Identification of Membrane-Contacting Loops of the Catalytic Domain of Cytochrome P450 2C2 by Tryptophan Fluorescence Scanning†

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ABSTRACT: The catalytic domain of cytochrome P450 is thought to contact the lipid core of the endoplasmic reticulum membrane based on antibody epitope accessibility, protease susceptibility, and hydrophobic surfaces present on P450 structures of solubilized forms of the proteins. Quenching by nitroxide spin label-modified phospholipids of the fluorescence of tryptophan residues substituted into cytochrome P450 2C2, modified to contain tryptophan only at position 120, was used to identify regions of P450 inserted into the lipid core and to estimate the depth of penetration. Consistent with the proposed models of cytochrome P450-membrane interaction, the fluorescence of tryptophans inserted at residues 36 and 69 in the two segments of P450 2C2 flanking the A-helix and at residue 380 in the β2-2 strand was quenched by nitroxide spin labels on carbon 5 of the fatty acid tails of the phospholipids within the lipid bilayer. The fluorescence of tryptophan at 380 was also strongly quenched by a spin label on carbon 12 of the fatty acids suggesting it was deepest in the membrane. However, fluorescence of tryptophan substituted at residue 225 in the F−G loop, which was predicted to be in the lipid bilayer, was not quenched by the spin labels at carbons 5 and 12 of the fatty acids. The pattern of quenching of fluorescence for tryptophans at the other positions tested, 80, 189, 239, and 347, was similar to the parent protein indicating they were not inserted into the lipid bilayer as expected. The results are consistent with an orientation of cytochrome P450 2C2 in the membrane in which positions 36, 69, and 380 are inserted into the lipid bilayer and residues 80 and 225 are near or within the phospholipid headgroup region. In this orientation, the F−G loop, which contains residue 225, could form a dimerization interface as was observed in the P450 2C8 crystal structure (Schoch, G. A., et al. (2004) *J. Biol. Chem. 279*, 9497).

Cytochromes P450 (P450s)[1] are integral membrane proteins in the endoplasmic reticulum. The 29-amino acid N-terminal signal anchor sequence targets P450 2C2 to the endoplasmic reticulum and also serves as a stop-transfer sequence so that the remainder of the P450 is on the cytoplasmic side of the membrane (*1*−*4*). The hydrophobic region of the signal anchor sequence is the only transmembrane helix in the protein with its N-terminus on the luminal side of the membrane. Multiple lines of evidence have suggested that the cytoplasmic domain also penetrates partially into the lipid core of the membrane. P450s with the signal anchor sequence deleted are associated with the membrane at physiological ionic strength and in some cases resistant to extraction by high ionic strength (*5*−*8*). Studies of the rotation of P450s in membranes by decay of absorption anisotropy, *r*(t), after photolysis of the heme−CO complex were consistent with penetration of the catalytic domain in the membrane (*6*, *9*). The residue equivalent to 37 in P450 2C2 was protected from trypsin in P450 1A2 (*10*), and trypsin digestion of P450 resulted in recovery of expected peptides after residue 75 to the C-terminus, suggesting that the first 75 N-terminal residues were in the lipid core of the membrane (*11*). Studies with a panel of antibodies to P450 2B1 indicated that regions of the molecule corresponding to residues 23−37, 39−48, and 210−222 of P450 2C2 were not accessible to antibodies when P450 2B1 was associated with microsomal membranes (*12*). These observations suggested that the N-terminal region from residues 23−48 and an internal position of P450s interacted with the lipid bilayer. A model based on the distribution of hydrophobicity in the structure determined for P450 2C5 and on the antibody accessibility studies was proposed (*13*) in which a tip of P450 is inserted into the membrane which consists of amino acids in segments before (30−45) and after (60−69) the A-helix, in β strand 2-2 (379−382) and the F−G loop (213−223) (Figure 1A).

