Engineering Microsomal Cytochrome P450 2C5 to Be a Soluble, Monomeric Enzyme

MUTATIONS THAT ALTER AGGREGATION, PHOSPHOLIPID DEPENDENCE OF CATALYSIS, AND MEMBRANE BINDING*

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Deletion of the N-terminal membrane-spanning domain from microsomal P450s 2C5 and 2C3 generates the enzymes, 2C5dH and 2C3dH, that exhibit a salt-dependent association with membranes indicating that they retain a monofacial membrane interaction domain. The two proteins are tetramers and dimers, respectively, in high salt buffers, and only 2C5dH requires phospholipids to reconstitute fully the catalytic activity of the enzyme. Amino acid residues derived from P450 2C5dH between residues 201 and 210 were substituted for the corresponding residues in P450 2C5 to identify those that would diminish the membrane interaction, the phospholipid dependence of catalysis, and aggregation of 2C5dH. Each of four substitutions, N202H, I207L, S209G, and S210T, diminished the aggregation of P450 2C5dH and produced a monomeric enzyme. The N202H and I207L mutations also diminished the stimulation of catalytic activity by phospholipid and reduced the binding of P450 2C5dH to phospholipid vesicles. The modified enzymes exhibit rates of progesterone 21-hydroxylation that are similar to that of P450 2C5dH. These conditionally membrane-bound P450s with improved solubility in high salt buffers are suitable for crystallization and structural determination by x-ray diffraction studies.

This study employed homology modeling and site-directed mutagenesis to identify a region in microsomal P450s 2C3 and 2C5 that is involved in substrate binding and that also affects membrane binding and protein oligomerization. P450s 2C3 and 2C5 hydroxylate progesterone with distinctly different regiospecificities. P450 2C5 catalyzes the hydroxylation of the C-21 methyl group, whereas P450 2C3 catalyzes 16α-hydroxylation of the steroid nucleus. A closely related variant of 2C3 that differs at 5 of 489 amino acids, 2C3v, catalyzes both the 6β-hydroxylation and 16α-hydroxylation of progesterone with a higher catalytic efficiency than 2C3. Both 2C3 and 2C5 have been expressed in Escherichia coli as modified proteins in which residues 3–21 that constitute a transmembrane anchor were deleted. When expressed in E. coli, the resulting proteins, 2C3d and 2C5d, exhibit a salt-dependent association with the inner membrane suggesting that removal of the membrane anchor domain converted these intrinsic membrane proteins to peripheral membrane proteins (1). The truncated P450s were also modified to include a histidine tag at the C terminus, 2C3dH and 2C5dH, allowing extensive purification of the enzymes from high salt lysates of E. coli without the use of detergents (1). The modified enzymes exhibit their native catalytic activity and regiospecificity for progesterone hydroxylation. However, P450 2C3dH no longer depends on DLPC to achieve optimal progesterone hydroxylase activity when reconstituted with purified human P450 reductase. The deletion of the membrane anchor sequence also reduced the aggregation of the purified proteins and resulted in predominantly dimers and tetramers for P450s 2C3dH and 2C5dH, respectively, compared with the octamers or larger aggregates that are seen for the full-length proteins.

The presence of a hydrophobic, monotonic membrane-binding site that leads to the salt-dependent association of the truncated proteins with membranes could contribute to the aggregation exhibited by the purified enzymes and to the DLPC requirement seen for reconstitution of the catalytic activity of P450 2C5dH. We reasoned that characterization of chimeric proteins derived from P450s 2C3dH and 2C5dH could identify regions that contribute to the observed differences between these two proteins in aggregation and the DLPC stimulation of catalytic activity.

Peterson and colleagues (2) have suggested that the region between helices F and G, the F-G loop, could contribute to membrane binding, which would orient the substrate access channel toward the membrane surface. This is concordant with available information on the topology of microsomal P450s that has recently been reviewed (3). In addition, an anti-peptide antibody produced to this region, residues 211–223 of P450 2C1, does not bind to the enzyme when it resides in the microsomal membrane but does bind to the solubilized enzyme. Thus, membrane interactions or the close proximity of this region to the membrane surface may mask residues 211–223 from the antibody (4).

