# Differential Effects of Mutations in Substrate Recognition Site 1 of Cytochrome P450 2C2 on Lauric Acid and Progesterone Hydroxylation†

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ABSTRACT: Mutations at amino acid positions 107–120, which are part of a predicted substrate recognition site [Gotoh, O. (1992) *J. Biol. Chem. 267*, 83–90], were analyzed in C2MstC1, a chimera of P450 2C2 and P450 2C1. This hybrid protein has a new activity for progesterone C21-hydroxylation in addition to the lauric acid (ω-1)hydroxylase activity present in both parent proteins. Various substitutions for highly conserved glycines at positions 111 and 117 and tryptophan at position 120 strongly decreased the lauric acid hydroxylase activity of P450 2C2 and C2MstC1 and the progesterone hydroxylase activity of C2MstC1. Activities of mutant proteins with substitutions at 107, 108, and 112–115 were also strongly reduced. Modest or no decreases in activity were observed for substitutions at 109, 110, 116, 118, and 119. Lauric acid hydroxylase activity decreased more in most C2MstC1 mutants than in those of P450 2C2, particularly at positions 107 and 108. A substitution of phenylalanine for valine-112 reduced progesterone hydroxylation by 30-fold while only moderately reducing lauric acid hydroxylase by 40%. This differential effect on two dissimilar substrates demonstrates the importance of residue 112 for substrate interactions. The results are consistent with a model in which residues 107–110 align with the B′-helix of the bacterial proteins P450cam and P450BM-3. This helix is followed by a substrate-contacting loop from 111 to 116, and residues 117–120 align with the C-helices of the bacterial proteins. In this alignment, Trp-120 is positioned behind the heme such that it could participate in electron transfer from the reductase. A glycine followed by three or four hydrophobic amino acids, comparable to residues 111–114 of P450 2C2, is conserved in all family 2 cytochromes P450 and in steroid and fatty acid hydroxylases of other families. This, therefore, may be a motif that mediates hydrophobic interactions with the substrate in many distantly related P450s.

Cytochromes P450 (P450)[1] form a large superfamily of monooxygenases that are present in species ranging from bacteria to man (Nelson & Strobel, 1987; Nelson et al., 1993). Their ubiquitous presence and similarities in primary and secondary structure as well as physicochemical characteristics suggest that all P450s evolved from a common ancestor gene by gene duplications, followed by specific mutations that altered substrate specificity (Nelson & Strobel, 1987, 1989; Edwards et al., 1989; Nelson et al., 1993). Today multiple P450s are present in each mammalian species, performing quite different tasks. The evolutionarily older P450s are integral to the biosynthesis of steroid hormones, prostaglandins, and other endogenous lipophilic compounds (Guengerich, 1987). Other P450s, however, evolved to metabolize and usually inactivate foreign compounds such as phytotoxins, carcinogens, and environmental pollutants (Nelson & Strobel, 1987).

In the absence of an X-ray structure for microsomal P450s, the experimentally determined three-dimensional structure of the bacterial P450, P450cam (Poulos et al., 1987; Poulos, 1991), has served as a basis for predicting probable structural features of the mammalian enzymes that determine their diverse substrate specificities. Alignment of the amino acid sequences of mammalian P450s with that of P450cam on the basis of sequence similarity, predicted secondary structure, and hydrophobicity profiles, as well as three-dimensional computer-assisted molecular modeling based on the P450cam structure, led Zvelebil et al. (1991) and Laughton et al. (1990, 1993) to predict several regions of the different P450s that would be likely to form the substrate binding sites of these enzymes. Gotoh (1992) generated an alignment of multiple amino acid sequences from family 2 P450s using similar criteria. Six regions of the amino acid sequences of these enzymes were predicted to be potential substrate recognition regions (SRSs) on the basis of their alignment with residues occurring within 10Å of the bound camphor molecule in P450cam as identified by Laughton et al. (1990). Gotoh (1992) expanded the SRS regions to include three additional amino acids in each direction. Several amino acid substitutions that had been shown in other studies to alter substrate specificities of family 2 enzymes were then found to cluster within three of the expanded SRSs. It was also noted that family 2 genes exhibit higher ratios of nonsynonymous to synonymous nucleotide substitutions within the SRS regions that may be related to selection for functional diversity within this family of enzymes during evolution. The first substrate

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[1] Abbreviations: P450, cytochrome P450; SRS, substrate recognition site.

