Site-directed mutagenesis of a domain (amino acids 299–338) aligning to the 1-helix region of P450cam, P450BM3, and P450terp was used to investigate the different regioselectivities displayed in the hydroxylation reactions performed by human aldosterone synthase (P450aldo) and 11β-hydroxylase (P45011b). The two enzymes are 93% identical and are essential for the synthesis of mineralocorticoids and glucocorticoids in the human adrenal gland. Single replacement of P450aldo residues for P450cam-specific residues at positions 296, 301, 302, 320, and 335 only gave rise to slightly increased 11β-hydroxylase activities. However, a L301P/A320V double substitution increased 11β-hydroxylase activity to 60% as compared with that of P450cam. Additionally substituting Ala-320 for Val-320 of P45011b further enhanced this activity to 85%. The aldosterone synthase activities of the mutant P450aldo proteins were suppressed to a varying degree, with triple replacement mutant L301P/E302D/A320V retaining only 10% and double replacement mutant L301P/A320V retaining only 13% of the P450aldo wild type activity. These results demonstrate a switch in regio- and stereoselectivities of the engineered P450aldo enzyme due to manipulation of residues at three critical positions, and we attribute the determination of these features in P450aldo to the structure of a region analogous to the 1-helix in P450cam.

In the adrenal gland essential steroid hormones such as glucocorticoids, mineralocorticoids, and androgens are produced. Cortisol, the major glucocorticoid in humans, is synthesized in the zona fasciculata/reticularis under control of pituitary derived adrenocorticotropic hormone, whereas the most sized in the zona fasciculata/reticularis under control of pituitary derived adrenocorticotropic hormone, whereas the most highly biosynthesized glucocorticoid in humans is cortisol, the major glucocorticoid in humans, is synthesized in the zona fasciculata/reticularis under control of pituitary derived adrenocorticotropic hormone, whereas the most highly biosynthesized glucocorticoid in humans is cortisol, the major glucocorticoid in humans. In bovine (9), porcine (10), and frog (11) adrenal cortex, synthesis of glucocorticoids is catalyzed by a single enzyme. Conversely, synthesis of human, rat (12), and mouse (13) glucocorticoids are separated in evolution and are carried out by distinct enzymes, yet the reason for these interspecies differences is enigmatic. We intended to gain insight into the principles underlying the different regioselectivities involved in 11β-hydroxylation in 18-hydroxylation/oxidation in the human enzymes. Since both proteins are 93% identical yet carry out separate reactions to yield different steroid hormones, it remained elusive on which structure-function relationships these diversities could be based. Recently, the cause of glucocorticoid-mediated aldosteronism, an autosomal dominant disorder in humans, was reported to arise from unequal crossing-over events between the CYP11B1 and CYP11B2 genes (14). The resulting chimeric genes comprise a 5’ CYP11B1 portion and a 3’ CYP11B2 portion under control of the CYP11B1 regulatory region. Pascoe et al. (15), through the analyses of hybrid proteins, determined the C-terminal 247 amino acids of P450aldo as crucial for aldosterone synthesis. Keeping this in mind, we carried out a computer-based sequence and structure alignment with three of the four by now crystallized P450 proteins, namely P450cam, Pseudomonas putida (16), P450BM3 from B. megaterium (17), and P450terp from another Pseudomonas species (18). We performed site-directed mutagenesis on a region supposedly analogous to the P450cam 1-helix and subsequent analyses of the mutants by transient transfection experiments using COS-1 cells. This led to the identification of mutant P450aldo proteins having dramatically increased 11β-hydroxylase activity, which in the P450aldo wild type protein is considerably lower than in the P45011b wild type protein (19). Concomitantly, aldosterone synthase activity in these mutants was lost to a substantial degree, indicating that regioselectivities have successfully been switched from one position to the other.
Site-directed Mutagenesis of P450<sub>aldo</sub> 8029

EXPERIMENTAL PROCEDURES

Materials

COS-1 cells were obtained from the American Type Culture Collection. Culture media and antibiotics were from Seromed (Berlin), and fetal bovine serum was from Life Technologies, Inc.

Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer at BioTex (Berlin). Restriction enzymes, Klenow fragment of DNA polymerase I, T4 polynucleotide kinase, bovine alkaline phosphatase, T4 DNA ligase, and DH5α cells were purchased from New England Biolabs Inc. or Boehringer Mannheim. T4 DNA polymerase was obtained from Perkin-Elmer. pALTER-1 plasmid, helper plasmid ρR408, J M109, and E1351 mutS cells were obtained as part of the Altered Sites in vitro mutagenesis system (Promega Co., Madison, WI). pRc/CMV was from Invitrogen, and pBS SK(−) was from Stratagene. The Δ Taq<sup>cyc</sup> cycling kit was purchased from U.S. Biochemical Corp., and [γ<sup>32</sup>P]dATP was from Amersham Corp. [3H]Deoxycorticosterone and [4<sup>3</sup>H]Deoxycorticosterone were obtained from DuPont NEN. Deoxycorticosterone, corticosterone, deoxycorticosterol, cortisol, chloroquine, cell culture-tested HEPES, and dimethyl sulfoxide were purchased from Sigma. DEAE-dextran was purchased from Pharmacia Biotech Inc. Radioimmunoassays were performed with Active<sup>™</sup> coated radiiodimma-

Assay kits from Diagnostic System Laboratories Inc. ECL Western blotting reagents were obtained from Amersham, polyclonal hemagglu- tinin (HA) 11 antibody was from the BABCO Berkeley Antibody Company, and the polyclonal anti-bovine adrenodoxin (Adx) antibody was raised in rabbit by Eurogentec SA. Nitrocellulose membrane was used from Schleicher & Schuell.

Methods

Epitope-Tagging of the CYP11B1 and CYP11B2 cDNAs and Construction of the Parent Plasmids Expressing P450<sub>aldo</sub> and P450<sub>11β</sub>—To be able to detect proteins in transfected COS-1 cells an HA epitope (32) was C-terminally fused to the P450<sub>aldo</sub> and P450<sub>11β</sub> encoding cDNAs via polymerase chain reaction. A common 5′-oligonucleotide, 5′-GGGCTCTAGAATCTGGAAAAGGGCC-3′, harboring an Xba site and the translation initiation site was used. BamH1-linked 3′-oligonucleotides 5′-GGGGGCACTACTACCGGTAAAGGTCAG for CYP11B1 and 5′-GGGGGCACTACTCAAGGGCTAAAGGTCAG for CYP11B2. The stop codons are underlined, and the HA epitope encoding sequence is in italics. The 548-base pair fragments were subcloned into pBS SK (+) to yield pBS-B1-ha and pBS-B2-ha. pRc/CMV was digested with BglII, blunt with Klenow fragment from DNA-polymerase I and further cut with XbaI to be subsequently ligated with XbaI EcoRl fragment from pBS-B1WT and pBS-B2WT, respectively. The sequences were verified by nucleotide sequencing using the chain termination method (20) and the Δ Tqa<sup>cyc</sup> sequencing kit.

Insertion of Mutations into the CYP11B2 cDNA by Site-directed Mutagenesis—The XbaI/BamH1 fragment from pBS-B2-ha was inserted into the pALTER-1 phagemid and transformed into J M109. Site-directed mutagenesis was performed according to Kunkel et al. (21) following instructions by the supplier (Promega). For the generation of multiple mutants several cycles of the mutagenesis procedure were performed by amp knock-out and tet repair or vice versa using the same vector molecule. The base substitutions were confirmed by sequencing first in pALTER-1 and a second time in pRc/CMV, into which they were inserted. All standard procedures were carried out as described by Sambrook et al. (22).

