Membrane-Protein Interactions Contribute to Efficient 27-Hydroxylation of Cholesterol by Mitochondrial Cytochrome P450 27A1*

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Mitochondrial cytochrome P450 27A1 (P450 27A1) catalyzes 27-hydroxylation of cholesterol, the first step in the alternative bile acid biosynthetic pathway. Although several crystal structures of P450s are known, no structural information is available for the mammalian, membrane-bound enzymes involved in the removal of cholesterol from the body. We prepared a three-dimensional model of P450 27A1 based on the structure of P450 BM-3. Conservative and non-conservative mutations were introduced at hydrophobic and positively charged residues in the putative F-G loop and the adjacent helix G (positions 219–237). Subcellular distribution of the mutant P450s expressed in Escherichia coli was used as a measure of membrane-protein interactions. Conservative substitutions of residues located on the surface, according to our model, L219V, L219F, Y220F, F223Y, L224I, R229K, Y231L, F234Y, R236R, and R237K, weakened the association of the mutant P450s with the membrane and led to the appearance of up to 21% of P450 27A1 in the bacterial cytosol. It is likely that the mutated side chains are involved in binding to membrane phospholipids. Substitutions in the F-G loop did not significantly affect the $K_m$ value for cholesterol hydroxylation. However, non-conservative mutants, L219N, Y220A, Y220S, F223A, K226R, and R229A, had significantly impeded catalytic properties, indicating strict requirements for the size and polarity of the side chains at these positions for the catalysis. The results provide insight into the membrane topology of mitochondrial P450s and indicate the importance of membrane-protein interactions in the efficiency of reactions catalyzed by P450 27A1.

Conversion of cholesterol into bile acids is the principal route of cholesterol removal from the body in mammals (1). Cholesterol is degraded to bile acids either through the classical (hepatic) or alternative (extrahepatic) bile acid biosynthetic pathways, involving several cytochrome P450 (P450)3 enzymes. P450 7A1 catalyzes 7α-hydroxylation of cholesterol, the first and rate-limiting step in the classical bile acid biosynthetic pathway (2, 3), whereas P450 27A1 carries out 27-hydroxylation of cholesterol, the first step in the alternative bile acid biosynthesis (4, 5). P450 27A1 also catalyzes multiple oxidation reactions at the C-27 atom of bile acid intermediates in the classical bile acid biosynthetic pathway. P450 7A1 seems to be vital for life because no mutations have yet been identified in the gene for this protein. Mutations in P450 27A1 gene correlate with a hereditary disease, cerebrotendinous xanthomatosis (CTX) (6), which is characterized by the abnormal deposition of cholesterol and cholestanol in body tissues. If untreated, CTX leads to tendon xanthomas, serious neurological defects, premature arteriosclerosis, osteoporosis, and even death (7). P450s 7A1 and 27A1 are membrane-bound proteins located in the endoplasmic reticulum and inner mitochondrial membrane, respectively. Both mitochondrial and microsomal members of the cytochrome P450 superfamily are proposed to have a similar mode of association with the membrane via a large hydrophilic domain anchored to the lipid bilayer through several noncontiguous portions of the polypeptide chain (8, 9). The membrane interacting areas have not yet been identified in mitochondrial P450s, and it is not clear at present whether they are the same in different enzymes. Microsomal P450s share at least one common membrane-binding site, an N-terminal signal anchor of about 20 amino acid residues. Another possible common site of association with the endoplasmic reticulum is an area between the F and G helices, the F-G loop (10–14). The F-G loop was also proposed to form a mouth of the channel that allows the substrate to pass from the surface of the P450 molecule to the buried active site (10, 11). Our recent studies indicate that membrane binding and substrate access merge in cholesterol-metabolizing enzyme, microsomal P450 7A1, and the putative F-G loop is simultaneously the site of attachment to membrane and cholesterol entry in this P450 (14). It is likely that another cholesterol-metabolizing enzyme, mitochondrial P450 27A1, also recruits cholesterol from the lipid bilayer, as neither the enzyme nor its substrate are soluble in the aqueous environment of the cell. Previously, we have studied the role of the putative helices F and G in P450 27A1, regions that flank the F-G loop (15). Our data indicate that the putative helices F and G form the sides of the of the substrate access channel in this P450, and residues lining the interior of the channel control the orientation of the substrate as it enters