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recognition site, SRS1, was also expanded to encompass two substrate-contacting loops of P450cam (Laughton et al., 1990, 1993) that are interrupted by α-helix B′. Substitutions for the amino acid equivalent to residue 113 in P450 2C proteins, which is in the C-terminal region of SRS1, have been shown to affect enzymatic activity in members of the 2A, 2B, and 2C P450 subfamilies. Mutations at this position were shown (1) to change the $K_m$ and $V_{max}$ values for progesterone hydroxylation (Kronbach et al., 1989, 1990), (2) to create novel substrate specificities (Lindberg & Negishi, 1989; Kronbach et al., 1991), and (3) to alter regioselectivity of steroid hydroxylation (Halpert & He, 1993; Richardson & Johnson, 1993). A series of substitutions for amino acid 113 in P450 2C2 and C2MstC1, a chimera of P450 2C2 and P450 2C1, indicated that a hydrophobic residue was required at position 113. In addition, the requirement for the size and hydrophobicity of the side chain was more stringent for progesterone, which is a relatively rigid molecule, than for lauric acid, a more flexible unsaturated fatty acid (Straub et al., 1993a). In the P450cam sequence corresponding to SRS1, three amino acids, Phe-87, Tyr-96, and Phe-98, contact the substrate (Poulos et al., 1987), implying that amino acids in addition to 113 for mammalian P450s also might be involved in the determination of substrate specificity. Cassette mutagenesis of amino acids 107–120 of P450 2C2 has been used to identify residues that affect lauric acid hydroxylation (Straub et al., 1993b). In P450 2C2, mutations of Gly-111 and Gly-117 and residues 112–115 caused substantial reductions in activity, which is consistent with an alignment with P450cam in which Gly-111 is aligned with the end of α-helix B′, where it may serve to terminate the helix, and Gly-117 is aligned with the beginning of α-helix C. This alignment locates amino acids 112–115 in close proximity to the substrate.

The appropriateness of this alignment was based only on the effects of amino acid substitutions on lauric acid hydroxylase activity of the mutant enzymes after transient expression in COS1 cells. However, effects other than substrate–enzyme interactions, which cannot be detected in the COS1 cell system, might affect the catalytic activity. Differential effects of mutations on structurally unrelated substrates or changes in the regiospecificity of hydroxylation have been used to strengthen the argument that a residue affects the interaction of the substrate with the enzyme (Lindberg & Negishi, 1989; Chen et al., 1993; Halpert & He, 1993; Richardson & Johnson, 1993).

In this study, we have exploited the fact that a chimeric protein of P450 2C2 and P450 2C1 has a new progesterone C21-hydroxylase activity while retaining lauric acid hydroxylase activity (Straub et al., 1993a). We now report the effect of mutations in the SRS1 region of the chimeric protein on these two dissimilar substrates.

## EXPERIMENTAL PROCEDURES

*Materials.* COS1 cells were obtained from the American Type Culture Collection. Cell culture media and antibiotics were from Gibco-BRL (Grand Island, NY), and fetal bovine serum was from Intergen Co. (Purchase, NY). Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA synthesizer at the Biotechnology Center of the University of Illinois. Restriction enzymes and bovine alkaline phosphatase were purchased from Gibco-BRL (Grand Island, NY). [1-$^{14}$C]lauric acid, [1,2,6,7-$^{3}$H]progesterone, and [α-$^{32}$P]-dATP were purchased from Amersham (Arlington Heights, IL). [$^{35}$S]Translabel was purchased from ICN Biomedicals (Irvine, CA). DEAE-Dextran and the helper phage M13K07 were obtained from Pharmacia Biotech, Inc. (Piscataway, NY). Chloroquine and dimethyl sulfoxide were from Sigma Chemical Co. (St. Louis, MO). The expression vector, pCMV5 (Andersson et al., 1989), was obtained from Dr. M. F. Stinski (University of Iowa, Iowa City).

