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Substitution at Residue 473 Confers Progesterone 21-Hydroxylase Activity to Cytochrome P450 2C2

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SUMMARY

The carboxyl-terminal 28 amino acids of rabbit cytochrome P450 2C2 are markedly different from those of other rabbit cytochrome P450 2C family members and, substitution of the equivalent amino acids of other cytochrome P450s can confer novel steroid hydroxylase activity to P450 2C2 while the normal lauric acid hydroxylase activity is retained. To determine the basis for the novel steroid hydroxylase activity, amino acids of cytochrome P450 2C1 were substituted for those of cytochrome P450 2C2 and the mutants were expressed in COS-1 cells. There are 13 differences between the sequences of cytochrome P450 2C2 and P450 2C1 in this region, including five nonconservative exchanges of charged and uncharged amino acids. However, only substitution of valine for Ser-473 increased steroid hydroxylase activity to the maximum level expected in a modified cytochrome P450 2C2, which contained additional substitutions in the 368–388 region to maximize progesterone hydroxylase activity. Introduction of this single substitution into cytochrome P450 2C2 resulted in 21-progesterone hydroxylase activity similar to that resulting from substitution of all 28 carboxyl-terminal cytochrome P450 2C1 amino acids. None of the substitutions, with one exception, substantially affected either lauric acid hydroxylase activity or the amount of immunologically reactive cytochrome P450 that was expressed. A glycine substitution for Val-477 reduced activity of both lauric acid hydroxylase and progesterone hydroxylase and altered the regioselectivity of the hydroxylation for both. Homology modeling of cytochrome P450 2C2, based on the cytochrome bacterial P450cam sequence, indicated that the side chains of residue 473 and the other five residues previously shown to affect substrate specificity face the substrate pocket. For four of the six residues, smaller and more hydrophobic residues increased progesterone relative to lauric acid hydroxylation.

P450s comprise a superfamily of monooxygenases that metabolize a large variety of endogenous and exogenous substrates (1). The diverse substrates of these enzymes result in part from the large number of P450s but also from the relatively relaxed substrate specificity of individual P450s (2). Mutagenesis studies guided by differences in related P450s with different substrate specificities have demonstrated that mutations at several positions along the molecule can alter substrate-P450 interactions but also that a single amino acid change can dramatically alter substrate specificity (3). The structural basis for the specificity of the interaction of the substrate with the active site of mammalian P450s has not been defined. Three-dimensional X-ray structures, however, have been determined for three bacterial P450s, which share sufficient similarity to the mammalian P450s to allow alignment of the sequences (4–6). Such alignments with family 2 P450s have predicted that six distinct regions, SRSs, are present in these P450s (7). In general, identification by mutagenesis of amino acids that alter substrate-P450 interactions have been in or near the predicted SRSs.

An example of closely related P450s with different substrate specificities is the rabbit subfamily 2C. Most of the P450s in this subfamily are steroid hydroxylases with a variety of regiospecific sites, which include the 21, 6β, and 16α positions of progesterone (8). Two of these P450s, P450 2C1 and P450 2C2, have no detectable steroid hydroxylase activity but instead are fatty acid hydroxylases with lauric, arachidonic, and other fatty acids as substrates (8–12). Interestingly, a chimeric protein containing amino-terminal sequence from P450 2C2 and carboxyl-terminal sequence from P450 2C1 retained lauric acid hydroxylase activity but also exhibited a new progesterone 21-hydroxylase activity, suggesting that the amino-terminal region of P450 2C1 and the carboxyl-terminal region of P450 2C2 were inhibitory for steroid substrates (13). Substitution of alanine for Val-113,

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ABBREVIATIONS: P450, cytochrome P450; SRS, substrate recognition site; PCR, polymerase chain reaction; TLC, thin layer chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; P450cam, cytochrome P450 101; P450BM-3, cytochrome P450 102; P450terp, cytochrome P450 108.

within SRS1, conferred low progesterone 21-hydroxylase activity to P450 2C1, suggesting that this region was at least part of the amino-terminal inhibitory region of P450 2C1 (14). Substitutions of P450 2C1 sequence for P450 2C2 sequence at positions 368, 369, 374, and 386, 388 were also able to confer progesterone hydroxylase activity on P450 2C2, indicating that these residues in P450 2C2 were in part responsible for inhibiting steroid substrate P450 interactions (15). However, to obtain maximal progesterone 21-hydroxylase activity, in addition to these five substitutions of P450 2C1 amino acids, the carboxyl-terminal 28 amino acids of P450 2C2 had to be replaced with those of P450 2C1. Conversely, substitution of only the carboxyl-terminal 28 amino acids conferred progesterone 21-hydroxylase activity to ~40% of that of the maximally active hybrids.

The sequence of the carboxyl-terminal 28 amino acids of P450 2C2 is distinct from that of other members of the rabbit 2C subfamily, so P450 2C1 is more similar to P450 2C3, P450 2C5, P450 2C14, and P450 2C16, which are steroid hydroxylases (16). P450 2C2 and P450 2C1 differ at 13 positions in the 28 carboxyl-terminal amino acids. In addition to the P450 2C2/2C1 hybrids, similar substitution of P450 2C14 carboxyl-terminal sequences in P450 2C2 also conferred steroid hydroxylase activity (17, 18). These residues from 463 to 490 include residues 470–477, which correspond to the SRS6 (7). In the present study, we demonstrate that only a single substitution of valine for Ser-473 in P450 2C2 substantially increases progesterone 21-hydroxylase activity and is sufficient to confer progesterone 21-hydroxylase activity similar to that obtained by substitution of the 28 carboxyl-terminal amino acids.