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The atomic coordinates and structure factors (code IMFX) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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The abbreviations used are: P450, cytochrome P450; CTX, cerebrotendinous xanthomatosis; PDB, protein data base.
membrane P450 27A1 with microsomal P450 7A1. Here we describe the effect of the enzyme active site (15). Here we describe the effect of the enzyme active site (15). Here we describe the effect of the enzyme active site (15).

| Template | Mutation | Oligonucleotides |
|----------|----------|------------------|
| Wild type | L219N | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | L219V | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | L219I | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | Y220A | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | Y220S | 5'-ATGTCTGAAATCTATCTCCACCTTC-3' |
| Wild type | Y220F | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | F223A | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | F223L | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | F223Y | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | F224A | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | F224I | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | P235A | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | P235V | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | P235I | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R236A | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R236K | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R236F | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R237A | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R237K | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R237F | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R237V | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R237Y | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R237L | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R237I | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |
| Wild type | R237S | 5'-GGTTAATTTGGCTCAAGAAATATCAATCCACCTTC-3' |

The mutated nucleotides are underlined.

Site-directed Mutagenesis—This was carried out using an in vitro QuikChange™ Site-directed Mutagenesis Kit (Stratagene) according to the instructions. The templates and complementary mutagenic oligonucleotides are shown in Table I. Mutations were confirmed by DNA se-

EXPERIMENTAL PROCEDURES
Subcellular Fractionation of the Mutant P450s in Escherichia coli—E. coli cultures were grown and harvested as described previously (16, 17), and the amount of the functional P450 protein in the E. coli cells was assessed by the reduced CO difference spectrum (18). Spheroplasts were prepared by suspending cells in 50% of the original culture volume in 10 mM potassium phosphate buffer (KP), pH 7.4, containing 20% glycerol. The cell suspension was incubated with lysozyme (0.5 mg/ml) for 30 min at 4 °C. Spheroplasts were pelleted at 5,000 × g for 10 min and then resuspended in 10 mM KP, pH 7.4, containing 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 1 μg/ml pepstatin. After sonication on ice using six 20-s pulses at 1 min intervals, cell debris was removed at 5,000 × g (10 min). The supernatant and pellet obtained after subsequent ultracentrifugation at 106,000 × g for 60 min were used as cytosolic and membrane fractions, respectively.

Quantification of P450 27A1 Subcellular Distribution—Proteins in subcellular fractions were separated by SDS-PAGE (5 μg of the total protein/lane) and transferred to a nitrocellulose membrane. Western blot analysis was carried out using rabbit antiserum against P450 27A1 and an ECL detection system (Amersham Biosciences) according to the manufacturer’s instructions. X-ray films were scanned, and the immunoreactive signal was quantified using NIH Image 1.52 software. Only exposures lying within the film’s linear range of sensitivity were used. Statistical analysis was carried out using Microsoft Excel software.

Determination of the Kinetic Parameters for Cholesterol 27-Hydroxylation—Enzymatic activities of wild type and mutated forms of P450 27A1 were determined as described (16, 19) using E. coli membrane fractions. The reaction conditions were optimized for the formation of cholesterol 27-hydroxylation. Reconstituted systems (wild type or mutant) contained varying concentrations of cholesterol. In all studies no more than 15% of radioactive cholesterol was converted at a zero point. To determine kinetic parameters for cholesterol 27-hydroxylation, enzymatic activity was measured at a zero point, when no cold cholesterol was present in the reaction mixture, and the concentration of 3Hcholesterol was very low and equal to 1.9 nM, and in the presence of increasing concentrations of cold cholesterol (2.5–60 μM). The Kν value was calculated from a double-reciprocal plot of a difference between the maximal spectral response. The assays were performed using partially purified preparations of P450 27A1 with the specific heme content of 6–8 nmol/mg protein. Titrations with cholesterol (1 μM P450 27A1) and 27-hydroxycholesterol (2 μM P450 27A1) were carried out in 1 ml of 50 mM KP, pH 7.2, and 50 mM KP, pH 7.2, containing 20% glycerol, respectively. Both ligands were added from a stock solution (1–10 mM) in 45% 2-hydroxypropyl-β-cyclodextrin. After each experiment, P450 content was quantified to confirm that there was no denaturation during titration. Under the experimental conditions used, the apparent Km of P450 27A1 wild type for the cholesterol was 0.19 μM.