Although reasonable models of 2C3 and 2C5 could be made using the structure of the soluble, bacterial P450 BM3 as a template, the fit and quality of the model in the region of helices F and G are significantly poorer than for other regions of the protein. The region of P450s 2C3dH and 2C5dH that aligns with the sequence of helix F to helix G of P450 BM3 includes a significant insertion of additional, predominantly hydrophobic amino acids (Fig. 1). This suggests that the structure of the P450 BM3 template may not be appropriate for modeling this region. This possibility is bolstered by secondary
structure predictions that the F and G helices in 2C5dH are shorter and that the F-G loop is longer than those found in P450 BM3 or in the structures of other soluble P450s. P450s 2C5dH and 2C3dH exhibit extensive differences in their amino acid sequences between the end of helix F predicted by the secondary structure prediction algorithm SPOMA (5), and the end of helix F predicted from alignment of the sequences with that of P450 BM3 (Fig. 1). In addition, a consensus for the alignment of this region of mammalian family 2 P450s with that of P450 BM3 is not evident when published homology models (6–9) are compared. The F helix also forms a side of the substrate binding cavity in experimentally determined structures of soluble microbial P450s. Mutations in the corresponding region of mammalian P450s 2A (10) and 2B (11) can alter both the regiospecificity of steroid hydroxylation as well as the substrate specificities of these enzymes suggesting that this region may be similarly placed. Molecular modeling using the approaches described under “Experimental Procedures” suggested that differences in this region could contribute to the distinct binding orientations of progesterone in homology models of 2C3 and 2C5. As will be discussed later, these modeled substrate-binding orientations correspond to the distinct regiospecificities for progesterone hydroxylation exhibited by the enzymes.

In order to determine whether amino acid differences between the two proteins in the region of helix F and the F-G loop contribute to the differences in catalytic properties and aggregation exhibited by the two truncated proteins, chimeras were constructed from P450s 2C5dH and 2C3dH. The results of this study were expected to provide insight regarding the topology of residues in this region as some of the properties examined reflect surface characteristics of the enzyme, whereas others reflect interactions with the substrate in the interior of the proteins. We anticipated that the selection of alignments for modeling would be aided by this information and that identification of the mutations that decreased the aggregation and phospholipid interactions of the enzymes would render the proteins more amenable to crystallization and structure determinations.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutants**—Two natural restriction endonuclease sites, HindIII and NesI, in the cDNA for P450 2C5, were used for the construction of reciprocal chimeras in pCW2C3dH (1). Two silent restriction sites, PvuII and KpnI, were introduced by site-directed mutagenesis into the DNA for P450 2C3 to construct the corresponding reciprocal chimeras in pCW2C3dH. Double-stranded oligonucleotides containing the appropriately altered codons and compatible single-strand overlaps were used to replace the native sequences in the two expression plasmids.

**Expression and Purification of P450s and P450 Reductase from E. coli**—The expression and purification of the P450s from high salt lysates without the use of detergents followed the procedures previously described by von Wachenfeldt et al. (1). The average specific contents of the preparations of P450s 2C3dH, 2C5dH, 2C3/5LVdH, and 2C5/3LVdH used in these experiments were 18.6, 16.5, 15.4, and 15.0 nmol/mg, respectively. The preparations of single substitutions in each of P450 2C5dH exhibited specific contents of P450 that ranged from 10 to 15 nmol/mg protein. The lower specific activities of the latter reflected the omission of the CM-Sepharose chromatography step for these preparations as well as differences in their levels of expression in E. coli.

**Enzyme Assay**—Concentrations of P450s were estimated spectrophotometrically from difference spectra determined for the formation of the carbon monoxide complex with the protein after reduction with sodium dithionite (14). The activity of the purified reductase preparation was determined spectrophotometrically by monitoring the NADPH-dependent reduction of cytochrome c at 550 nm. One unit of activity is defined as the reduction of 1 μmol of cytochrome c per min. Protein concentrations were determined using the BCA protein kit (Pierce) with bovine serum albumin as the standard. Phospholipid concentrations were determined colorimetrically as described (15). NADH oxidase activity, which was used as a marker for E. coli inner membranes, was determined for 20–100-μl aliquots of subcellular fractions prepared from E. coli lysates in a final volume of 1 ml of 50 mM KPi, pH 7.4, containing 0.1 mM EDTA. The reaction was initiated by the addition of 100 μM NADH, and the rate of NADH depletion was monitored spectrophotometrically at 340 nm using an extinction coefficient of 6,250 cm⁻¹⁻¹⁻¹.

