 with P450$_{terp}$, P450$_{cam}$, and SRS-2 of P450 2A4 and P450 2B1 may be fairly accurate for this region. Met$^{185}$ of P450$_{BM-3}$ is not aligned with P450 3A4 position 210, although it is not aligned with the corresponding residues in P450$_{terp}$ or P450$_{cam}$ either. Another published alignment has the residues from positions 185 of P450$_{cam}$, 188 of P450$_{terp}$, and 185 of P450BM-3 aligned with position 206 of P450 2B1 (46).

It was previously noted with polycyclic aromatic hydrocarbon substrates (13) that α-NF differentially stimulates P450 3A4 hydroxylation of the narrower portions of the substrate. Our
tered substrate metabolism by mutant enzymes (47, 48). The effects of cytochrome b5 will also be examined in light of the previous finding that some mutations at position 209 in SRS-2 of the P450 2A subfamily altered the coupling efficiency of electron transport to substrate oxidation (49).

Acknowledgments—We thank Dr. Gilbert H. John for isolating the P450 3A4 cDNA and David Stepp for technical assistance.

REFERENCES

1. Guengerich, F. P. (1992) in Cytochrome P450: Structure, Mechanism, and Biochemistry (Ortiz de Montellano, P. R., ed) pp. 473–515, Plenum Press, New York
2. Guengerich, F. P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 462–465
3. Raney, K. D., Grogan, J. D., Harris, T. M., and Guengerich, F. P. (1991) Chem. Res. Toxicol. 5, 202–210
4. Huang, M.-T., Johnson, E. F., Muller-Eberhard, U., Koop, D. R., Coon, M. J., Fischer, V., Tyndale, R., Inaba, T., Kalow, W., Gelboin, H. V., and Korzekwa, K. R. (1994) Biochemistry 33, 493–499
5. Shou, M., Grogan, J., Mancewicz, J. A., Kraus, K. W., Gonzalez, F. J., Gelboin, H. V., and Korzekwa, K. R. (1994) Biochemistry 33, 6450–6455
6. Yamano, S., Mancewicz, J. A., Friedman, J. H., and Guengerich, F. P. (1995) Chem. Res. Toxicol. 8, 218–225
7. Gotow, O. (1992) J. Biol. Chem. 267, 83–90
8. Liu, J., He, Y. A., and Halpert, J. R. (1996) Arch. Biochem. Biophys. 327, 167–173
9. Luo, Z., He, Y., and Halpert, J. R. (1994) Arch. Biochem. Biophys. 309, 52–57
10. Kedzie, K. M., Balfour, C. A., Escobar, G. Y., Grimm, S. W., He, Y., Pepperl, D. J., Regan, J. W., Stevens, J. C., and Halpert, J. R. (1991) J. Biol. Chem. 266, 22455–22461
11. He, Y., Luo, Z., Klokotka, P. A., Burnett, V. L., and Halpert, J. R. (1994) Biochemistry 33, 4419–4424
12. He, Y., Luo, Z., Klokotka, P., Burnett, V. L., and Halpert, J. R. (1994) Biochemistry 33, 9229–9232
13. Hasler, J. A., Harlow, G. R., Sklarz, G. D., John, G. H., Kedzie, K. M., Burnett, V. L., He, Y.-A., Kaminsky, L. S., and Halpert, J. R. (1994) Mol. Pharmacol. 46, 338–345
14. Harlow, G. R., and Halpert, J. R. (1990) Arch. Biochem. Biophys. 286, 85–92
15. Wu, N., Iwasaki, M., Darden, T. A., Pedersen, L. G., Davis, D. G., and Halpert, J. R. (1993) J. Biol. Chem. 268, 16433–16435
16. Lindberg, R. L. P., and Negishi, M. (1989) Nature 339, 632–634
17. Haseman, C. A., Kurumbail, R. G., Bode, S. S., Jenner, P., and Deinzenlofer, J. (1995) Curr. Biol. 2, 41–42
18. Imai, Y., and Nakamura, M. (1989) Biochem. Biophys. Res. Commun. 158, 717–722
19. Kronbach, T., Kemper, B., and Johnson, E. F. (1991) Biochemistry 30, 6097–6102
20. Kronbach, T., and Johnson, E. F. (1991) Biochemistry 30, 6215–6220
21. Kaminsky, L. S., de Morais, S. M. F., Faletto, M. B., Dunbar, D. A., and Goldstein, J. A. (1992) Mol. Pharmacol. 43, 234–239
22. Imaoka, S., Imai, Y., Shimada, T., and Funae, Y. (1992) J. Biol. Chem. 268, 21997–22003
23. Haseman, C. A., Kurumbail, R. G., Bode, S. S., Peterson, J. A., and Deinzenlofer, J. (1995) Curr. Biol. 2, 41–42
24. Imai, Y., and Nakamura, M. (1989) Biochem. Biophys. Res. Commun. 158, 717–722
25. Kronbach, T., Kemper, B., and Johnson, E. F. (1991) Biochemistry 30, 6097–6102
26. Kronbach, T., and Johnson, E. F. (1991) Biochemistry 30, 6215–6220
27. Kaminsky, L. S., de Morais, S. M. F., Faletto, M. B., Dunbar, D. A., and Goldstein, J. A. (1992) Mol. Pharmacol. 43, 234–239
28. Imaoka, S., Imai, Y., Shimada, T., and Funae, Y. (1992) Biochem. Biophys. Res. Commun. 158, 717–722
29. Kronbach, T., Kemper, B., and Johnson, E. F. (1991) Biochemistry 30, 6097–6102
30. Kronbach, T., and Johnson, E. F. (1991) Biochemistry 30, 6215–6220
31. Kaminsky, L. S., de Morais, S. M. F., Faletto, M. B., Dunbar, D. A., and Goldstein, J. A. (1992) Mol. Pharmacol. 43, 234–239
32. Imaoka, S., Imai, Y., Shimada, T., and Funae, Y. (1992) Biochem. Biophys. Res. Commun. 158, 717–722
33. Haseman, C. A., Kurumbail, R. G., Bode, S. S., Peterson, J. A., and Deinzenlofer, J. (1995) Curr. Biol. 2, 41–42
34. Imai, Y., and Nakamura, M. (1989) Biochem. Biophys. Res. Commun. 158, 717–722