*Construction of C2MstC1.* Mutants of P450 2C2 cDNA in pCMV5-C2 at positions 107–120 (Figure 1) were obtained by cassette mutagenesis (Straub et al., 1993b), and pCMV5-C2MstC1, which encodes the chimera of P450 2C2 and P450 2C1 (C2MstC1), was constructed as has been described (Straub et al., 1993a). C2MstC1 contains the N-terminal 306 amino acids of P450 2C2 and the C-terminal 184 amino acids of P450 2C1. In order to transfer the mutated cassettes into C2MstC1, pCMV5-C2 mutant vectors were digested with *Kpn*I at bp −40 (relative to the A in the initiator codon ATG) and *Mlu*I at bp 372. The resulting 412-bp fragment, which encoded 124 N-terminal amino acids of P450 2C2 with the mutated cassette at the C-terminus, was isolated from a low melting point agarose gel and cloned into the corresponding restriction sites of pCMV5-C2MstC1. Plasmid DNA for expression assays was purified by selective binding to resins as described by the supplier (Qiagen, Inc., Chatsworth, CA). The enzymatic activities expressed in cells transfected with different preparations of the same plasmid were within the standard error of the assay (data not shown). The mutations were verified by sequencing using the dideoxy chain termination method and the Sequenase version 2.0 kit (United States Biochemicals, Cleveland, OH) (Sanger et al., 1977).

*Enzymatic Assays in Transfected COS1 Cells.* COS1 cells were maintained at 37 °C and 6% $CO_2$ in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 10 units of penicillin/mL, and 0.1 mg of streptomycin/mL. Cells grown to 80% confluence were transfected by the DEAE-Dextran/chloroquine method as described previously (Straub et al., 1993b). Lauric acid hydroxylation was assayed in whole-cell extracts (Straub et al., 1993b), and progesterone hydroxylation was assayed in whole cells (Straub et al., 1993a).

*Immunoprecipitation of Expressed Proteins.* The levels of expressed proteins were determined by immunoprecipitation of proteins from lysates of transfected cells with polyclonal antisera raised against P450 2C3 (Kronbach & Johnson, 1991) as described previously (Straub et al., 1993b). Cells were incubated for 4 h in 50 μCi/mL [$^{35}$S]Translabel (ICN) in methionine- and cysteine-free minimal essential medium (Gibco-BRL, Grand Island, NY) before lysis.

## RESULTS

*Generation of Mutations.* Random mutations were generated by insertion of an oligonucleotide cassette degenerate in the sequence encoding amino acids 107–120 in P450 2C2 (Straub et al., 1993b). Of a potential 78 mutations, 21 mutations were obtained with at least one mutation at each amino acid position, and three or more at positions 107 and 119. Two mutations, one a relatively conservative change and the other nonconservative, for positions 107 and 119 and all the remaining mutations at other positions were selected for analysis in C2MstC1 (Figure 1).

*Effects of the Mutations on the Levels of Expression.* Sequence alignment of 54 P450s in family 2 (not shown) reveals that Gly-111 is invariant and Gly-117 is present in 50 of the sequences. Similarly, Trp-120 is nearly always conserved in mitochondrial as well as microsomal P450s, exceptions being subfamily 2A members and cholesterol 7α-hydroxylase, which have an Ala at this position. The conservation of these three amino acids suggests that the choice of amino acids at these