**Reconstitution of P450 and P450 Reductase**—Each P450 (10 pmol) was reconstituted with 0.3 units of reductase in 50 mM HEPES, pH 7.6, 1.5 mM MgCl₂, 0.1 mM EDTA in the presence or absence of 30 μg of DLPC in a total reaction volume of 1 ml at 37 °C for 3 min. Reactions were initiated by the addition of 1 mM NaN₃P. The method used for the separation of [¹⁴C]progesterone and its metabolites by thin layer chromatography has been previously reported (16). The amount of product formation was determined using a PhosphorImager SI following exposure of the screens to the thin layer chromatography plates.

**Determination of Enzyme Oligomerization**—The apparent molecular weight of each enzyme was estimated by size exclusion chromatography employing a Superdex 200HR 10/30 column (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml/min at 25 °C. The mobile phase was 10 mM NH₄PO₄, pH 7.4, containing 150 mM NaCl and 0.1 mM EDTA. The apparent size of each P450 construct was estimated from a standard curve determined for the following protein standards: bovine thyroglobulin, 669 kDa; sweet potato β-amylose, 200 kDa; yeast alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; carbonic anhydrase, 27 kDa; and RNase A, 14 kDa. The elution of the P450s was monitored at 280 nm and that of the protein standards at 280 nm.

**Subcellular Distribution of the P450 Mutants in E. coli**—Cultures of E. coli were grown and harvested as described previously for the isolation of heterologously expressed P450s (1). Spheroplasts were prepared by suspending cells in 10% of the original culture volume in a 20 mM KP₇ buffer, pH 7.4, containing 20% glycerol, 1 mM PMSF, and 10 mM β-mercaptoethanol. The cell suspension was incubated with lysozyme (0.2 mg/ml) for 30 min at 4 °C. An equal volume of cold water was added, and the incubation was continued for an additional 10 min. Spheroplasts were pelleted by centrifugation at 5000 × g for 10 min. The spheroplasts were suspended in 10 mM KP₇, pH 7.4, containing 20% glycerol and 1 mM PMSF. Incompletely disrupted spheroplasts were removed by centrifugation at 5000 × g for 10 min, and membrane fractions were separated from the resulting supernatant by centrifugation at 123,000 × g for 90 min. An equal volume of each fraction was determined spectrophotometrically as described earlier. In additional experiments, 1.5-mI aliquots of E. coli lysate prepared in either 10 or 500 mM KP₇, pH 7.4, containing 10% glycerol, 1 mM PMSF, and 10 mM β-mercaptoethanol, were fractionated using a discontinuous sucrose gradient. The gradient was formed from 1 ml of 70% sucrose, 4 ml of 30% sucrose, and 3.5 ml of 15% sucrose prepared in
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RESULTS

Catalytic Activity and DLPC Dependence of the P450 2C3dH and 2C5dH Chimeras Reconstituted with P450 Reductase—Initially, two chimeras were constructed between P450s 2C3dH and 2C5dH. The segment exchanged between the two proteins, residues 201 to 210, is depicted in Fig. 1. Expression, purification, and reconstitution of the two chimeric proteins indicated that P450 2C3/5dH exhibited the regiospecificity and catalytic activity of P450 2C3dH. In contrast, P450 2C5/2C3dH did not exhibit appreciable progesterone hydroxylase activity (Fig. 3).

Examination of homology models in which progesterone had been docked suggested that the loss of activity displayed by the 2C5/3dH chimera might reflect changes to the active site that interfered with the productive binding of progesterone. As shown in Fig. 4, when progesterone is docked in the model of P450 2C5dH (Fig. 4), with this in mind, two of the clusters that exhibited significant negative energy interactions were examined to determine whether the binding orientation would place a potential site for hydroxylation within 6 Å of the heme iron (21).