Cell Culture and Transfection—COS-1 cells were maintained at 37°C and 8% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml benzyl penicillin, and 0.1 mg/ml streptomycin. Transfections were done as described by Zuber et al. (23) except for some minor modifications. One day prior to transfection cells were plated at a density of 350,000/cm². For transfection cells were starved for 1 h in medium containing 20 mM HEPES and lacking fetal bovine serum, which was then aspirated and substituted for the same medium containing 5 μg of pBAdx4 (a kind gift from M. Waterman, Nashville, TN), and 0.5 mg of DEAE-dextran (M, 500,000) in starvation medium. After another incubation of 1 h, chloroquine was added in 2 ml of complete medium to a final concentration of 52 μM, and cells were kept under these conditions for 4 h. Finally, a 2-min treatment with dimethyl sulfoxide in Dulbecco’s modified Eagle’s medium was carried out; cells were washed twice in Hank’s balanced salt solution and grown for 24 h before substrate addition. Hydroxylase Assays—Transfected cells were incubated for 48 h with either [1,2-3H]cortisol or [3H]deoxycorticosterone. For extraction of ste- roids the medium was combined with 4 volumes of an ethanol/aceton mixture (1:1) and incubated at room temperature (24). After pelleting the debris, the supernatant was transferred to a fresh tube and evaporated to the original volume. Steroids were extracted with 2 volumes of dichloromethane and the organic phase was evaporated. The residue was dissolved in 0.5 ml of hexane and centrifuged at maximal speed, giving a small pellet. Then the supernatant was rotated under vacuum to dryness, and the extraction products were redissolved in 150 μl of 10% (v/v) isopropranol alcohol in hexane. After the addition of a combina- tion of internal steroid standards the samples were subjected to normal phase HPLC using a Lichrosorb® Diol column (Merck, Darmstadt, FRG). A gradient solvent system was run starting with 15% (v/v) isopropranol alcohol in n-hexane at a flow rate of 1.3 ml/min. The standards were monitored by UV detection at 254 nm, while the radioactivity was assayed with a Betascan Sc radiation detector fitted to the HPLC.

Alternatively, steroids were measured using an Active<sup>™</sup> aldosteron or Active<sup>™</sup> cortisol radioimmunoassay.

Immunodetection of Expressed Proteins—Transfected cells were incubated twice with ice-cold phosphate-buffered saline and scraped in 0.5 ml of phosphate-buffered saline. Lysis was done in radioimmuno pre- precipitation buffer (10 mM Tris-Cl, pH 7.4, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) supplemented with 0.04 units of aproatin/ml (Sigma) and 0.1 μg/ml leupeptin (Sigma) for 10 min on ice followed by 6 s of sonication, and lysates were boiled for 5 min in SDS-polyacryl- amide gel electrophoresis sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis according to Laemmli (25), blotted to a nitrocellulose filter, and detected by hybridization with an anti-HA or anti-bovine Adx antibodies using the ECL system from Amersham.

Alignment of P450 Sequences—An initial alignment of the P450<sub>aldo</sub> and P450<sub>11β</sub> sequences was performed using HOMOL, a data base created by D. Nelson in which he related 155 different P450 proteins to P450<sub>cam</sub>. The mode of alignment takes the identity and resemblance of single amino acid residues as well as secondary structure predictions into consideration. We added the structural alignments of P450<sub>11β</sub> and P450<sub>cam</sub> to our assignation in order to increase possible predictions for structural entities in P450<sub>aldo</sub> and P450<sub>11β</sub>. Updating of the sequences and further optimization was carried out by hand. In addition, we included the sequences of P450<sub>cam</sub> and P450<sub>11β</sub> from other species by using CLUSTAL. The first amino acid of the different ste- roidogenic proteins to be considered was the first residue of the mature sequences.

**RESULTS**

Alignment of P450<sub>aldo</sub> and P450<sub>11β</sub> with P450<sub>cam</sub>, P450<sub>11β</sub>mp, and P450<sub>11β</sub>—Secondary structure is much better conserved in cytochrome P450 proteins than would be expected from an unprocessed proteins, respectively. Considering the distribu-

D. Nelson, personal communication.
Furthermore, Thr-252 of P450cam is highly conserved in P450 corresponding to the I-helix in P450cam. P450cam I-helix extends from amino acids 299–338, which is labeled on the right side. Amino acids differing between the two proteins are printed in boldface type. P450cam residues at positions 296, 301, 302, 320, and 335 were mutated to P450alo residues, and the HA epitope was fused to the C termini of the proteins as described under “Experimental Procedures.”