Experimental Procedures

Materials. All PCR reagents and the enzyme *Taq* DNA polymerase were purchased from Promega Corp. Oligonucleotides for PCR and sequencing were synthesized with an Applied Biosystem Model 380A DNA synthesizer at the Biotechnology Center of University of Illinois at Urbana-Champaign. COS-1 cells were obtained from the American Type Cell Collection. Cell culture media, other reagents, and antibiotics were purchased from Life Technologies. DEAE-dextran was purchased from Pharmacia LKB Biotechnology, and calf serum, chloroquine, and dimethylsulfoxide were obtained from Sigma Chemical Co. Deoxyadenosine 5′-[α-^{32}P]triphosphate, [1,2,6,7-^{3}H]progesterone, and 1[^{14}C]lauric acid were purchased from Amersham Corp. Tran^{35}S-label was purchased from ICN Biomedicals. Polyclonal antiserum raised against P450 2C3 was a gift from Dr. Eric Johnson (Scripps Research Institute, La Jolla, CA).

Plasmid constructions and mutagenesis. pCMV5-C2, pCMV5-C2 pm, and pCMV5-C2 pmHincC1, which express P450 2C2, P450 C2 pm, and P450 C2 pmHincC1, respectively, were constructed as described (15). All of the mutations were carried out by the PCR method with pCMV5-C2 pm DNA as a template. One of the two primers used for the PCR was the oligonucleotide, 5′CCACCCGGGGATCC3′, which hybridizes to vector sequence to the 3′ side of the P450 cDNA insert. The sequence of the other primer was one of the following oligonucleotides that introduced the mutation in the underlined codons: O463/465, 5′-AAACCTCTGGTC*G*/*AA*CCC*AAAG*/*C*AATG-3′; O466/467, 5′-AAACCTCTGGTCAACCC*AAACG*/*AATA*/*GTT*GA-3′; O469, 5′-AAACCTCTGGTCAACCCAAACAATGTTGA-TA*C*AAAT-3′; O470, 5′-AAACCTCTGGTCAACCCAAACAATGTT-GATGAAA*C*/*AT*CCATTC-3′; O472, 5′-AAACCTCTGGTCAACCCA-AACAATGTTGATGAAAATCCA*TTG*TCCA-3′; O473, 5′-AAACCTC-TGGTCAACCCAAACAATGTTGATGAAAATCCATTC*GTC*AGT-3′; O476/477, 5′-AAACCTCTGGTCAACCCAAACAATGTTGATGAAA-ATCCATTCTCCA-GTGGA*C*/*ATTGG*/*TC*CGT-3′. PCR was carried out as described (15) with 250 ng of each primer, 20 ng of the template DNA, and 1 unit *Taq* DNA polymerase at 94° for 1 min, 50° for 1 min, and 72° for 2 min for 30 cycles. The PCR product with the mutations was digested with *Hinc*II and *Bam*HI, and this fragment was substituted for the *Hinc*II/*Bam*HI fragment of C2 pm. Each mutation was confirmed by sequencing of the entire PCR fragment by Sanger's dideoxy chain termination method (19) with Sequenase Version 2 (U.S. Biochemicals) as recommended by the manufacturer, using the primer 5′CGCATGGAGCTGTTT3′, which hybridizes to the 5′ side of the *Hinc*II site.

To construct pCMV5-C2 S473V, pCMV5-C2 was digested with *Kpn*I and *Bam*HI. The fragment containing the P450 2C2 cDNA was then digested with *Hinc*II. *Kpn*I/*Bam*HI-digested pCMV5, the *Kpn*I/*Hinc*II fragment of P450 2C2 cDNA, and the *Hinc*II/*Bam*HI fragment containing the S473V mutation, which was obtained by digesting C2hm cDNA with *Hinc*II and *Bam*HI, were ligated in a single step.

Expression in COS-1 cells and assay of enzymatic activity. Transfection of COS-1 cells by the DEAE-dextran method, immunoprecipitations, and enzymatic assays using progesterone and lauric acid as substrates were carried out as described (15).

Molecular modeling. The sequence of P450 2C2 was aligned to that of P450cam according to Gotoh (7). All molecular modeling studies were done with InsightII, Version 2.1.2 software (Biosym Technologies, San Diego, CA) on a Silicon Graphics IRIS workstation. With the three-dimensional structure of P450cam as a template for modeling, the substrate binding pocket of P450 2C2hm was constructed. The side chains of residues in the entire B′-helix/loop/C-helix (residues 84–126 of P450cam, 96–139 of P450 2C2), which includes SRS1; F-helix/loop/G-helix (residues 173–214 of P450cam, 190–254 of P450 2C2), which includes SRS2 and SRS3; I-helix (residues 234–267 of P450cam, 283–316 of P450 2C2), which includes SRS4; β strand 3/β sheet 4/β strand 3 (residues 292–323 of P450cam, 359–392 of P450 2C2), which includes SRS5; and β sheet 5 (382–405 and 146–150 of P450cam, 459–483 of P450 2C2), which includes SRS6, were replaced with those of P450 2C2hm (hm indicates hexamutation). Hydrogen atoms were added using the polar hydrogen atom description. To minimize overlapping contacts due to the incorrect orientation of the side chains, the initial model was energy minimized using the Optimize command from the Builder module. This was followed by energy minimization from the Discover module, first with steepest gradient algorithm for the initial relaxation of the poorly refined molecule and then by minimization with a conjugate gradient algorithm until the energy of the entire system was <0.001 kcal/mol.

An energy-minimized structure of the progesterone was manually docked into the substrate binding pocket of C2hm in such a way that the carbon-21 of progesterone aligned with the C5-H groups of camphor, a substrate for P450cam. Fixing the above carbon atom, the substrate was rotated to obtain a least energy state, in which a best fit of the substrate into the pocket was assumed. All of the hydrogen bonds and electrostatic interactions were reconstructed, and the entire complex was further energy minimized. The energy-minimized three-dimensional model of the substrate binding pocket of P450 2C2hm had a potential energy of −746 kcal/mol.