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substituted residues, Phe-201 and Phe-205, present in the P450 2C5dH chimera, were converted back to the native Leu-201 and Val-205 residues found in P450 2C5dH to produce the chimera P450 2C5/3LVdH. As shown in Fig. 3, this chimera displayed a rate of 21-hydroxylation activity in the absence of DLPC that was roughly one-third of that exhibited by the wild-type P450 2C5dH. Examination of the model indicated that a third substitution, N202H, might also interfere with a progesterone binding orientation suitable for 21 hydroxylation (Fig. 4) and contribute to the lower turnover number exhibited by 2C5/3LVdH. When residues Phe-201, His-202, and Phe-205 derived from P450 2C5dH were restored to the native residues Leu, Asn, and Val found in P450 2C5dH, the resulting chimera, P450 2C5/3LVNdH, exhibited the activity seen for wild-type P450 2C5dH when either is reconstituted with P450 reductase in the absence of DLPC. Examination of the dependence of the catalytic rate on progesterone concentration in the absence of DLPC indicates that P450s 2C5dH, 2C5/3LVdH, and 2C5/3LVNdH exhibit $K_{\text{m}}$ values of 4.0, 2.8, and 4.6 $\mu$M and $k_{\text{cat}}$ values of 16.5, 5.2, and 20.2 nmol/min/nmol P450, respectively. These differences in the $k_{\text{cat}}$ of the three enzymes could reflect the effects of the substitutions on reconstitution with the reductase. In contrast to P450 2C5dH, the catalytic activities of reconstituted P450 2C5/3LVdH and 2C5/3LVNdH were not significantly stimulated by the addition of DLPC. This suggests that residues derived from 2C3, other than 201, 202, and 205, confer independence from the DLPC stimulation of the reconstituted activity of these 2C5 chimeras. Taken together, these results indicate that residues within the segment exchanged between the two parental enzymes contribute to differences in the catalytic activity and in the DLPC dependence of the two enzymes.

Rates of Progesterone 21-Hydroxylation Catalyzed by Single Mutants of P450 2C5dH—In order to examine the role of the individual amino acids in the segment that was exchanged between the chimeras on the catalytic activity of P450 2C5dH, individual mutants were constructed, expressed in *E. coli*, and purified. Following reconstitution with P450 reductase in the presence or absence of DLPC, turnover numbers for the 21-hydroxylation of progesterone were determined for each mutant (Fig. 5). In general, the single mutants exhibited rates that were similar to or greater than that of P450 2C5dH and also displayed a similar dependence on DLPC with three notable exceptions. The V205F mutant did not exhibit significant catalytic activity. This suggests that the V205F mutation could account for most of the loss of activity seen for the P450 2C5/3dH chimera and underline the restoration of activity that occurred when the F201L and F205V reverse mutations were made to generate the P450 2C5/3LVdH chimera. This is consistent with observations that residues at this alignment position are key determinants of substrate specificity and the regiospecificity of catalysis by P450 2A, residue 209 (10), and by P450 2B, residue 206 (11). In addition, two single mutants, N202H and I207L, did not exhibit the stimulation of catalysis by DLPC that is seen for P450 2C5dH or the other mutants. The N202H and I207L mutations are likely to underlie the restored activity that occurred when the F201L and F205V reverse mutations were made to generate the P450 2C5/3LVdH chimera. This is consistent with observations that residues at this alignment position are key determinants of substrate specificity and the regiospecificity of catalysis by P450 2A, residue 209 (10), and by P450 2B, residue 206 (11). In addition, two single mutants, N202H and I207L, did not exhibit the stimulation of catalysis by DLPC that is seen for P450 2C5dH or the other mutants.

Aggregation of the 2C5dH/2C3dH Chimeras and Single Mu-
The retention times (min) for 2C5/3LVdH, 2C3dH, and 2C5dH are 30.1, 24.6, and 28.3 min, respectively. The scale indicates the elapsed time in minutes from injection of the sample.

The absorption of visible light at 417 nm by the eluant.

The mutants of 2C5dH—Size exclusion chromatography revealed that the aggregation of the purified P450 2C5/3LVdH chimera was reduced in a high salt buffer as evidenced by a longer retention time, 30.1 min, than that of either of the parental enzymes. As shown in Fig. 6, P450s 2C5dH and 2C3dH exhibit retention times of 24.6 and 28.3 min, respectively. When compared with a standard curve, these values indicate that P450s 2C5dH and 2C3dH are predominantly tetramers and dimers, respectively. In contrast, preparations of P450 2C5/3LVdH appear to contain predominantly monomers under these conditions. Preparations of P450s 2C5/3dH and 2C5/3LVNdH also appeared to be predominantly monomers, whereas the distribution of aggregates in purified preparations of P450 2C3/5dH did not differ significantly from that of P450 2C3dH (Fig. 7).