The interaction of the catalytic domain with the membrane could have important functional significance. During their catalytic cycle, P450s accept electrons from P450 reductase

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[1] Abbreviations: P450, cytochrome P450; PA, phosphatidic acid; POPC, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; 12-Doxyl, 1-palmitoyl-2-stearoyl-(12-doxyl)-*sn*-glycero-3-phosphocholine; 5-Doxyl, 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine; TEMPO, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(TEMPO)choline; pFB1, pFastBack1; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Sf9, *Spodoptera frugiperda*; TN5, *Trichoplusia ni.*

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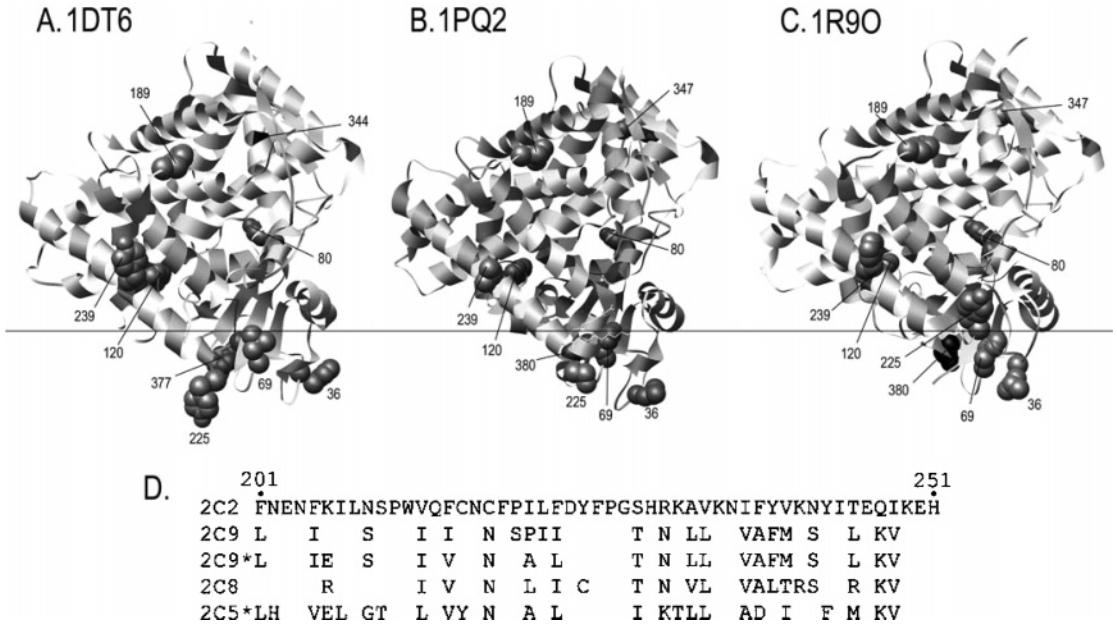

FIGURE 1: Models of subfamily 2C cytochromes P450 associated with the membrane. The orientation of the P450s in the membrane are based on that proposed for P450 2C5/3LVdH by Williams et al. (*13*). The P450 2C5/3LVdH (1DT6) structure is shown in panel A, and those of P450 2C8 (1PQ2) (*28*) and P450 2C9 (1R9O) (*29*) are shown in panels B and C, respectively. The solid lines indicate the boundaries of the lipid bilayer core of the membrane approximately to scale with the protein structures. The proteins are displayed in a ribbon representation with space filling representation of the atoms of residues for which Trp was substituted. The N-terminal signal anchor was deleted from the proteins crystallized for the structures and is not shown. Because of a three-amino acid deletion in the sequence of P450 2C5 compared to that of P450 2C2, residues 344 and 377 of P450 2C5 are equivalent to residues 347 and 380, respectively, of P450 2C2. The structures were rendered with the program Chimera from the University of California, San Francisco. In panel D, the sequences of P450 2C2, P450 2C5/3LVdH (2C5*), P450 2C8, P450 2C9, and the modified P450 2C9 used for structure 1OG5 (2C9*) from residues 201−251 which includes the F−G loop are compared with only the differences in amino acids shown for the last four sequences relative to the sequence of P450 2C2.