Modeling of P450 27A1—The secondary structure of P450 27A1 was predicted using the program Jpred, and those of P450 BM-3 were taken from the PDB code 1FAG. The region in bold was modeled using constraints extracted from a similar region of PDB code 1QTG (see “Experimental Procedures”).

![Fig. 1. Alignment of P450 27A1 with P450 BM-3 (PDB code 1FAG) used for modeling. Underlined regions represent α-helices.](Image 314 to 548x728)
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RESULTS

Modeling and Analysis—Our working model of the P450 27A1 is shown in Fig. 2. Some of the residues studied here, which are probably involved in the F–G loop region, are indicated.

Mutagenesis Strategy—Initially, non-conservative and mainly alanine substitutions were introduced at each position occupied by the hydrophobic, positively charged or proline side chains within the putative F-G loop and the N-terminal part of the G helix in P450 27A1 (residues 219–237, Fig. 1), and the effects on subcellular distribution and kinetic parameters for cholesterol hydroxylation were investigated. Because the observed effects could be the result of conformational changes introduced by non-conservative replacement, additional, conservative substitutions were then generated for each position under the study except for that occupied by the proline residues. Where possible, the conservative mutations reproduced amino acid residues found at the corresponding positions in P450s 27A1 from other species (L219V, V231L, P234Y, and K236R). The K226R substitution reproduces one of the mutations that underlies CTX. Trp-235 and Tyr-238 were studied earlier and the properties of the W235A and Y238A mutants are described elsewhere (15).

Effect of Mutations on Expression Levels in E. coli—As shown in Table II and Fig. 3, conservative and non-conservative substitutions at positions 219, 220, 223, and 236 did not affect the levels of expression of P450 27A1, as assessed by the CO spectra and Western blot analysis. In contrast, both conservative and non-conservative substitutions at positions 224, 226, 229, and 232 reduced by 2–5-fold the amount of the functional P450 form. The decrease in the P450 form of the L224A, L224I, R229K, and L232I mutants correlated with the decrease in the immunoreactive protein on the Western blot, whereas the K226A, K226R, R229A, and L232A mutants showed about 1.5–2.5-fold more immunoreactive protein than the functional P450 form, indicating either reduced stability of the mutant enzyme or a conformational change that affects protein folding and heme binding or both. Conservative substitutions at positions 231, 234, and 237 resulted in the same amounts of functional P450 form as the wild type enzyme, whereas non-conservative alanine mutations decreased the amount of the P450 form to the same extent as the level of the immunoreactive protein. No functional P450 was found in the cells expressing the P225A, W227A, and P233A mutants; however, immunoreactive protein was still detected on a Western blot. In our model structure, both Pro-225 and Pro-233 play important structural roles; Pro-225 is located within one of the turns in the F-G loop, whereas Pro-233 terminates the F-G loop and initiates the helix G. It is likely that the P225A and P233A mutations affected P450 27A1 conformation.