Results

Sequence correction and requirement of proline at position 471. The initial sequence reported for P450 2C2 cDNA predicted the presence of leucine at position 471 (16). During sequencing mutations generated by PCR in these studies, the sequence CCA, encoding proline, rather than CTA, was determined for codon 471, which confirms the sequence for this codon reported by Imai *et al.* (20). In the process of introducing mutations at nearby positions by PCR,

leucine was also erroneously introduced at position 471. In each case, mutants with Leu-471 were devoid of both progesterone and lauric acid hydroxylase activities in COS-1 cells, suggesting that Pro-471 is important for expression of a functional P450.

Analysis of protein stability. To identify the individual residues contributing to the progesterone 21-hydroxylase activity, 10 of the P450 2C2 residues near SRS6 in C2 pm were mutated to P450 2C1 residues (Fig. 1). C2 pm is P450 2C2 in which five substitutions of P450 2C1 amino acids (H368R, T369A, L374V, D386A, and L388I) have been made. These substitutions provide maximal progesterone 21-hydroxylase activity in C2 pmHincC1, which also contains substitutions of the 28 carboxyl-terminal amino acids of P450 2C1. A total of 15 mutations were made, which included single mutations at the 10 different sites, four double mutants, and one triple mutant.

Each of the mutant proteins was expressed to approximately the same level. After labeling with radioactive amino acids for 4 hr, which is sufficient to estimate steady state levels, the amount of the radioactive proteins immunoprecipitated did not differ by >2-fold (Fig. 2A).

Lauric acid (ω-1)hydroxylase activity of C2 pm mutants. Results from six independent transfections for lauric acid and progesterone hydroxylase activity of P450 C2 pm mutants are summarized in Fig. 3. The activities of the mutants are expressed as a percentage of the activity of C2 pmHincC1. C2 pm expressed in COS-1 cells has 15% and 60% of the progesterone and lauric hydroxylase activities, respectively, of C2 pmHincC1. The substitution of glycine for Val-477 resulted in unique hydroxylation products and is discussed later.

The lauric acid (ω-1)hydroxylase activities of the various mutants of C2 pm from six different experiments are summarized in the Fig. 3B. All of the mutants tested retained lauric acid hydroxylase activity in amounts ranging from ~60% to 150% of that retained by P450 2C2. This result indicates that the expression of functional protein, like the total immunoreactive protein, is approximately the same for all the mutants. Changes in the ratio of lauric acid to progesterone hydroxylase activity (Fig. 3C) resulting from a mutation provide strong evidence that the mutation is affecting substrate-P450 interactions and not expression of functional proteins or changes in general catalytic activity.

Identification of amino acids in the carboxyl-terminal region of P450 2C1, which contribute to progesterone 21-hydroxylase activity. A representative autoradiogram of a thin layer chromatogram, which separated progesterone metabolites produced by the mutant P450s, is shown in Fig. 2B, and the results of six experiments are summarized in Fig. 3A. Of the 10 substitutions made, 8 had little or no effect on progesterone 21-hydroxylase activity. Surprisingly, these included substitutions of charged for uncharged residues at positions 463, 465, and 466 of P450 2C2.

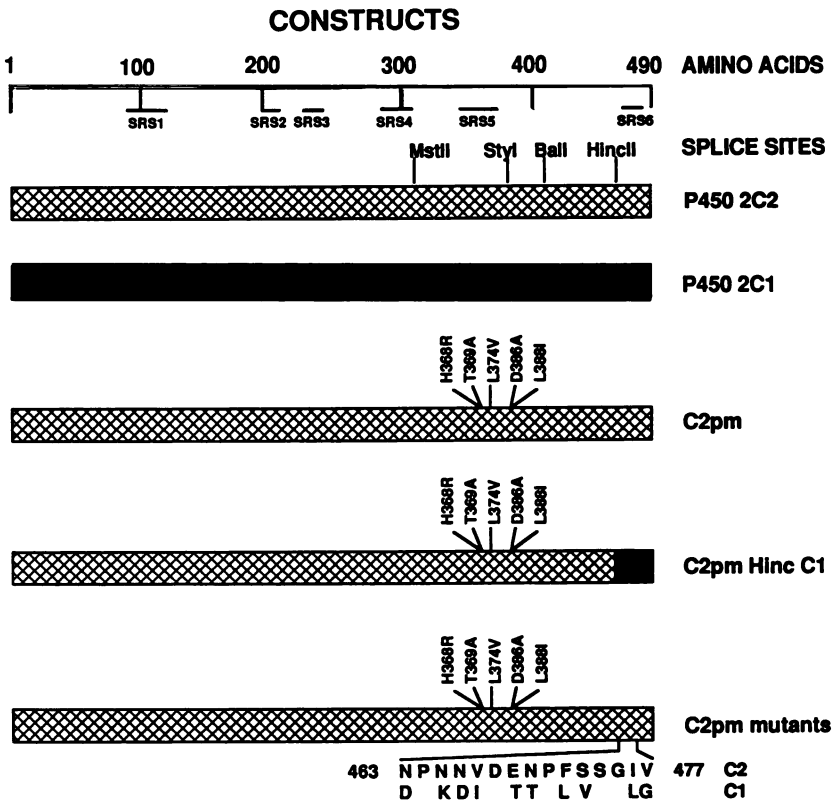

Fig. 1. Schematic representation of the cDNAs of the parental, chimeric, and the mutant P450s that were analyzed. ■, P450 2C1 cDNA; ■, P450 2C2 cDNA; *numbering at top*, the amino acid positions of the coding region of the protein; *horizontal line*, SRSs (7) at amino acid positions. Splice sites represent the restriction enzyme sites that were used to make chimeric P450s. The protein encoded by each of the cDNAs is labeled on the *right* of the diagram. *C2pm*, a mutant form of P450 2C2 with five mutations in the 368–388 region as indicated. *C2pm Hinc C1*, the chimeric form of C2 pm with an additional substitution of the carboxyl-terminal 28 residues of P450 2C1. The carboxyl-terminal P450 2C2 residues of C2 pm were mutated to P450 2C1 residues by PCR, as described in the Experimental procedures. The differences in the amino acid sequence of P450 2C2 and P450 2C1 from position 463 to 477 are shown.