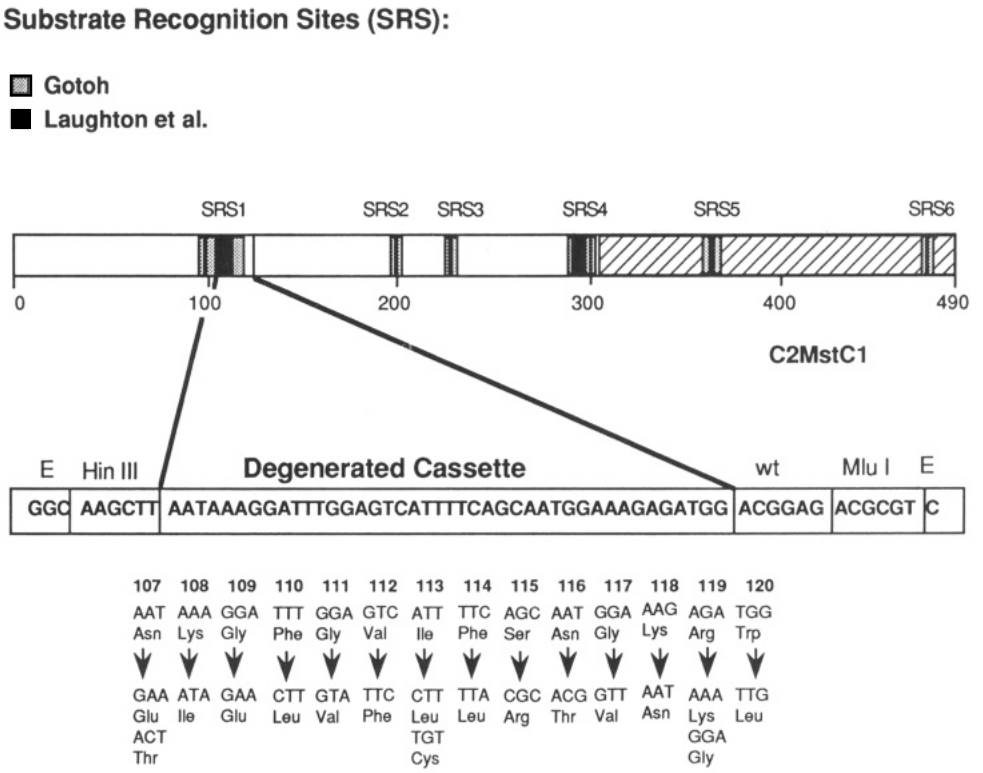


FIGURE 1: Schematic representation of the structure of C2MstC1 and mutations that were analyzed. At the top is a schematic diagram of the chimeric C2MstC1 with P450 2C2 sequence represented by the open region and P450 2C1 by the hatched region and the number of amino acid residues indicated below. The six SRSs predicted by Gotoh (Gotoh, 1992) are stippled, and the regions in $P450_{17\alpha}$ aligned with P450cam residues within 10 Å of the substrate (Laughton et al., 1990) overlay the SRS regions in solid black. The sequence of the oligonucleotide used for cassette mutagenesis is shown below the schematic diagram and includes extensions (E) to enable cleavage by *Hin*dIII and *Mlu*I. At the positions delimited by the lines extending from the schematic diagram, 97% of wild type (sequence shown) and 1% of each of the other nucleotides were present during oligonucleotide synthesis (Straub et al., 1993b). At the bottom, the individual codons and the nucleotide and corresponding amino acid changes for the mutants characterized are shown.

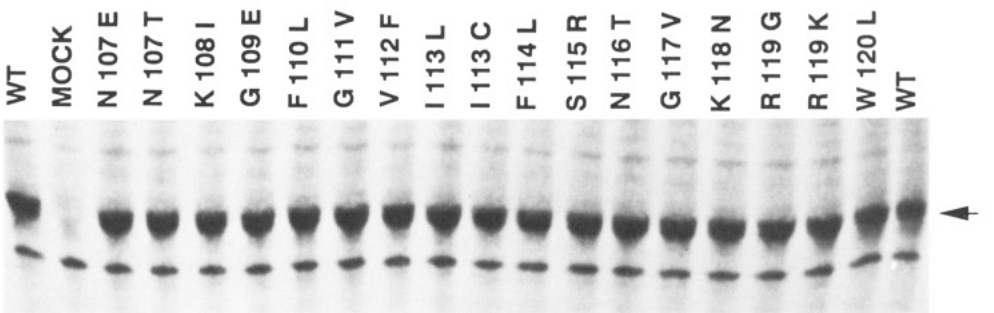


FIGURE 2: Expression of immunoreactive C2MstC1 mutants in COS1 cells. Forty-eight hours after transfection, COS1 cells were labeled for 4 h with 50 $\mu$Ci/mL [$^{35}$S]Translabel. After lysis, P450s were immunoprecipitated using a polyclonal antiserum raised against P450 2C3 (Kronbach et al., 1991). Immunoprecipitated proteins were analyzed by SDS–PAGE and fluorography. P450 protein bands are marked by an arrow at the right. Individual lanes are labeled by the designation for each mutant as wild-type amino acid, position, and mutant amino acid in that order. The lane showing immunoprecipitated products of cells transfected with the empty vector is labeled MOCK.

positions is restricted either by catalytic functions or by structural determinants of protein folding and stability. In the latter case, mutations might result in a decrease in the amount of protein expressed. Abnormal proteins generated by mutations have been shown to be rapidly degraded in cultured mammalian cells (Capecchi et al., 1974). In general, mutations studied previously in the region from 107 to 120 of P450 2C2 have not affected the stability of the proteins, one exception being the substitution of Gly at residue 113 in both P450 2C2 and C2MstC1 (Straub et al., 1993a).

The levels of the mutant C2MstC1 proteins were assessed by immunoprecipitation of radioactive protein after incorporation of [$^{35}$S]methionine for 4 h, which equals at least 5 half-lives of the protein in COS1 cells (Straub et al., 1993b) and provides a good estimate for steady-state levels. No significant differences could be detected in the expression levels of any of the mutants (Figure 2). The stability of these mutants indicates that the region under investigation is not critical with respect to protein folding and stability and that the highly conserved amino acids are probably subject to functional rather than gross structural constraints.