Subcellular Distribution of the Mutant P450s—The wild type P450 27A1 is localized exclusively to the E. coli membrane fraction in both low (10 mM KPi, 1 M KCl, 20% glycerol) and high (400 mM KPi, 1 M KCl, 20% glycerol) ionic strength buffers. This P450 is associated with the E. coli membrane relatively tightly because only 30–40% of the enzyme can be solubilized from the membrane by using 0.8% sodium cholate. For comparison, 80% of P450 11A1, another mitochondrial enzyme, can be recovered from the membrane by using 0.5% sodium cholate. Conservative substitutions of residues that lie on the surface of the P450 27A1 model (Fig. 2), including the L219V, L219I, Y220F, P223Y, L224I, R229K, V231L, P234Y, K236R, and R237K mutations, resulted in appearance from 7.1–21% of P450 27A1 in the cytosol in low ionic strength buffer (Table II and Fig. 3). Supported by the homology model, the subcellular distribution data thus indicate the involvement of Leu-219, Tyr-220, Phe-223, Leu-224, Arg-229, Val-231, Phe-234, Lys-236, and Arg-237 in the interaction with membrane phospholipids. Non-conservative and conservative substitutions of Leu-219 and Tyr-220 provided insight into whether it is the hydrophobicity or the size of the side chain that is important for membrane-protein interactions. In the case of the Leu-219 mutants, the amount of the P450 in the cytosol rose with the increase in size of the replacing amino acid residue (L219N < L219V < L219R). A similar tendency was observed for the Tyr-220 mutants. Replacement of Tyr-220 with Ala or Ser resulted in less cytosolic P450 than mutation to Phe. For Phe-223, substitutions with Ala, Val, and Tyr, which have different...
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| P450 27A1   | Expression in E. coli (nmol/liter) | % in cytosol |
|------------|------------------------------------|--------------|
| Wild type  | 600–800                            | 0            |
| L219N      | Same                               | 7.7 ± 1.4    |
| L219I      | Same                               | 21.0 ± 1.7   |
| Y220A      | Same                               | 7.1 ± 1.2    |
| Y220F      | Same                               | 14.0 ± 0.4   |
| F223L      | Same                               | 15.6 ± 3.1   |
| F223Y      | Same                               | 17.1 ± 1.7   |
| L224A      | ↓ 2-Fold                           | 15.1 ± 1.9   |
| L224I      | ↓ 3-Fold                           | 12.9 ± 1.7   |
| K226A      | No functional P450                 | 3.6 ± 1.2    |
| K226R (underlies CTX) |                   | 15.6 ± 0.8   |
| W227A      | No functional P450                 | 5.6 ± 1.9    |
| R229A      | ↓ 10-Fold                          | 2.5 ± 0.5    |
| R229K      | ↓ 3-Fold                           | 8.0 ± 1.9    |
| P230A      | Same                               | 11.4 ± 3.5   |
| V231A      | ↓ 2-Fold                           | 8.6 ± 1.7    |
| V231L (as in pig P450 27A1) |               | 7.1 ± 0.6    |
| L232A      | ↓ 3-Fold                           | 11.4 ± 2.0   |
| L232I      | ↓ 3-Fold                           | 2.4 ± 0.4    |
| P233A      | No functional P450                 | 4.9 ± 1.3    |
| F234A      | ↓ 3-Fold                           | 4.6 ± 1.1    |
| F234Y (as in pig P450 27A1) |               | 14.9 ± 1.4   |
| K236A      | Same                               | 9.5 ± 2.7    |
| R236R (as in pig P450 27A1) |               | 12.6 ± 1.3   |
| R237A      | ↓ 3-Fold                           | 13.5 ± 1.8   |
| R237K      | Same                               | 13.9 ± 3.9   |
| L219I/F223Y/F234Y/K236R/R237K |           | 15.6 ± 2.0   |
| L219I/F223Y/V231L/F234Y/K236R |           | 20.6 ± 2.4   |

Assessed by Western blot analysis. P450 27A1 in cytosol is expressed as a percentage of the total recovered in the membrane fraction and the cytosol. In all cases, two independent expression experiments were carried out to isolate two sets of membrane fraction and cytosol. SDS-PAGE was then carried out for each set independently, and one or two different gels were run independently for each set following by Western blot analysis. The results represent the average of three to four different Western blot analyses ± S.D.

Conservative substitution K226R also resulted in a cytosolic P450; however, as discussed earlier, this substitution likely affected either the enzyme stability or conformation. The homology model suggests that the side chain of Lys-232 can form a hydrogen bond with the Phe-223 carbonyl oxygen, and replacement of this side chain may lead to conformational changes in the loop region and consequently weaken the interaction with the membrane. The homology model also provides an explanation of why only small amounts of the L232I mutant were seen in the cytosol. The side chain of Leu-232 is partially buried, and most probably does not contribute significantly to the interaction with membrane phospholipids.

To test whether the F-G loop is the only site of attachment to membrane in P450 27A1, two penta mutants were generated. Multiple mutations resulted in only 15–21% of P450 in the cytosol, amounts comparable with those generated by single substitutions.