In order to better characterize the extent of P450 binding to membranes, discontinuous gradients composed of 15, 30, and 45% sucrose in 50 mM potassium phosphate, pH 7.4, were employed. In general, this procedure reduced the yield of P450 in the membrane fraction from that observed in the previous experiments. Under these conditions, cytoplasmic proteins were retained in the 15% sucrose fraction, whereas the inner and outer membranes formed a band at the 30–70% interface following centrifugation at 150,000 g for 4 h. The bulk of E. coli proteins were retained at the top of the gradient, whereas most of the phospholipid was found in the fractions containing the membranes, as shown for P450 2C5/3LVdH in Fig. 9. Examination of the gradient material at 5,000 g for 90 min following the removal of particulate material at 150,000 g for 10 min (1). The retention of the individual mutants and of the P450 2C5/3LVdH and 2C5/3LVNdH chimeras in the membrane fraction was examined in order to determine whether mutations that altered the aggregation of the enzymes also affected the binding of the P450s to membranes in in salt buffers. Although a substantial fraction of each of these P450s sediments with the membrane fraction, the amount of the 2C5/3LVdH and 2C5/3LVNdH chimeras and several of the single mutants that was recovered in the membrane fraction was significantly reduced (p < 0.01) relative to that exhibited by P450 2C5dH (Fig. 8). The largest differences were observed for P450 2C5/3LVdH and three of the single mutants, I207L, S209G, and S210T where the membrane-associated P450 was reduced to as low as 22%. As described earlier, these proteins were predominantly monomers in high salt buffers when characterized by size exclusion chromatography.

The distribution of monomers, dimers, and tetramers observed in purified preparations of the various single mutants is summarized in Fig. 7. Several of the single substitutions produced predominantly monomeric enzymes. These included the N202H, I207L, S209G, and S210T mutations. In contrast, the L201F, V205F, and R206E mutations did not diminish the aggregation of the resulting proteins relative to P450 2C5dH. Thus, several single substitutions were sufficient to produce significant changes in oligomerization, and these included two, N202H and I207L, that reduced the DLPC dependence of reconstituted catalytic activity.

Subcellular Distribution of P450 2C5dH Mutants Expressed in E. coli—When lysates of E. coli expressing P450 2C3dH or 2C5dH are prepared in low ionic strength buffers, the two proteins are distributed roughly equally between the membrane and the soluble fractions obtained by sedimentation at 150,000 x g for 90 min following the removal of particulate material at 5,000 x g for 10 min (1). The retention of the individual mutants and of the P450 2C5/3LVdH and 2C5/3LVNdH chimeras in the membrane fraction was examined in order to determine whether mutations that altered the aggregation of the enzymes also affected the binding of the P450s to membranes. Although a substantial fraction of each of these P450s sediments with the membrane fraction, the amount of the 2C5/3LVdH and 2C5/3LVNdH chimeras and several of the single mutants that was recovered in the membrane fraction was significantly reduced (p < 0.01) relative to that exhibited by P450 2C5dH (Fig. 8). The largest differences were observed for P450 2C5/3LVdH and three of the single mutants, I207L, S209G, and S210T where the membrane-associated P450 was reduced to as low as 22%. As described earlier, these proteins were predominantly monomers in high salt buffers when characterized by size exclusion chromatography.

In order to better characterize the extent of P450 binding to membranes, discontinuous gradients composed of 15, 30, and 70% sucrose in 50 mM potassium phosphate buffer were employed. In general, this procedure reduced the yield of P450 in the membrane fraction from that observed in the previous experiments. Under these conditions, cytoplasmic proteins were retained in the 15% sucrose fraction, whereas the inner and outer membranes formed a band at the 30–70% interface following centrifugation at 150,000 x g for 4 h. The bulk of E. coli proteins were retained at the top of the gradient, whereas most of the phospholipid was found in the fractions containing the membranes, as shown for P450 2C5/3LVdH in Fig. 9. Examination of the gradient fractions indicated that in low ionic strength lysis buffer a substantial amount of P450s 2C3dH, 2C5dH, and 2C5/3LVdH sediments more slowly than inner membranes but faster than soluble proteins. Table I summarizes the distribution of 2C5/3LVdH, 2C5dH, and 2C5/3LVNdH in three fractions that were collected from the sucrose gradient in the following manner: a 2-mL lower fraction containing the inner and outer membranes, a 4-mL upper fraction containing the cytosolic proteins that included the 15% interface, and the remaining 4-mL intermediate fraction. The amounts of P450 recovered in the lower fraction containing membranes (Table I) is significantly reduced relative to the amount (Fig. 8) obtained by centrifugation in 20% glycerol for 90 min at the same force in the absence of
a separation layer between the cytosolic proteins and membranes. A comparison of these results (Table I) indicates that the amount of P450 2C5dH in this membrane fraction is roughly 50% of the amount found for P450s 2C5dH or 2C3dH. This suggests that the association of the chimeric protein with the inner membrane is diminished by the mutations as was seen in the experiment shown in Fig. 8.