*Effects of the Mutations on Progesterone Hydroxylation.* A representative analysis of the metabolites of progesterone formed by COS1 cells transfected with the C2MstC1 mutants is shown in Figure 3, and a summary of four independent experiments is presented in Figure 4B. The strongest effects on progesterone hydroxylation were found with mutations in the region between amino acids 111 and 115 (Figure 4B). In this region all mutations result in a 5-fold or greater reduction of progesterone hydroxylation even though most of the mutations characterized were conservative in nature. A substitution of Phe for Val-112 resulted in a decrease in activity

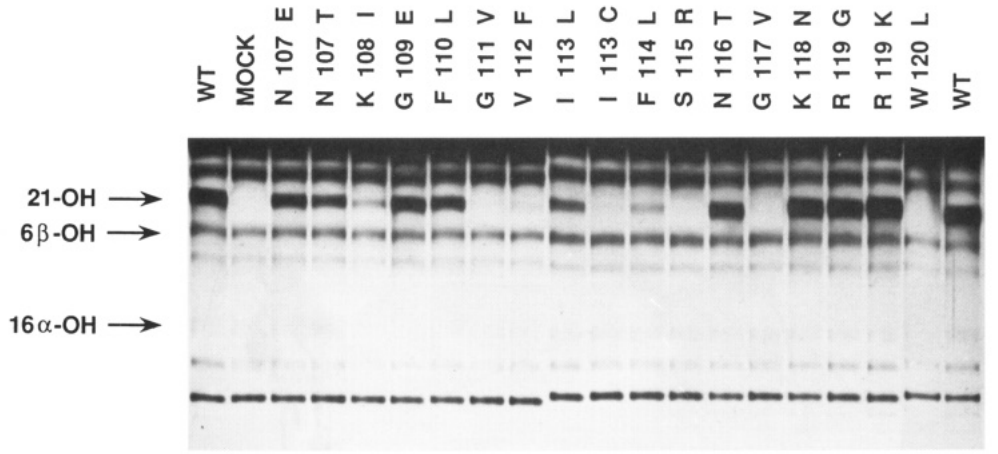


FIGURE 3: Autoradiogram of progesterone metabolites produced by intact COS1 cells after transfection with C2MstC1 mutants. Progesterone was added to the medium to a final concentration of 20 $\mu$M, with 1 $\mu$Ci of 1,2,6,7[$^3$H]progesterone (81 Ci/mmol) added per 0.6 mL. After 8 h, progesterone and its derivatives were extracted with chloroform and separated by thin-layer chromatography. An autoradiogram of the thin-layer chromatography plate is shown, and the positions of the unlabeled markers, 21-hydroxyprogesterone (21-OH), 16$\alpha$-hydroxyprogesterone (16$\alpha$-OH), and 6$\beta$-hydroxyprogesterone (6$\beta$-OH), are indicated. At the top, the mutants are designated as described in the caption to Figure 2.