Kinetic Parameters for Cholesterol 27-Hydroxylation of the Mutant P450s—As is seen from Table III, conservative and non-conservative substitutions within the F-G loop did not significantly affect the $K_m$ value for cholesterol. The L232A and F234A mutants exhibited the highest, 2.1-fold, increase in $K_m$. The Y220F and F223L mutants showed the lowest, 2.2-fold, decrease in $K_m$. However, the $k_{cat}$ values for the mutants varied greatly. On the basis of catalytic properties, the mutants could be arbitrarily placed into two groups. The first group is formed by the L219V, L219I, Y220F, L224A, L224I, R229K, P230A, V231A, V231L, L232I, F234A, F234Y, K236A, K236R, R237A, and R237K mutants. The $k_{cat}$ and catalytic efficiency ($k_{cat}/K_m$) of these mutants were either unchanged or changed up to 2.9- and 4.6-fold, respectively. The 2.9- and 4.6-fold values were chosen as a cut-off limit for this group of mutants based on the properties of the K226R mutant, a substitution that affects metabolism of cholesterol in vivo and results in the disease CTX. Minor effects of both non-conservative and conservative substitutions at positions 224, 231, 234, and 236 (the L224A, L224I, V231A, V231L, F234A, F234Y, K236A, K236R, R237A, and R237K mutants) and non-conservative substitution of Pro-230 to Ala implies that requirements for the size of the side chains at these positions are relaxed for efficient cholesterol hydroxylation.

The second group includes the mutants with significantly (L219N, Y220A, Y220S, F233A, and R229A) and moderately (F233L, F233Y, K236R, and L232A) altered catalytic properties. The L219N, Y220A, and R229A substitutions resulted either in undetectable or very low enzymatic activity, making it difficult to determine the kinetic parameters. The Y220S and F233A replacements decreased the catalytic efficiency more than 50-fold, and the K226R substitution led to a 4.6-fold decrease in catalytic efficiency. The $k_{cat}$ of the F223L, F223Y, and L232A mutants was changed 3.1–3.8-fold. However, the catalytic efficiency was changed only up to 1.8-fold because of the simultaneous change of the $K_m$. In contrast to the R229A, K236R, and L232A mutants, undetectable or extremely low enzymatic activity of the L219N, Y220A, Y220S, and F233A mutants and the decreased $k_{cat}$ of the F223L and F223Y mutants are not the result of structural perturbations around the heme, because the membrane fractions of these mutants showed a large peak at 450 nm and a small peak at 420 nm in the reduced CO difference spectrum (Fig. 4), indicating retention of the integrity of the P450 heme environment. The data therefore indicate that size and polarity of the side chains at positions 219, 220, and 223 are crucial for the efficient catalysis of cholesterol.

**DISCUSSION**

The results obtained in this study indicate that the putative F-G loop and the N-terminal part of the putative helix G (residues 219–237) are involved in the association with membrane in P450 27A1. Analysis of the effects of amino acid substitutions on expression and subcellular distribution of mutant enzymes and examination of the homology model allowed identification of residues that are likely to participate in the interaction with membrane phospholipids (Leu-219, Tyr-220, Phe-223, Leu-224, Arg-229, Val-231, Phe-234, Lys-236, and Lys-237) and residues whose mutations might cause structural
rearrangements, resulting in a change of the shape of the putative F-G loop and membrane binding properties (Pro-225, Lys-226, Trp-227, and Pro-233). The putative F-G loop is predicted to contribute to membrane association in microsomal P450s (10, 11) and shown to contain residues involved in the interaction with the lipid bilayer in microsomal P450s 2B1, 2C5, and 7A1 (12–14). The present work is the first study showing that the putative F-G loop and the N-terminal part of the putative G helix are also the sites of membrane attachment in mitochondrial P450 27A1. As this region is amphipathic and about the same size in different mitochondrial P450s (15), it is very likely that the putative F-G loop and a part of the helix G are also involved in membrane binding in other mitochondrial P450s. Thus, mitochondrial and microsomal P450s appear to have an overlapping membrane-binding site. This finding supports the notion that mitochondrial P450s most probably arose from mistargeting of microbial enzymes during evolution (26). Introducing five different mutations simultaneously in the loop had no more effect on membrane attachment than a single mutation suggesting that there are additional site(s) of association with the membrane in P450 27A1. Weakening of the membrane-protein interactions in the putative F-G loop may lead to a tighter binding to the membrane at the other side(s).