Surprisingly, the amount of each P450 in *E. coli* lysates that was recovered in the intermediate fraction in 50 mM buffer exceeded the amounts found in either the soluble or membrane fractions. When analyzed on sucrose gradients in 500 mM KPi buffer, the majority of the P450 from the lysates is found in the upper fraction with almost none detected in the intermediate and lower fractions. Thus, elevated ionic strength prevents the sedimentation of the P450s with membranes. When purified protein is analyzed on the sucrose gradient in 50 mM KPi, the P450 is not found in the intermediate and lower fractions of the gradient even at the lower ionic strength. This suggests that the presence of P450 in the intermediate and lower fractions prepared from *E. coli* lysates at low ionic strength is dependent on some other component in the lysates such as membranes. When the P450 in the intermediate fraction of the lysate in 50 mM KPi was recovered, concentrated, and applied to another sucrose gradient in 50 mM KPi, the P450 was recovered in the upper, soluble fraction. Thus, the initial presence of the P450 in the intermediate and lower fractions obtained using an *E. coli* lysate was unlikely to reflect enzyme precipitation in low salt conditions but could result from the dissociation of the P450 from membranes while traversing the sucrose gradient.

Reversible Binding of P450s to DLPC Liposomes—The salt-dependent binding of the purified proteins to liposomes prepared from DLPC was also examined. Following incubation of P450 2C5dH with DLPC liposomes in 50 mM buffer containing 20% glycerol and centrifugation, both the liposomes and the P450 were recovered in a pellet at the bottom of the tube. In contrast, when either the P450 or the liposomes were centrifuged using the same conditions, each remained in the soluble fraction. This indicated that P450 2C5dH interacts with the DLPC vesicles and that this binding increases the density of the vesicles and causes the liposomes incorporating P450 to form a pellet during centrifugation in 20% glycerol. Similar experiments were performed with lower density liposomes prepared from egg yolk phosphatidylcholine. The binding of P450 2C5dH to these vesicles was also observed. However, P450-bound vesicles did not form a pellet but rather formed a band at a position that was dependent on the density (glycerol concentration) of the medium and the duration of the centrifugation (data not shown). Thus, the sedimentation rate of the P450 is dependent on liposome density and is indicative of an association with phospholipid vesicles. When the pellet containing both DLPC liposomes and P450 2C5dH was suspended in 500 mM buffer containing 20% glycerol and subjected to centrifugation, the P450 2C5dH and liposomes remained soluble indicating the reversibility of the interaction between the P450 and the DLPC liposomes in high salt conditions.