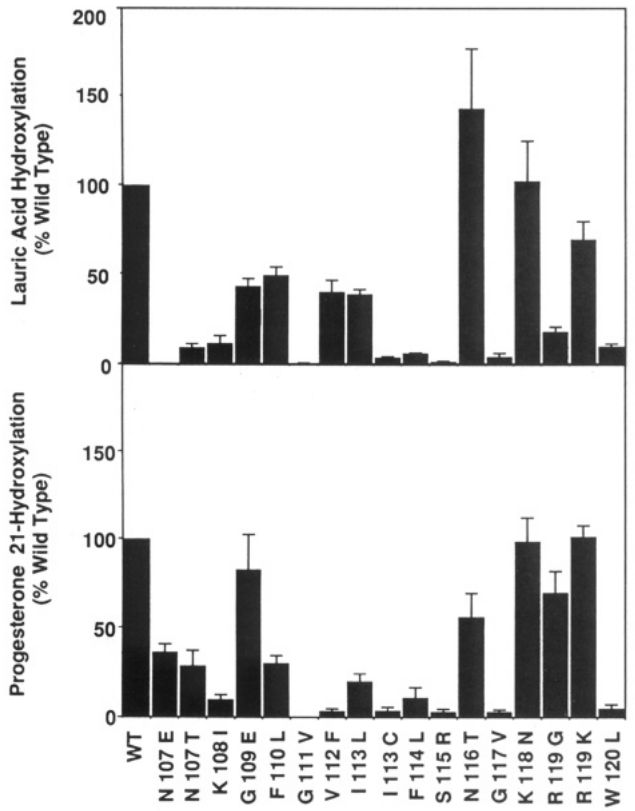


FIGURE 4: Lauric acid and progesterone hydroxylation in COS1 cells transfected with mutations in C2MstC1. The means of four independent transfections for each of the proteins are plotted with the standard error indicated. Designations for the mutants at the bottom are as described in the caption to Figure 2. Values for the individual hydroxylase activities were normalized against activity of the wild type. (A, top) Cell lysates from COS1 cells, transfected with the indicated mutants, were incubated with 20 $\mu$M [1-$^{14}$C]lauric acid for 90 min at 37 °C. Lauric acid and its hydroxylated derivatives were extracted with ethyl acetate and separated by high-pressure liquid chromatography. (B, bottom) Progesterone hydroxylase activities were determined as described in the caption to Figure 3.

to about 3% of the activity exhibited by C2MstC1, which is near the detection limit of the assay. A substitution of Leu for Ile-113 caused a 5-fold decrease in progesterone C21-hydroxylation, and changing Phe-114 to Leu resulted in a 10-fold decrease in activity. Changing Ile-113 to cysteine, a smaller hydrophilic amino acid, resulted in a decrease in activity to near the limits of detection. The substitution at position 115 from Ser to Arg also resulted in a similar reduction of activity. Because of their nonconservative nature, these two mutations might indirectly affect substrate interactions by altering protein structure, so that the information provided is limited. Similarly, the substitution of Gly for Arg may indirectly affect substrate interactions resulting in decreased lauric acid hydroxylase activity (Figure 4A).

Glycines are present in P450 2C2 at positions 109, 111, and 117. As noted above, Gly-111 and Gly-117 are highly conserved. In contrast, 18 of 54 P450s in family 2 contain other amino acids at position 109. As might be predicted from this pattern of conservation, mutation of Gly-109 to Glu, a larger negatively charged residue, did not significantly affect progesterone hydroxylase activity (Figure 4B). However, mutation of Gly-111 or Gly-117 to Val, a more conservative change than the change to Glu, reduced activities to near the limits of detection. Either the small size of Gly or its $\alpha$-helix-destabilizing property might be important at positions 111 and 117.

Mutations at positions 107–110 moderately reduced progesterone hydroxylation. Substitution of Glu or Thr for Asn-107 resulted in an approximately 3-fold reduction of progesterone C21-hydroxylation. Although the Thr mutation was a relatively conservative substitution of an uncharged polar amino acid, the Glu substitution introduces a negative charge. The similar effects of both substitutions and the moderate reduction in activity with a nonconservative change suggest that residue 107 is not likely to directly influence progesterone binding. At position 108, the positively charged hydrophilic residue Lys was replaced by Ile, an uncharged hydrophobic amino acid, resulting in a 20-fold decrease in activity. Although the decrease in activity suggests that this residue is important for activity, the nonconservative nature of the exchange may result in substantial changes in the local structure and indirectly affect the active site. Mutation of Phe-110 to Leu resulted in a 3-fold decrease in activity. Interestingly the same mutation at position 114 resulted in a 30-fold inhibition. This modest effect of the substitution at position 110 suggests that this residue does not play a major role in substrate recognition.

On the C-terminal end of the cassette, nonconservative mutations at positions 116, 118, and 119 did not cause pronounced reductions in progesterone C21-hydroxylation.

Replacing Asn-116 by Thr resulted in only a 40% reduction in progesterone hydroxylation, whereas changing the positively charged Lys-118 to a neutral Asn did not reduce progesterone hydroxylation. Finally, a mutation of the charged, bulky Arg-119 to Gly resulted in only a 30% reduction in progesterone hydroxylation, while a conservative substitution by lysine had no effect. These results clearly demonstrate that the amino acids at positions 116–119, with the exception of Gly-117, can be exchanged without major effects on the ability of the protein to hydroxylate progesterone.

*Effects of the Mutations on Lauric Acid Hydroxylation.* Many of the mutations produced similar effects on lauric acid hydroxylation compared to progesterone hydroxylation (Figure 4A). Strong decreases in the activity of both reactions resulted from mutations at Gly-111, Gly-117, Lys-108, Phe-114, Ser-115, and Trp-120 and from hydrophilic substitutions at 113. Decreased activity for both substrates could result from altered substrate–enzyme interactions or other effects on the enzymatic activity of the protein. Modest reductions in the activity of both reactions were observed with relatively nonconservative substitutions at Gly-109, Phe-110, Asn-116, and Lys-118 and conservative substitutions at Arg-119. These modest effects on two dissimilar substrates suggest that these positions do not directly affect substrate–enzyme interactions.