Previously, we have shown that conservative and non-conservative substitutions of the three residues within the putative F-G loop, which forms a part of the enzyme-membrane interface in microsomal cholesterol hydroxylating P450 7A1, decreased significantly the \( K_m \) value for cholesterol but did not alter the apparent \( K_d \) value (14). We proposed that these residues are adjacent to the entry of the substrate access channel and that they participate in substrate recognition, which involves initial docking of cholesterol and determines orientation of the substrate as it enters the substrate access channel and then the enzyme active site (14). In mitochondrial cholesterol hydroxylating P450 27A1, mutations within the putative F-G loop did not significantly affect the \( K_m \), indicating that either cholesterol is not recognized by the surface residues in P450 27A1 or the putative F-G loop is not the site where cholesterol recognition occurs. The \( K_m \) value for cholesterol of P450 27A1 (7.0 \( \mu \)M) is more than 30-fold higher than the \( K_m \) value (0.19 \( \mu \)M), indicating that binding and release of the substrate is slower than product formation and release (\( k_{cat} \)) and are thus the rate-limiting steps in the 27-hydroxylation of cholesterol (Table IV). This is in contrast to P450 7A1, where \( K_m \) and \( K_p \) values of which are similar (6.9 \( \mu \)M and 9.5 \( \mu \)M, respectively). Possibly, these two P450s have different mechanisms for substrate recruitment and retaining in the active site.

P450s 7A1 and 27A1 are also different in terms of their catalytic efficiencies because the \( k_{cat} \) value of P450 7A1 is more than 20-fold higher than that of P450 27A1 (349 versus 15.6 \( \text{min}^{-1} \)), whereas the \( K_m \) values are approximately the same (Table IV). Non-conservative substitutions of Leu-219, Tyr-220, and Phe-223, the side chains which are likely involved in the interaction with membrane phospholipids, significantly impaired catalytic activity of mutant P450s. This finding raises a question as to how the mutations of the surface residues located outside the enzyme active site influence the catalysis inside the buried active site. There are two possible explanations in our opinion. The first is that weakening of the membrane-protein interactions increases water access to the substrate access channel and enzyme active site. Water molecules can serve as the proton donor for catalysis, and changes in the active site solvation may affect the proton-transfer pathway to bound oxygen and consequently catalysis. The second explanation is that alteration of the membrane-protein interactions slows down exit of product, thus decreasing the \( k_{cat} \). To test whether the mutations affected product binding, we tried to determine the apparent \( K_p \) value for 27-hydroxycholesterol by using a spectral binding assay. Unfortunately, the spectral shift of the wild type P450 27A1 induced by 27-hydroxycholesterol was very low, thereby precluding a reliable determination of the constant.

Kinetic differences between P450s 27A1 and 7A1 are likely to reflect different physiological requirements for the two enzymes. Up to 400 mg of cholesterol is degraded daily to bile acids through the classical bile acid biosynthetic pathway in which P450 7A1 catalyzes the first and rate-limiting step (3), and only about 4% of cholesterol (16 mg) is converted to bile acids through the alternative (extrahepatic) bile acid pathway initiated by the P450 27A1 (26). Because of the high quantities of cholesterol that must be metabolized, P450 7A1 should be a
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Kinetic parameters for 27-hydroxylation of cholesterol were determined as described under “Experimental Procedures.” The results are the mean ± S.D. of three to four experiments.