Titration of a fixed quantity of purified P450 with increasing amounts of phospholipid indicated that almost all of the P450 2C5dH, about 85%, could be recovered in the pellet (Fig. 10A). The molar ratio of phospholipid to P450 that yielded maximum incorporation in the pellet was 1500:1. This ratio is similar to
that used to stimulate reconstitution of catalytic activity with the reductase. Similar experiments using P450s 2C3dH and 2C5/3LVdH indicated that the maximum amount of P450 incorporated into the pelleted DLPC liposomes was much lower, 10 and 25% respectively (Fig. 10A). Examination of the binding of each of the mutants to DLPC liposomes under conditions that yielded maximal binding for P450 2C5dH indicated that the L201F, V205F, R206E, S209G, and S210T exhibited extensive binding to the liposomes (Fig. 10B). On the other hand, several of the mutants and chimeras exhibited lower extents of liposome binding, <30%. These were the N202H and I207L mutations and the 2C5/3LVdH and 2C5/3LVNdH chimeras. However, the extent of incorporation for these P450s into the liposomes was greater than that seen for P450 2C3dH. Interestingly, like P450 2C3dH, the mutants and chimeras that exhibit lower extents of binding to DLPC liposomes also do not exhibit stimulation of reconstituted catalytic activity by DLPC (Fig. 3 and Fig. 5). To better understand the basis for the difference in the extent of membrane binding, purified P450s 2C5dH and 2C5/3LVdH were incubated with DLPC liposomes as described above (Fig. 11). The mixture was then diluted to 5% glycerol and applied to a discontinuous gradient composed of 10, 15, and 20% glycerol in 50 mM KPi. Following centrifugation for 150 min at 150,000 × g, a pellet was obtained for liposomes containing either P450 but not for DLPC liposomes alone. Under these conditions almost all (88%) of the liposomes were found in the pellet after incubation with either P450. In each case, a small amount of P450 was seen at the top of the gradient. However, the distribution of the two P450s differed across the gradient (Fig. 11). Almost all of the P450 2C5dH was found in the pellet. In contrast, P450 2C5/3LVdH was distributed relatively evenly across the gradient suggesting that this P450 dissociated from the liposomes while traversing the glycerol gradient. When these fractions were recovered, concentrated, and subjected to centrifugation again, the P450 remained at the top of the gradient. These results indicate that P450 2C5/3LVdH associates with the liposome with a lower affinity than is seen for P450 2C5dH. Taken together, these results indicate that the salt-dependent reversible binding of P450 2C5dH to phospholipid membranes can be altered by modifications of several residues to the corresponding residues found in P450 2C3dH. These mutations reduce the binding of the protein with DLPC liposomes to levels that approach those seen for P450 2C5dH.

**DISCUSSION**

The results obtained in this study indicate that residues thought to reside either in helix F or in the region between helix F and helix G, the F-G loop, can determine the aggregation state in solution and membrane interactions of P450 2C5dH. It is likely that the residues in this region that affect the aggregation of the enzyme, that modify the effects of phospholipids on catalytic activity, and that alter the binding of P450 2C5dH to DLPC liposomes are located on the surface of
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...the enzyme. In contrast, mutations that do not affect these properties but that do affect catalytic activity are likely to identify residues that are located in the interior of the active site. This topological information should aid in modeling this region of the microsomal enzymes. In addition, these results indicate that residues in helix F and the F-G loop affect membrane interactions and are likely to contribute to microsomal membrane association. Moreover, appropriate changes in this region can dramatically enhance enzyme solubility while retaining catalytic activity. These alterations have also enabled subsequent structural studies (22).

Purified preparations of the 2C5/3LVdH and 2C5/3LVNdH chimeras as well as 4 single substitution mutants, N202H, I207L, S209G, and S210T, were monomeric with little evidence for significant aggregation in high salt buffers. Thus, these constructs differed from P450 2C5dH, a tetramer, and P450 2C3dH, a dimer. Each of the substituted residues is likely to contribute to the lower aggregation seen for P450 2C3dH. With the exception of the N202H substitution, these changes are relatively conservative. However, small differences in the volume of residues may permit greater hydration at the interface between interacting proteins that alters the strength of the interactions that lead to aggregation (23). The ability of several single mutations to prevent aggregation strongly suggests that each of these residues are likely to have a surface exposure that should be considered when modeling corresponding residues in this region of other microsomal P450s.

The catalytic activities of P450 2C5/3LVdH, P450 2C5/3LVNdH, as well as of the N202H and I207L single mutants were no longer stimulated by DLPC. It has been proposed that DLPC facilitates the redistribution of aggregates of full-length P450s and of full-length reductase into mixed aggregates that permit catalysis. Optimal activity is obtained with a roughly equal stoichiometry of the two proteins in these multimeric complexes, and DLPC liposomes do not appear to incorporate the full-length enzymes under the conditions of the assay system (24). In contrast, the incorporation of full-length microsomal P450s into liposomes generally requires high initial concentrations of detergent with gradual removal of the detergent to achieve membrane incorporation (25–27). It is interesting to note that the binding to liposomes of mitochondrial P450s, which lack an N-terminal transmembrane domain, does not require detergents to facilitate binding to liposomes (28). This is similar to the truncated P450s used in this study. Thus, mutations that render the enzyme more soluble might be expected to diminish the requirement for DLPC. Surprisingly, the greater monodispersity exhibited by the S209G and S210T single mutants did not eliminate the effects of DLPC on reconstitution with P450 reductase. In addition, P450 2C5dH and the S209G and S210T single mutants associate extensively with DLPC liposomes. As the residual membrane-binding domain found in the truncated P450s is monofacial, association of these proteins with the membrane is also likely to be more facile than for the full-length proteins that are more highly aggregated and that require detergents to achieve significant incorporation.