In contrast, mutations at some positions had distinctly different effects on progesterone and lauric acid hydroxylation. Mutation of Val-112 to Phe resulted in a 30-fold inhibition of progesterone hydroxylation, but only a 2–3-fold inhibition of lauric acid hydroxylation. Likewise, changing Ile-113 to Leu was better tolerated by lauric acid than by progesterone, although the difference in the magnitude of inhibition was only 2-fold. Mutation of Asn-107 reduced activity more for hydroxylation of lauric acid than for hydroxylation of progesterone and was particularly dramatic for the Glu substitution, which reduced lauric acid hydroxylation to undetectable levels (Figure 4A). Substitution of Gly for Arg-119 reduced lauric acid hydroxylation 5-fold but had little effect on the progesterone reaction. These differential effects indicate that changes at these positions directly or indirectly affect substrate–enzyme interactions.

## DISCUSSION

Seventeen mutations in the SRS1 region, previously analyzed in P450 2C2 (Straub et al., 1993b), have now been studied in C2MstC1, a chimera of P450 2C2 and P450 2C1 that exhibits progesterone C21-hydroxylase activity which is absent in the wild-type proteins. In the chimera, the effects of a given mutation on the hydroxylation of two substrates, one a relatively rigid steroid molecule and the other a flexible saturated fatty acid, can be compared. Differential effects of a mutation on hydroxylation of two dissimilar substrates by the same P450 provides strong evidence that the residue is important, directly or indirectly, for interactions of the substrate with the enzyme. In P450 2C2 (Straub et al., 1993b) and C2MstC1, substitution of Phe for Val-112 decreased lauric acid hydroxylation by only 40%. Since the level of expression of functional P450 cannot be determined in the transfected COS1 cells, reductions of this magnitude might be due to altered expression rather than inherent activity of the mutated enzyme. However, progesterone hydroxylation in C2MstC1 was reduced by this mutation to 3%, near the detection limits of the assay. This differential effect on the two substrates provides strong evidence that the level of functional enzyme does not account for the decrease and that residue 112 influences substrate-enzyme interactions. This conclusion is consistent with its position adjacent to residue 113, which has been shown to affect substrate binding in several P450s (Kronbach et al., 1989, 1990, 1991; Lindberg & Negishi, 1989; Halpert & He, 1993; Richardson & Johnson, 1993; Straub et al., 1993a). The I113C mutation also appeared to affect the progesterone C21-hydroxylase activity to a greater extent than the hydroxylation of lauric acid, although the difference was not as great as that seen for the V112F mutant.

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        B'              C       Helices
    |------|          |--
 89 PREAGEAY-DFIPTSM-DPPEQ- 108  P450cam
107       NKGFGVIFS--NGKRW  120  P450 2C2
 75 LKFVRDFAGDGLFTSWTHEKNW  96  P450BM-3
    --------|          |---
        B'              C       Helices
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FIGURE 5: Alignment of P450cam and P450BM-3 sequences with residues 107–120 of P450 2C2. P450cam and P450BM-3 were aligned on the basis of their tertiary structure (Ravichandran et al., 1993). The positions of $\alpha$-helicies B′ and C in P450cam and P450BM-3 are indicated.

Mutation of residues 107, 108, and 119 affected lauric acid hydroxylation more strongly than progesterone 21-hydroxylation. This is surprising, as these mutations had little effect on the lauric acid hydroxylase activity of P450 2C2. In addition, nearly all the mutations tested were more detrimental in C2MstC1 than they were in P450 2C2, although the effects of mutations at other residues in C2MstC1 were qualitatively similar to those in P450 2C2. Asn-107 and Lys-108 of P450 2C2 align within $\alpha$-helix B′ of P450cam and P450BM-3 (Figure 5), and this helix is in close apposition to $\beta$-sheet 3 in P450cam and $\beta$-strand 1–4 in P450BM-3. Each of these $\beta$-sheets forms part of the substrate binding pocket and is derived from the P450 2C1 portion of the C2MstC1 chimera. These nearby P450 2C1 sequences could influence the effects of mutations in the $\alpha$-helix B′ region derived from P450 2C2.