| P450 27A1 | \( K_m \) \( \mu M \) | \( k_{cat} \) \( min^{-1} \) | \( k_{cat}/K_m \) \( min^{-1} \mu M^{-1} \) |
|-----------|----------------|-------------------|---------------------|
| Wild type | 7.0 ± 1.0 | 15.8 ± 2.6 | 2.2 ± 0.3 |
| L219N | Undetectable | | |
| L219V (as in rabbit P450 27A1) | 12.1 ± 0.3 | 28.3 ± 2.5 | 2.3 ± 0.3 |
| L219I | 7.2 ± 0.8 | 24.7 ± 4.5 | 3.4 ± 0.3 |
| Y220A | Undetectable | | |
| Y220S | 6.9 ± 1.6 | 0.3 ± 0.1 | 0.04 ± 0.01 |
| Y220F | 3.2 ± 0.7 | 8.7 ± 1.9 | 2.7 ± 0.1 |
| F223A | 19.8 ± 6.0 | 0.9 ± 0.3 | 0.04 ± 0.003 |
| F223L | 3.2 ± 0.5 | 4.2 ± 0.4 | 1.3 ± 0.1 |
| F223Y | 4.0 ± 0.5 | 4.9 ± 0.9 | 1.2 ± 0.1 |
| L224A | 5.2 ± 0.4 | 9.4 ± 0.2 | 1.8 ± 0.1 |
| L224I | 12.0 ± 0.1 | 13.8 ± 2.2 | 1.1 ± 0.2 |
| P225A | No functional P450 | | |
| K236A | 13.4 ± 1.1 | 38.3 ± 4.2 | 2.9 ± 0.1 |
| K236R (underlies CTX) | 11.3 ± 0.5 | 5.4 ± 0.1 | 0.48 ± 0.01 |
| W227A | No functional P450 | | |
| R229A | 13.6 ± 2.1 | 29.9 ± 1.2 | 2.2 ± 0.2 |
| R292K | Very low activity | 10.1 ± 0.9 | 2.3 ± 0.01 |
| P230A | 6.6 ± 0.4 | 22.0 ± 2.3 | 2.6 ± 0.2 |
| V231A | 6.6 ± 0.6 | 21.0 ± 0.9 | 3.2 ± 0.2 |
| V231L (as in pig P450 27A1) | 14.6 ± 1.2 | 49.7 ± 5.8 | 3.4 ± 0.4 |
| L232A | 13.8 ± 3.7 | 31.6 ± 6.0 | 2.4 ± 0.6 |
| L232I | No functional P450 | | |
| P233A | 14.5 ± 0.9 | 22.1 ± 3.0 | 1.53 ± 0.1 |
| P234A | 9.9 ± 1.4 | 27.8 ± 1.6 | 2.9 ± 0.5 |
| P234Y (as in pig P450 27A1) | 6.6 ± 0.6 | 13.7 ± 1.2 | 2.1 ± 0.3 |
| R237A | 12.6 ± 1.2 | 32.0 ± 6.1 | 2.5 ± 0.3 |
| R237K | 7.9 ± 1.2 | 34.8 ± 7.1 | 4.4 ± 0.2 |
| L219/F223Y/F234Y/K236R/R237K | Not determined | | |
| L219/F223Y/V231L/F234Y/K236R | Not determined | | |

Table III

Effect of mutations on kinetic properties of P450 27A1

| Source | \( K_m \) \( \mu M \) | \( k_{cat} \) \( min^{-1} \) | \( k_{cat}/K_m \) \( min^{-1} \mu M^{-1} \) |
|--------|----------------|-----------------|---------------------|
| M* | 14.0 | 24.9 | 14 |
| M (P.P.* | 9.5 | 111 | 14 |
| 27A1 (M*) | 7.0 | 15.8 | 2.3 |
| 27A1 (P.P.) | 0.19 | ND | ND |

Table IV

Comparison of kinetic and cholesterol binding properties of P450s 7A1 and 27A1

| P450 | \( K_d \) \( \mu M \) | \( K_m \) \( \mu M \) | \( k_{cat} \) \( min^{-1} \) | \( k_{cat}/K_m \) \( min^{-1} \mu M^{-1} \) |
|-------|----------------|----------------|-----------------|---------------------|
| 7A1 (M*) | ND | 14.0 | 349 | 24.9 |
| 7A1 (P.P.*) | 9.5 | 6.9 | 111 | 16.1 |
| 27A1 (M) | ND | 7.0 | 15.8 | 2.3 |
| 27A1 (P.P.) | 0.19 | ND | ND | ND |

* M, membrane fraction; P.P., partially purified enzyme.

To summarize, the novelty of the present work is that it provides insight into membrane topology of mitochondrial P450s by mapping for the first time the secondary structural elements involved in binding to the membrane, presents the first experimental verification of the concept that microsomal and mitochondrial P450s have a similar mode of association with the membrane (8, 9), establishes that intact membrane-protein interactions are important for efficient catalysis in P450s, and ascertains kinetic differences between P450s 7A1 and 27A1, two major enzymes in degradation of cholesterol.

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