The binding to liposomes of the 2C5/3LVdH and 2C5/3LVNdH chimeras and of the N202H and I207L single mutants is less extensive than that of P450 2C5dH. The changes at residues 202 and 207 appear to alter the interaction of 2C5dH with phospholipids and probably contribute to the inability of DLPC to stimulate the reconstituted catalytic activity of the LV and LVN chimera. This difference in the extent of binding to DLPC liposomes could reflect a lower partition ratio due to changes in the membrane-binding interface resulting from the mutations at positions 202 and 207.

Based on the helical topology of the model for the 201–210 region, some of the mutated residues studied were likely to be internal and contribute to the formation of the active site. Also, the properties affected by interior residues were likely to be distinct from those of surface residues that affected aggregation and membrane association. Only one of the single substitutions, V205F, had a large effect on the progesterone 21-hydroxylase activity of P450 2C5dH. This single substitution reduced the 21-hydroxylase activity of the resulting enzyme to insignificant rates. This observation is consistent with earlier studies indicating that changes in the residues that align with amino acid 205 of P450 2C5 alter the regiospecificity of steroid hydroxylation and the substrate specificities of P450s 2A (10) and 2B (11). The effects of substitutions at this site have been extensively studied, and the results are highly suggestive that residues at this alignment position are likely to be substrate contact residues in family 2 P450s. Based on the initial model presented here, two additional substitutions, L201F and R206E, appeared likely to reside near the substrate-binding site. However, these mutations did not significantly affect the...
turnover number for progesterone 21-hydroxylation when introduced as single substitutions. The interior location of residues 201, 205, and 206 in the model is consistent with the absence of effects on aggregation, DLPC stimulation of catalysis, and membrane binding seen for these single mutants. In the initial model, the substitutions at positions 201 and 206 are more distant from the docked substrate than the V205F substitution, and differences in these residues might be more easily accommodated.

Based on the model, the N202H substitution also appeared likely to affect substrate binding and the catalytic activity of the enzyme. The reverse substitution, H202N, increased the catalytic activity of 2C5/3LVdH 3-fold, and the resulting protein, 2C5/3LVNdH, displayed a $k_{\text{cat}}$ similar to that of 2C5dH. However, the activity of the single mutant N202H is only marginally lower than that of the other single mutants. Also, this single mutation produced a monomeric enzyme and reduced binding to DLPC liposomes suggesting that residue 202 may have a greater surface exposure than is apparent in our model. The difference in catalytic activity of the P450 2C5/3LVdH and 2C5/3LVNdH chimeras largely reflects a difference in $k_{\text{cat}}$, with little apparent difference seen for the $K_m$ for progesterone. The difference in $k_{\text{cat}}$ could reflect, in turn, the effects of the N202H substitution on the reconstitution of the P450s with the reductase and differences in DLPC interaction that reflect a surface location for the residue. Taken together these results suggest that the topology of the F helix differs from our model and may be more similar to that modeled for P450 2B4 by Chang et al. (7).

The results of this study suggest that the region between helices F and G contributes to the membrane association exhibited by P450s 2C5dH and 2C3dH. The effects of salt are more likely to affect the ionic interactions of the protein with the surface of the membrane where the phospholipid head groups are located. In contrast, the enzyme aggregation of 2C3dH and 2C5dH that is seen in high salt buffers is likely to reflect hydrophobic interactions that are promoted by the high salt concentrations. The region modified in this study resides in close proximity to the hydrophobic region of the F-G loop, and the ability of the mutations to reduce aggregation could result from disrupting intermolecular interaction of the loop between monomers that promoted self-aggregation in high salt. Additional intramolecular ionic interactions may override these effects in low salt buffers and stabilize the protein in a conformation that exhibits greater hydrophobicity and that interacts more strongly with membranes.

The changes made to P450 2C5dH dramatically alter the behavior of the protein in solution while retaining the catalytic activity of the enzyme. This has facilitated, in turn, the crystallization of the modified P450 2C5dH. The structure of the enzyme determined by x-ray diffraction studies confirms the topology inferred for the residues examined here (22). The resulting structural information should help to clarify the differences between mammalian microsomal P450s and P450 BM3 that hinder accurate model construction. The structure of P450 2C5 should provide a better template for modeling the structures of other microsomal cytochrome P450s.

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