The 107–120 region of P450 2C2 is aligned in Figure 5 with the corresponding sequences of P450cam and P450BM-3, which are aligned with each other on the basis of their tertiary structures (Ravichandran et al., 1993). In contrast to P450cam, P450BM-3 has substantial sequence similarity with P450 2C2, with 5 of 13 identical amino acids in the region aligned in Figure 5; and like P450 2C2, P450BM-3 is a lauric acid hydroxylase. In our previous alignment with P450cam (Straub et al., 1993b), residues 107–110 align with $\alpha$-helix B′, 112–116 are part of a substrate-contacting loop, and 118–120 are part of $\alpha$-helix C. Gly-111 and -117 are at either end of the substrate-contacting loop. Trp-120 is highly conserved in all mammalian P450s and is located behind the heme near basic residues proposed as part of a docking site for P450 reductase (Nelson & Strobel, 1987; Poulos et al., 1987; Stayton & Sligar, 1990; Gotoh, 1992; Koga et al., 1993), and it may facilitate the transfer of electrons from the reductase to the heme of P450 (Straub et al., 1993c). In agreement with this model, substitutions at Gly-111, Gly-117, Trp-120, and residues 112–116 all strongly reduced both the progesterone and the laurate hydroxylase activities of C2MstC1.

The alignment of P450 2C2 with P450BM-3 forces a shift of residues 110–115 of P450 2C2 by two positions, and of residues 107–108 by three positions, in the C-terminal direction compared to the alignment with P450cam proposed earlier (Straub et al., 1993b). Gly-109 of P450 2C2 aligns with a gap in P450cam introduced by the alignment of P450cam with P450BM-3 (Ravichandran et al., 1993). In this alignment, Val-112 and Ile-113 of P450 2C2 align close to Phe-87 of P450BM-3. Phe-87 is positioned near the end of the substrate binding pocket, and Ravichandran et al. (1993) proposed that it might be important for sequestering the $\omega$-end

of lauric acid, which is not hydroxylated. The model based on the alignment with P450BM-3 agrees well with the evidence suggesting that position 113 in the mammalian P450s interacts with the substrate as described above. Gly-111 of P450 2C2 aligns with Gly-85 of P450BM-3. This alignment suggests that the critical role of Gly-111 is not related to its $\alpha$-helix-breaking property as proposed previously (Straub et al., 1993b), as it is positioned two residues from the end of the B′-helix. Instead, its small size or the increased flexibility it confers to the loop at this position may be required for substrate access to the active site. Gly-85 in P450BM-3 is followed by two hydrophobic amino acids, including Phe-87, which would be analogous to Gly-111 followed by three hydrophobic residues in the mammalian P450s. These residues possibly could provide a hydrophobic region for substrate interaction. The mutational data with P450 2C2 and C2MstC1, which suggest that each of these hydrophobic residues is important in substrate interactions, are consistent with their alignment with substrate-contacting residues in P450BM-3.

The motif of Gly followed by three or four hydrophobic amino acids in SRS1 is conserved in all family 2 P450s. It is also conserved in P450 family 4 members for which lauric acid and prostaglandins are substrates and in other, but not all, steroid hydroxylases (Gotoh & Fujii-Kuriyama, 1989). Interestingly, the adrenal progesterone C21-hydroxylase does not have the Gly–hydrophobic amino acid motif. However, the substrate specificity of the natural liver progesterone C21-hydroxylase, P450 2C5, differs substantially from that of the adrenal enzyme (Trant et al., 1990), so that the active sites of these two enzymes are clearly different. C2MstC1 presumably has an active site more similar to that of its close relative, P450 2C5. In contrast, the steroid-metabolizing adrenal enzymes $P450_{17\alpha}$, $P450_{ssc}$, and $P450_{11\beta}$ all contain this motif, as does $P450_{AROM}$ with aromatase activity. These regions of both $P450_{17\alpha}$ and $P450_{AROM}$ have been aligned near the substrate-contacting amino acids of P450cam, Tyr-96 and Phe-98, and have been predicted by molecular modeling to form hydrophobic interactions with the substrate (Laughton et al., 1990, 1993). Using similar methods, Vijayakumar and Salerno (1992) aligned this region in $P450_{ssc}$ differently but also near another substrate-contacting residue of P450cam, Phe-87. The presence of Gly followed by three or four hydrophobic acids near the substrate binding site in these distantly related microsomal and mitochondrial P450s suggests that this motif is important for the binding and metabolism of fatty acids and steroids in many P450s.

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