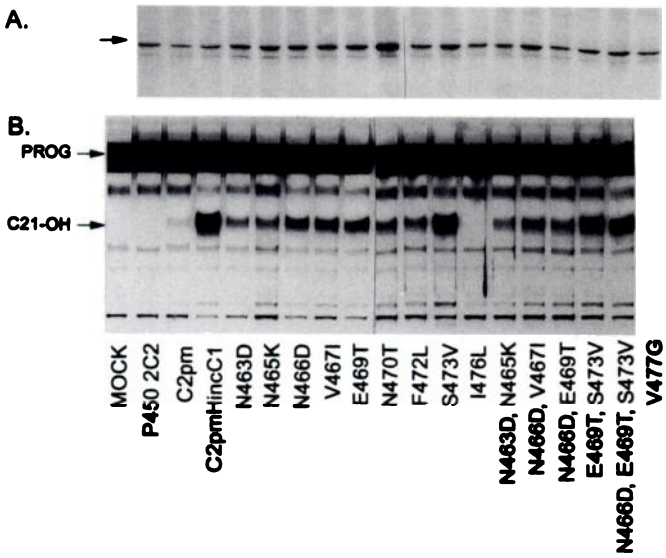

Fig. 2. Stability and progesterone 21-hydroxylase activity of the P450s 2C1, 2C2, their chimera, and mutant P450s expressed in COS-1 cells. Individual lanes are labeled by the P450s expressed in the COS-1 cells or by the mutations introduced into P450 C2 pm. Mutants are designated as wild-type amino acid, the position, and the mutant amino acid, in that order. A, Immunoprecipitation of the expressed proteins in COS-1 cells. Expression of P450 2C2, their chimeras, and mutants in COS-1 cells were analyzed by immunoprecipitation of proteins labeled by incubation of transfected COS-1 cells for 4 hr with Tran ^{35}S-label as described in Experimental Procedures. Immunoprecipitated proteins were analyzed by SDS-PAGE and fluorography. P450 protein bands are marked by an *arrow* at the left. B, Autoradiogram of progesterone (*PROG*) metabolites produced by the cell lysates of COS-1 cells. *MOCK* refers to the control cells, for which no DNA was used during transfection. The cell lysates were obtained and assayed for progesterone hydroxylation as described in Experimental Procedures. Progesterone and its metabolites were separated by TLC. An autoradiogram of the TLC plate is shown, and the positions of 21-hydroxyprogesterone and progesterone are indicated.

This cluster of charged amino acids is conserved in other P450s in family 2 but not in P450 2C2. More conservative mutations of V467I, N470T, or I476L also did not result in an increase in progesterone hydroxylation, indicating that these residues do not contribute to the change in this activity. Substitution of Thr for Glu-469 or leucine for Phe-472 resulted in progesterone 21-hydroxylase activities of ~30–35% of that of C2 pmHincC1, which were slightly higher than that of C2 pm.

The only mutation that resulted in a dramatic increase in the progesterone hydroxylase activity to a level comparable to that of C2 pmHincC1 was S473V. Presence of a hydrophobic amino acid at this position, in contrast to serine, resulted in a 7-fold increase in the progesterone hydroxylase activity of C2 pm. These results strongly suggest that there is a requirement for valine at position 473 for the progesterone 21-hydroxylation. C2 pm with the S473V mutation is referred to as C2hm.

Progesterone hydroxylase activity of multiple mutants of C2 pm. These results clearly indicated that a single mutation, S473V, could confer progesterone 21-hydroxylase activity to C2 pm comparable to that of C2 pmHincC1. The importance of this mutation was further confirmed in several multiple mutants. Mutants that did not contain the S473V mutation (N463D/N465K, N466D/V467I, or N466D/E469T) had little or no increase in progesterone 21-hydroxylase activity (Fig. 3). In contrast, multiple mutants that contained the S473V substitution (E469T/S473V and N466D/E469T/S473V) had activity similar to that of the single S473V mutation.

Activity of [Val-473]P450 2C2. The single mutation of S473V was introduced into P450 2C2 to determine whether this single change had effects equal to the substitution of the carboxyl-terminal 28 amino acids. COS-1 cells transfected with [Val-473]P450 2C2 had detectable progesterone 21-hydroxylase activity (Fig. 3, 4), which was equal to that of C2HincC1 in which all 28 carboxyl-terminal amino acids of P450 2C1 were substituted (Fig. 3, A and C). These results demonstrate that substitution of valine at position 473 alone can confer progesterone 21-hydroxylase activity to P450 2C2 at a level of ~40% of that obtained by substituting the entire carboxyl-terminal region of P450 2C1 from residue 368 to 490.