Of these mismatches, the alignment (Fig. 1) shows that five of them cluster to a region that could be analogous to the I-helix in P450cam (17, 18). This putative domain in P450alo and P45011b would be encoded by the second half of exon 5 and the first half of exon 6 of the genes, a region of amino acid 256, which was reported to be a critical breaking point in the study of hybrid P450aldo and P45011b proteins. The P450alo portion C-terminal region of this residue serves to be important for aldosterone synthase activity (15). Since the I-helix in P450amo apart from the heme binding site and a region containing Tyr-96 (26), contributes to the active site, it also may be, in P450alo and P45011b, a determinant of substrate binding and specificity. Furthermore, Thr-252 of P450cam is highly conserved in P450 proteins (27) and in P450cam, was reported to play a critical role in oxygen activation (28–30). Taking all these observations into account, positions 301, 302, 320, and 335 of P450alo and P45011b could be candidate amino acids for determination of the different regioselectivities in hydroxylation reactions performed by these proteins. Positions 296 and 339 in our analyses did not map to the very ‘putative’ I-helix region but should be flanking it (Fig. 1) and thus may have an influence on the positioning of the helix.

Construction of the P450alo and P45011b Parent Expression Plasmids and of P450alo Mutants—To obtain full-length fragments of P450alo and P45011b encoding cDNAs, we performed reverse transcription-polymerase chain reaction on total RNA from a human adrenal gland as described elsewhere (31). The P45011b cDNA sequence corresponded to the one published by Mornet et al. (4), whereas in the P450alo sequence, amino acid 173 was substituted from Arg to Lys, which was also found by Kawamoto et al. (3). After verifying the functionality of both cDNAs by expression studies, we further engineered them by adding an HA epitope tag (32) to both C termini in order to enable protein detection in subsequent expression experiments.

To investigate the role of the putative I-helix in the regioselectivity of P450alo and P45011b, we performed site-directed mutagenesis on the P450alo cDNA. Derived from the alignment studies, the region corresponding to the I-helix in P450cam extends from amino acid 299 to 338 (Fig. 2). This part of the total sequence includes four diverging amino acids at positions 301, 302, 320, and 335, but we also analyzed the role of Lys-296 in our investigations, since also a flanking amino acid could exert an influence on the positioning of the putative I-helix and thereby the regiospecificity of hydroxylation. Mutants were generated using the mutagenic oligonucleotides listed in Table I. Thus, P450alo-specific amino acid residues were substituted for P45011b-specific ones, and single, double, and triple replacement mutants of P450alo were created.

Expression and Analysis of P450alo Mutants—To analyze the mutant versus the wild type proteins with respect to their hydroxylation specificities we cotransfected the resultant plasmids together with pBAdx4 into COS-1 cells. Expression of bovine adrenodoxin stimulates the activity of mitochondrial steroidogenic P450 enzymes, since the intrinsic amount of adrenodoxin in COS-1 cells seems inadequate for maximum electron transfer between the reductase and the P450 component of the system (23). Since the level in 11β-hydroxylase activity toward 11-deoxycortisol is the most distinguishing feature of P450alo and P45011b, transfected cells were first assayed for their ability to convert 11-deoxycortisol to cortisol, and subsequently their aldosterone-synthesizing potential was estimated. Incubation with low substrate concentrations (0.25 μM 11-deoxycortisol) did not reveal significant differences among P450alo and P45011b wild type proteins under the conditions tested. The prior enzyme, although 11β-hydroxylating at a much lower rate than the latter one, was able to convert more than 90% of 11-deoxycortisol to cortisol within a 48-h interval. However, using higher substrate concentrations (2.5 to 5 μM), clear differences were observed among P450alo and P45011b, so that intermediate activity mutants could be arranged between a maximal and a minimal limit. These flanking values are indicated in representative HPLC profiles in Fig. 3. In addition, at higher substrate concentrations the endogenous ability of COS-1 cells to dehydrogenate cortisol to cortisone by the action of an 11β-hydroxysteroid dehydrogenase became negligible, and the addition of glycyrrhetinic acid, an inhibitor of this activity, was unnecessary. Each mutant was assayed in cell culture by incubation with [3H]11-deoxycortisol, and metabolites were either separated on HPLC or quantitated by radioimmunoassay. The results are summarized in Fig. 4A. The small cortisol values detected in mock-transfected COS-1 cells were due to the antibody used in radiomimune assays, which exerted a slight cross-reactivity to 11-deoxycortisol. Relative to P450alo wild type, the activities of the single replacement P450alo mutants substituted at positions 296 and 335 were only slightly increased, whereas inserting a P45011b residue at position 301, 302, or 320 did enhance the 11β-hydroxylation potential 1.5–2-fold, suggesting that the latter amino acids could be of relative importance for 11β-hydroxylase activity. Combination of single substitutions resulting in double and triple replacement mutants, however, did lead to much stronger effects.Triple mutant L301P/E302D/A320V showed the most pronounced increase in 11β-hydroxylation, accounted for about 85% of the 11β-hydroxylase activity. Also the combined substitutions L301P and A320V did result in an activity en-
hanced to 60% as compared with P450_11b wild type.