Activity of [Gly-477]C2 pm. C2 pm with glycine at position 477 did not show any progesterone 21-hydroxylase activity above the detection limits. Interestingly, however, a distinct unidentified product of progesterone metabolism appeared on the thin layer chromatogram, and its mobility was between that of 21-hydroxyprogesterone and that of 6β-hydroxyprogesterone (Fig. 5). Furthermore, this mutant did not catalyze the (ω-1)hydroxylation of lauric acid above detection limits, but another product of lauric acid metabolism, with a retention time different from that of (ω-1)hydroxylauric acid, was eluted (Fig. 6). These metabolites have not been identified; however, this mutation appears to change the regiospecificity of hydroxylation of both the steroid and fatty acid substrate. The activities of the V477G mutant were lower than those of C2 pm, C2 pmHincC1, or other mutants tested. The changes in the stereospecificity or regiospecificity of hydroxylation caused by this mutation suggest that the substitution of glycine for Ser-477 alters the orientation of the substrates in the substrate binding pocket.

Discussion

Altered substrate specificities of chimeric proteins have indicated that the carboxyl-terminal 28 amino acids are important for the interactions of the substrate with P450, and deletion analysis has indicated that this region is important for the formation of a stable protein. Deletion of the P450 2C2 carboxyl-terminal 28 amino acids or as little as 4 amino acids prevented the expression of stable enzymes in yeast, and mutation of conserved proline at 480 and 481 resulted in loss of lauric hydroxylase activity (17, 18). In our studies, replacing Pro-471 with leucine resulted in undetectable lauric or progesterone hydroxylase activity in transfected COS-1 cells. These results suggest that these prolines are important for proper folding of the catalytic site and that the carboxyl-terminal region plays a role in stabilizing the protein, as proposed (18). When the carboxyl-terminal 28 residues of P450 2C2 were replaced with the corresponding sequences of P450 2C14, the resultant chimera exhibited testosterone 16β-hydroxylase activity that was absent in the parental P450s (21). Likewise, substitution of the carboxyl-terminal 28 amino acids of P450 2C2 with P450 2C1 is required for maximal progesterone 21-hydroxylase activity (15). Replacing this carboxyl-terminal sequence of P450 2C2 with those of P450 2B5 and P450 2E1, however, did not confer steroid

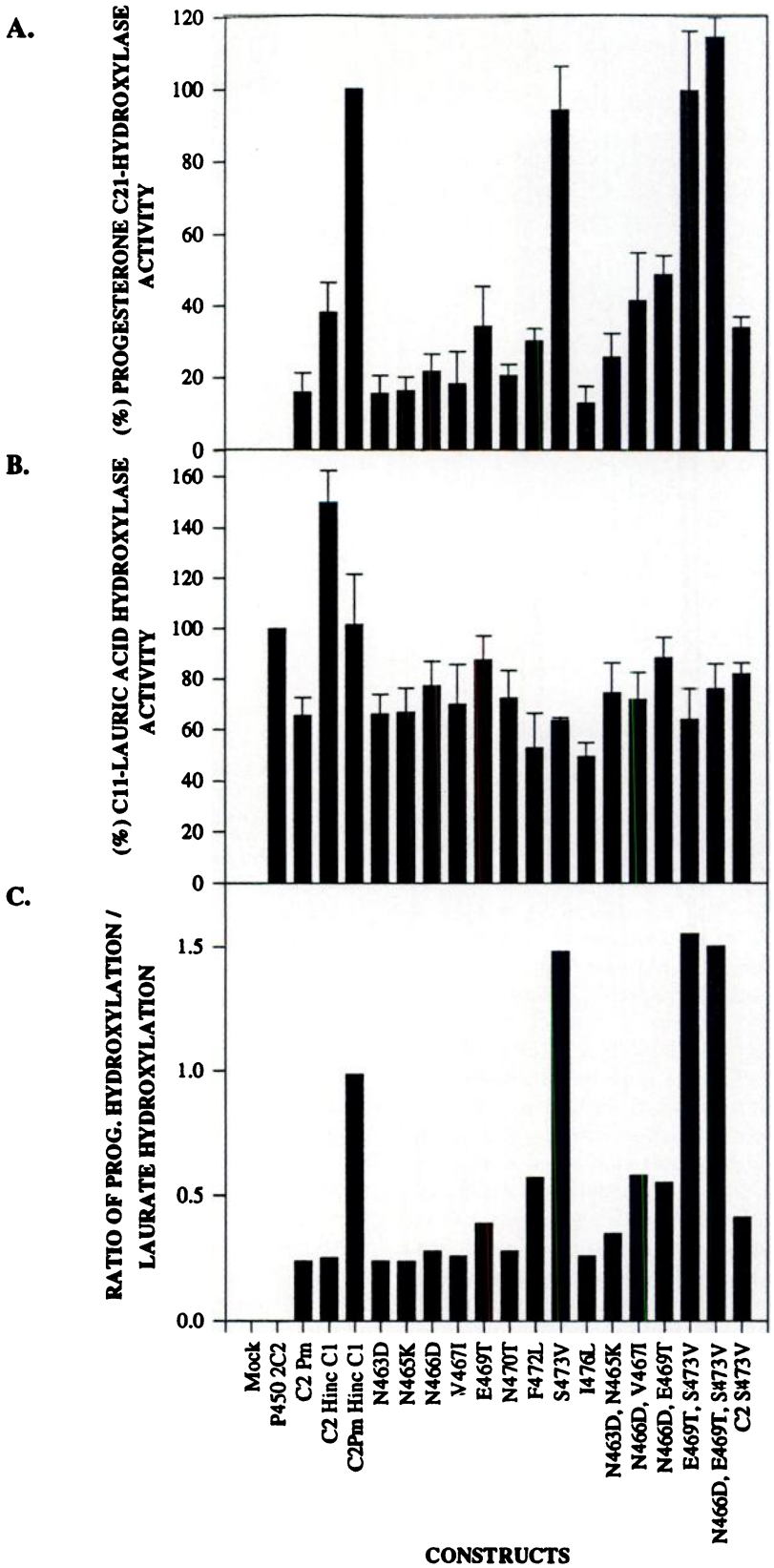

Fig. 3. Summary of progesterone and lauric acid hydroxylase activities in COS-1 cells transfected with P450s 2C1, 2C2, their chimeras, and mutants. Values plotted represent mean and standard errors for six independent transfections. Both the progesterone and lauric acid hydroxylase activity assays were performed on the same cell lysates for each transfection as described in Experimental Procedures. A, Progesterone hydroxylase activity. Position of the 21-hydroxylated product was determined by comigration with the unlabeled 21-hydroxyprogesterone. Bands containing the product were scraped, and radioactivity was assayed by scintillation counting. Values for the individual progesterone hydroxylase activities were normalized against that of C2 pmHincC1. B, (ω-1)-Lauric acid hydroxylation. Values for the individual lauric acid hydroxylase activities were normalized against activity of the wild-type P450 2C2. C, Ratio of the mean of the progesterone 21-hydroxylase activity to lauric acid hydroxylase activity.

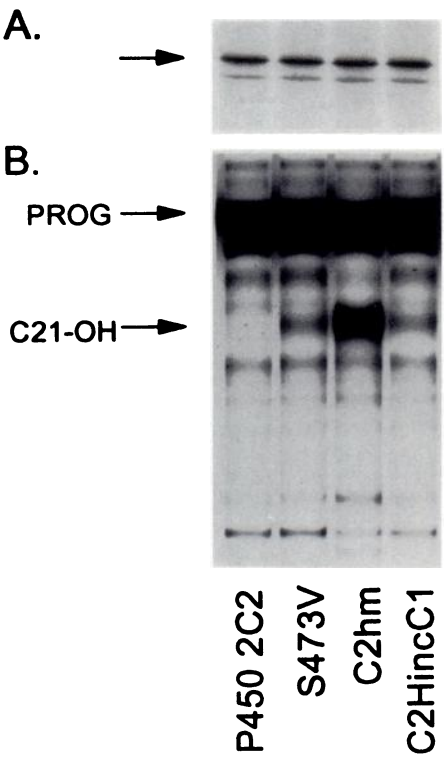

Fig. 4. Stability and progesterone 21-hydroxylase activity of P450 2C2, [Val-473]P450 2C2, C2hm, and C2HincC1 expressed in COS-1 cells. A, Immunoprecipitation of the expressed proteins in COS-1 cells. Synthesis of radioactive P450s expressed in COS-1 was analyzed as described for Fig. 2A. B, Typical autoradiogram of the TLC showing the separated products of progesterone (*PROG*) metabolism. Assays were done as described for Fig. 2B. The mobility of the marker for 21-hydroxyprogesterone is indicated.

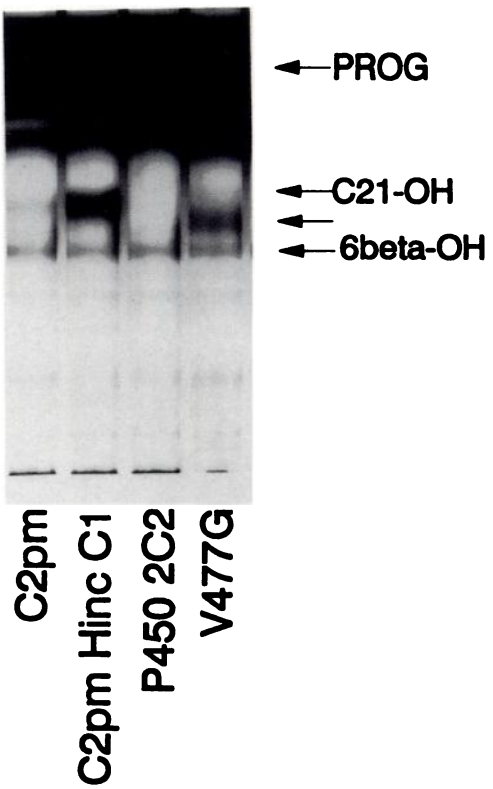

Fig. 5. A typical autoradiogram of the TLC showing the separated products of progesterone (*PROG*) metabolism produced by P450 2C2, C2 pm, C2 pmHincC1, and [Gly-477]C2 pm expressed in COS-1 cells. The assays were done as described for Fig. 2B. The mobilities of the marker containing the hydroxylated forms of progesterone are also indicated.

hydroxylase activity (21). This result suggested that the P450 2C2 carboxyl-terminal sequence is not just in general inhibitory to steroid substrates but that specific residues are important.

The carboxyl-terminal region of P450 2C2 differs substantially from other P450 2C members and has 10 differences from P450 2C1 in the 16 residues from 463 to 478 (16). However, the present study identifies residue 473 as the predominant determinant of progesterone 21-hydroxylase activity conferred on P450 2C2 by substitution of the carboxyl-terminal 28 amino acids of P450 2C1. Unexpectedly, substitutions exchanging charged and uncharged polar residues at 463, 465, 466, and 469 had little effect on either progesterone or lauric acid hydroxylation. These results suggest that these hydrophilic residues are surface residues that can accommodate substantial changes in the side chains without affecting the geometry at the active site. Consistent with this proposal, this region of P450 2C2 aligns with residues 385–391 of P450cam (Fig. 7), which are in a β strand on the surface of the protein.