Because of the dual ability of the P450_aldol wild type enzyme to produce both cortisol and aldosterone in vitro we investigated aldosterone synthase activity in this set of P450_aldol mutants. The primary question was whether one of the two activities (cortisol synthesis) could be increased without affecting the other activity (aldosterone synthesis) or whether there is a reciprocal behavior to be found, meaning no increase in one activity without loss in the other. Examination of the aldosterone-synthesizing abilities of the mutants showed that triple mutant L301P/E302D/A320V only retained about 10% and double mutant L301P/A320V about 13% of the P450_aldol wild type activity (Fig. 4B). Mutants L301P/E302D and A320V were only slightly compromised in their activities, but the combination of both resulting in the triple mutant synergized drastically to a severe loss in 18-hydroxylation or 18-oxidation activity. Paradoxically, double mutant L301P/E302D was less severely affected in its aldosterone-synthesizing capacity (87% retained as compared with P450_aldol wild type) than the respective single mutants. Introducing P450_11b residues at positions 296 and 335 decreased activity to about 40% in the single mutants. The combined double mutant is reduced to about 15% in its aldosterone-synthesizing capacity as compared with P450_aldol wild type, and additionally substituting Asp at position 335 for Asn totally destroys aldosterone synthase activity. These data show that every alteration made in this region did negatively affect the aldosterone synthase activity of the recombinant enzyme although to a varying degree.

Immunodetection of Proteins—To examine protein synthesis in transfected COS-1 cells we performed Western blotting of whole cell extracts. P450_11b and P450_aldol wild type proteins and the mutants created were detectable to a very similar amount, indicating that the amino acid substitution did not notably impair protein translation and stability (Fig. 5). This, of course, is not a means to distinguish between an apo- and a holoprotein, but one would expect that an apoprotein tends to be malfolded and thus readily would become a target of the mitochondrial degradation machinery. Due to the 11 amino acids comprising the HA tag fused to the C termini of the proteins the molecular masses of the proteins were increased to about 56 kDa as compared with the wild type proteins having molecular masses of 50 and 48.5 kDa, respectively (8). Sequences added to the C termini of the two P450 enzymes did not decrease their hydroxylation abilities (data not shown). We also detected the cotransfected bovine adrenodoxin molecule (molecular weight 14 kDa) in the cells, since this also is a critical parameter determining the electron transport of the system and thus hydroxylation efficiency, and we found it to be present in equal amounts (Fig. 5).

**DISCUSSION**

In pursuing structure to function relationships in mammalian P450 proteins, alignment to the bacterial P450cam and recently also to P450BM_3 and P450erp has proved to be useful approach. However, a relatively low amino acid sequence homology often hampers accurate alignment of specific residues
and ECL chemiluminescence served for visualization of bands. Detection of single replacement mutants in comparison with P450aldo wild type, P45011β hydroxylases. The I-helix in P450cam, like in P450BM3 and structural entities in P450aldo and P45011β (33). We therefore benefited from the HOMOL database developed by D. Nelson, which combines amino acid identities and secondary structure predictions. By including in our alignment also the P450BM3 and P450terp sequences, which (due to the revealed secondary structures) could be structurally aligned to P450cam (17, 18), we intended to improve the prediction of structural entities in P450aldo and P45011β. Since for P450cam it is well established that the I-helix critically participates in substrate binding and because the P450aldo and P45011β proteins show some diversities in a region corresponding to the P450cam I-helix, we hypothesized that these differences could be the basis for the different regioselectivities of the two steroid hydroxylases. The I-helix in P450cam, like in P450BM3 and P450terp, runs like a tube through the interior of the molecule (Fig. 6) and in the case of P450cam, together with the heme binding region, makes up part of the substrate binding pocket (16, 34). One critical feature of this conserved helix in P450 proteins is Thr-252 in P450cam, necessary for the activation of molecular oxygen (28–30). In P450cam the substrate camphor is found to be in tight association with the -VGGL- stretch, where the two Gly residues induce a bend in the helix and thus serve as a site where the substrate can fit into position correctly. There is only one Gly residue of the -VGGL- motif conserved in P450aldo and P45011β (Fig. 1) and it is questionable whether this is sufficient for bending the helix likewise.