The same substitution in P450 2C2, S473V, has been reported to increase the ratio of testosterone 16α-hydroxylase to lauric hydroxylase activity by ~10-fold (18), similar to the 7-fold relative increase in progesterone activity resulting in C2 pm from this mutation. The lauric acid hydroxylase activity, however, was reported to decrease 5-fold in contrast to the minor decrease observed in the present study. This may simply reflect the differences in the assay systems: a reconstituted system with yeast-expressed protein versus activity in microsomal membranes of protein expressed in mammalian cells. A 7-fold increase in testosterone 16α-hydroxylase activity with little effect on lauric hydroxylase activity was observed if additional mutations (E469T, N470T, and F472L) were made (18). Less dramatic but consistent with this finding were small increases in progesterone 21-hydroxylase activity observed with the mutations E469T and F472L. These results are consistent with the suggestion that residues in the region 469–473 participate in contacts between the substrate and protein (18) and indicate that residue 473, in particular, is critical for steroid hydroxylation.

Alignment of the P450 2C2 sequence with bacterial P450s, for which the three-dimensional structure has been determined, is consistent with a role of residue 473 in substrate enzyme interactions (Fig. 7). The X-ray crystallographic studies of P450cam have shown that the entrance of the substrate binding pocket is defined by the residues in the carboxyl terminus of the protein (22, 23). As aligned by Gotoh (7), Ser-473 of P450 2C2 corresponds to Ile-395 of P450cam, which is within 10 Å of the camphor substrate (6, 24). This residue also falls within SRS6, residues 470–477, as proposed by Gotoh (7). In P450BM-3, residue 473 of P450 2C2 corresponds to Leu-437 within β sheet 4, which is one of the segments that define the substrate binding pocket. Leu-437 is in close van der Waals' contact with Met-185, stabilizing the packing of β sheet 1–4 against the F helix (4). In P450terp, position 473 of P450 2C2 corresponds to Phe-414. The proposed model for the substrate binding pocket of P450terp suggests that Phe-414 and Val-415 are amino acids

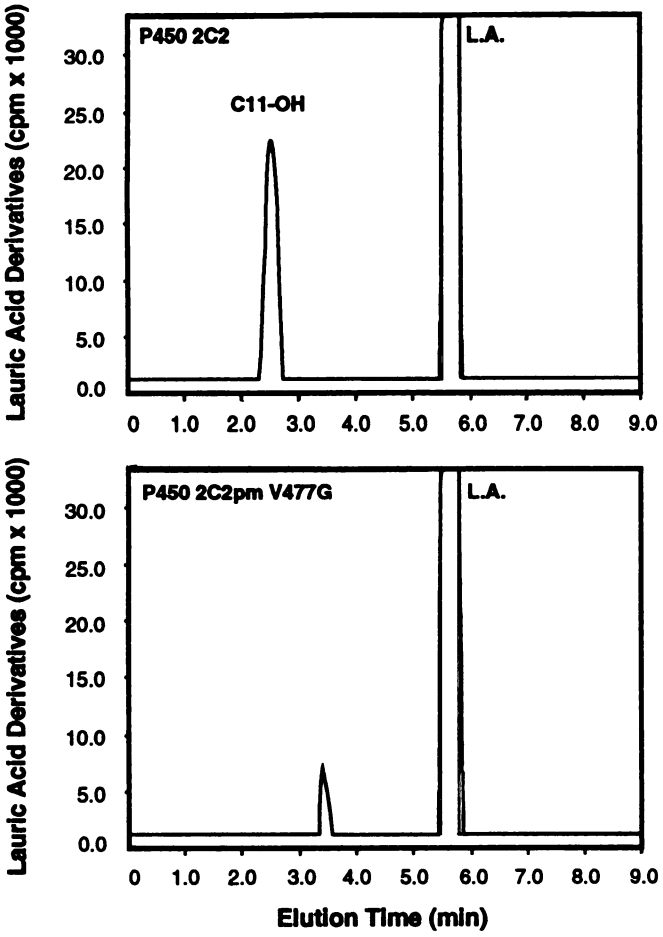

Fig. 6. The high pressure liquid chromatography profiles of the separated products of lauric acid metabolism produced by P450 2C2 and the [Gly-477]C2 pm mutant of C2 pm. The assay for lauric acid hydroxylase was done as described for Fig. 3. *Top*, Product (ω-1)-hydroxylauric acid produced by P450 2C2. This product has a retention time of 2.5 min; *bottom*, product obtained with the V477G mutant of C2 pm; the retention time is 3.4 min under the same conditions, indicating that this unidentified product is distinct from that of (ω-1)-hydroxylauric acid.

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460 PLVNPNNVDENPF.SSGIVRVP 480  C2
460 PLVDPKDIDTTPL.VSGLGRVP 480  C1
457 SLVEPKDLDITAV.VNGFVSVP 477  C5
459 PLVDPKDIDPTPV.ENGFVSVP 479  C3
382 SIAPGAQIQHKSG.IVSGVQAL 402  CAM
424 EDHTNYELDIKET.LTLKPEGF 434  BM-3
401 ELS..GPPRLVATNFVGGPKNV 420  TERP
                SRS-6
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Fig. 7. Comparison of amino acid sequences in the carboxyl-terminal region between P450s 2C1, 2C2, 2C3, 2C5, P450cam, P450BM-3, and P450terp. *Numbers*, the position of the corresponding residues in each of the protein. The amino acid sequences of P450s 2C1, 2C2, 2C3, and 2C5 were aligned to P450cam (7). The P450cam sequence was aligned to that of P450BM-3 according to Ravichandran *et al.* (4) and to that of P450terp according to Hasemann *et al.* (5). The amino acids corresponding to position 473 in P450 2C2 are boxed.

that contact the substrate α-terpineol (5). For each of the bacterial P450s, therefore, residue 473 of P450 2C2 aligns with an amino acid proposed to be near, and possibly contacting, the substrate.