A possible relevance of the putative I-helix region for the regioselectivity is also supported by the observations of Pascoe and colleagues (15). By studying artificially engineered hybrid proteins with variable N-terminal P45011β and C-terminal P450aldo portions reflecting an in vitro model of glucocorticoid-remediable aldosteronism, a genetic disorder, His-256, was defined as a critical breakpoint, and the sequence C-terminal of it was identified as essential for aldosterone synthesis. In performing site-directed mutagenesis on the P450aldo protein and assessing 11β-hydroxylase activities in transfected COS-1 cells, we found that single amino acid replacements by P45011β specific residues at positions 296 and 335 only slightly increased 11β-hydroxylase activity and that there was up to a 2-fold increase detected when position 301, 302, or 320 harbored P45011β residues. However, double substitution L301P/ E302D already conferred 60%, and triple replacement mutant L301P/E302D/A320V mounted up to an activity being about 85%, that of the P45011β wild type protein, given that under these experimental conditions the P450aldo wild type enzyme exhibited only 5% of the activity of the P45011β wild type (Fig. 4A). These data indicate a synergistic rather than a mere additive effect contributed by these three residues. A nonconservative change from Leu to Pro at position 301 alone had no substantial impact, although it was expected to exert other than size effects, since Pro in many cases distorts if not destroys helix continuity. In contrast, the overall effect on cortisol production could be drastically enhanced by two additional conservative substitutions at positions 302 and 320, suggesting that the size of the side chains at these positions is critical for 11β-hydroxylase activity.

In assaying the aldosterone-synthesizing activities of the mutants, we found mildly to strongly decreased activities. Interestingly, already single substitutions in some mutants markedly reduced aldosterone synthesis. Contrasting 11β-hydroxylase activity, P450aldo residues Lys-296 and Asp-335 obviously are important for maximum aldosterone synthase activity since single substitution at these positions lowered aldosterone synthesis to about 40%. Moreover, double replacement mutant L296N/D335N showed an aldosterone synthase activity decreased to 15%, which was completely abolished by adding the A320V mutation. Among the mutants still producing aldosterone, double replacement mutant L301P/A320V and triple replacement mutant L301P/E302D/A320V gave rise to the most reduced aldosterone levels (13 and 10% of the P45011β wild type), which reciprocally parallels their increase in 11β-hydroxylase activity. In conclusion, there is no strict correlation between 11β-hydroxylase increase and decrease in aldosterone synthesis to be seen in this set of mutants. Whereas Pro-301, Asp-302, and Val-320 clearly are major contributors to 11β-hydroxylation, aldosterone synthesis, apart from the residues at these positions in the P450aldo sequence also seems to be influenced by Lys-296 and Asp-335.
Aldosterone synthesis thus is dependent on a highly evolved structure in the P450_{aldo} protein and is susceptible to minor changes in this region.

Until now, no CYP11B2 defects have been linked to this region in patients suffering from hypoaldosteronism, but it is conceivable that their occurrence would deteriorate mineralocorticoid synthesis. Whether the positions targeted by site-directed mutagenesis directly contact the substrate or deci-

The I-helix also was postulated in modelling studies of P450_{cr}, by Graham-Lorence and co-workers (35) and P450_{17}, by Laughton and co-workers (36) to form part of the substrate binding pocket for the substrates androstenedion and pregnen-

In contrast, for P450 proteins that belong to the CYP2 family and participate in liver microsomal steroid hydroxylation, it has been shown that also the N-terminal part of these proteins contributes to the substrate specificities and regioselectivities of hydroxylations (reviewed in Refs. 37 and 38). Lindberg and Negeshi (39) have shown that only one substitution, F209L, was sufficient to confer steroid 15β-hydroxylase activity from P450 2A4 to P450 2A5, a coumarin hydroxylase. Recently, Halpert and He (40) were able to shift P450 2B1 androgen activating two positions, namely Ile-114 and Gly-478, of the en-

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