Three-dimensional structures have been proposed for mammalian microsomal P450s by homology to P450cam, including some with steroid substrates. In the structure proposed for human aromatase, P450arom (24), the amino acid corresponding to Ser-473 of P450 2C2 is His-475, which has been proposed to be a major contributor to the active-site surface area for the lower edge of the A- and B-rings of the steroid substrate. P450 2B1, which has been extensively analyzed with mutagenesis, has also been modeled in homology with P450cam (25). The alignment used for this model differs from that of Gotoh (7) shown in Fig. 7 in that the mammalian sequences are shifted three residues to the left. This alignment places residue 478 of P450 2B1, which corresponds to 477 of P450 2C2, near the substrate contacting residues Ile-395 and Val-396, Phe-414 and Val-414, and Leu-437 in P450s cam, terp, and BM-3, respectively. This alignment of residue 478 is the same as that proposed by Hasemann *et al.* (26), which incorporated structural characteristics of the three bacterial enzymes in the alignment procedure. Residue 478 of P450 2B1 has been implicated in substrate-P450 interactions as substitutions of multiple residues at this position resulted in changes of up to 10-fold in the ratios of 16α to 16β and of 15 to 16 hydroxylation of androstenedione (27). Interestingly, substitution of glycine for Val-477 of P450 2C2 had even more dramatic effects than the residue 473 substitution. Neither 21-hydroxyprogesterone nor (ω-1)hydroxylauric acid could be detected; however, novel unidentified hydroxylated products were produced. The effects of the substitution of residue 477 in P450 2C2 provide further evidence for the substrate interaction role of residue 478 of P450 2B1. However, this alignment with residue 477 near the substrate moves the residue corresponding to Ser-473 of P450 2C2 a substantial distance from the active site. The role of the

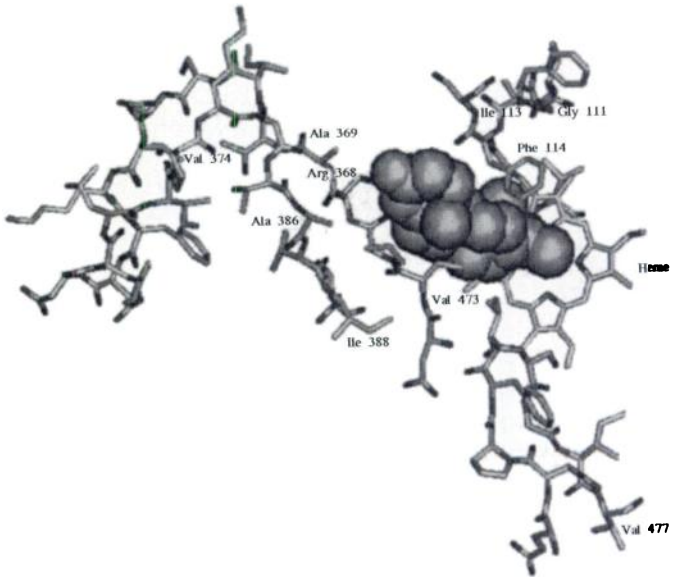

Fig. 8. Proposed model for the substrate binding pocket of P450 2C2hm. P450 2C2 hm contains the six mutations H368R, T369A, L374V, D386A, L381I, and S474V which contribute to increased progesterone 21-hydroxylation relative to lauric acid hydroxylation. The substrate pocket of P450 2C2hm was constructed based on the structure of P450cam, energy minimized, docked with progesterone and further energy minimized as described in Experimental Procedures. Selected residues, labeled by name and number, which were implicated in the increase in progesterone hydroxylation relative to lauric acid hydroxylation and neighboring residues are shown. The heme group and the van der Waals surface of the progesterone molecule are shown.

corresponding residue in P450 2B1, 474, is not clear because mutations were not made at this site; however, substitutions at 473 in P450 2B1 and at 473 and 475 in P450 2B11 did not markedly affect substrate specificity (28, 29). These results suggest that residue 477, rather than 473, may be close to the substrate. However, in P450 2C2, the substrate-specific effect of substitutions at 473 and nearby residues (18) suggests that this residue is near the substrate and that substitution of glycine for valine in a β strand at 477 might plausibly indirectly affect the position of residue 473 in the same β strand. These results illustrate the difficulties of aligning sequences within the varying substrate binding regions of P450s (26). It is possible that the structures of 2C and 2B P450s are substantially different, but this seems unlikely because of the sequence similarity between these two subfamilies in this region. It is more probable that either residue 473 is distant from the substrate and indirectly affects substrate interactions of residue 477, or vice versa. The present mutagenesis data for the 2B and 2C P450s do not resolve this issue.

The spatial relationship of the P450 2C2 residues that contribute to the progesterone hydroxylase activity are summarized in a model of the active site, based on homology to P450cam, with progesterone docked (Fig. 8). With the alignment shown in Fig. 7, each of the critical amino acids in the SRS5 and SRS6 regions have side chains facing the substrate binding pocket. The simplest rationalization of the mutagenesis data is that for the larger, more rigid steroid substrate (compared with lauric acid), a larger, more hydrophobic pocket or access channel is required. Thus, four of six changes (T369A, L374V, D386A, and S473V) resulted in amino acids with smaller and/or more hydrophobic side chains. The other two changes were relatively conservative (R368H and I383L). In addition, amino acids in SRS1, residues 111–115, are also important for maximal activity of both lauric acid and progesterone hydroxylation. Although mutations of C2MstC1 in this region in general affected both reactions, the substitution of phenylalanine for Val-112 resulted in greater inhibition of progesterone than lauric acid metabolism (13), which follows the same general rule that a larger, more hydrophobic pocket favors steroid over fatty acid metabolism. Thus, homology modeling based on P450cam has provided a reasonable rationalization of the mutagenesis data and a proposed structure for part of the substrate binding pocket.

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