and in some cases cytochrome b_5. These two redox partners are also integral membrane proteins, and the orientation relative to the membrane for these proteins and P450s must be compatible with their interactions. In addition, substrate access channels in models of P450s are inserted into the membrane so that the substrate can enter directly from the membrane (*13*, *14*). Since most substrates for P450s are hydrophobic molecules and will be concentrated in the lipid bilayer of the membrane, this would facilitate binding of the substrate to P450. Differences in susceptibility to trypsin digestion of P450s in microsomal membranes and in salt dependence of the binding to membranes of P450s with the N-terminal signal anchor deleted suggest that the catalytic domains of different P450s may penetrate the membrane to different depths (*15*). The depth of the membrane could be functionally significant if, for example, depth correlated with the hydrophobicity of substrates (*16*, *17*).

Understanding the nature of the interaction of P450s with the membrane is important for deciphering the function of these enzymes. In the present paper, we have used Trp fluorescence, combined with depth-selective quenching, to determine which regions of P450 2C2 interact with the membrane. Consistent with the proposed models of P450−membrane interaction, the fluorescence of Trp residues inserted into the β2-2 strand and two segments of P450 2C2 flanking the A-helix are quenched by nitroxide spin labels in the lipid bilayer. However, a Trp in the F−G loop is not quenched by a spin label in the lipid bilayer and possibly may be part of a dimerization surface external to the lipid bilayer.

MATERIALS AND METHODS

Reagents. Phosphatidic acid (PA), 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), 1-palmitoyl-2-stearoyl-(12-doxyl)-*sn*-glycero-3-phosphocholine (12-Doxyl), 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine (5-Doxyl), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho(TEMPO)choline (TEMPO) were purchased from Avanti Polar Lipids.

Site-Directed Mutagenesis. To prepare P450 2C2 with a single Trp at position 120, the vector pc2A (*18*), which contains the P450 2C2 cDNA, was used as template for single-stranded DNA site-directed mutagenesis (*19*) to produce mutants with single, double, and triple substitutions of Leu for Trp-20, Phe for Trp-212, and Phe for Trp-120. Active forms of the triple mutant could not be obtained, so the double mutant, W20L/W212F, was used as the parent for the Trp scanning. The W20L/W212F cDNA sequence was inserted into pFastBack1 (pFB1) or pCW vectors, and 6-His tags were inserted at the C-terminus in each case. Trp codons were then substituted at positions 36, 69, or 80 in pCW(W20L/W212F)-2C2 by site-directed mutagenesis using the Quick Change XL Kit (Stratagene, Inc., La Jolla, CA), and HindIII fragments containing the P450 cDNAs with His tags were substituted for the corresponding fragment in pFB1(W20L/W212F-2C2). Trp codons were substituted at positions 189, 225, 239, 347, or 380 in pFB1-(W20L/W212F)2C2 by site-directed mutagenesis using the Quick Change XL Kit. The characteristics of the mutants are shown in Table 1.

Table 1 Characterization of P450 2C2 Trp Mutants

mutants[a]	specific content[b] (nmol/mg)	enzymatic activity[c] (% of WT)
wild-type	16.0	100
Trp-120	14.4	82.0 ± 3.0[d]
Trp-36/120	13.2	71.8 ± 3.5
Trp-69/120	12.8	73.1 ± 8.1
Trp-80/120	14.1	84.2 ± 3.9
Trp-189/120	12.5	61.9 ± 1.1
Trp-225/120	9.6	72.0 ± 2.1
Trp-239/120	16.6	79.1 ± 1.7
Trp-347/120	15.1	79.5 ± 7.4
Trp-380/120	11.9	67.9 ± 3.9

[a] Mutant proteins were expressed in insect cells and purified as described in Material and Methods. W20L/W212F (Trp-120) is the parent protein for the remainder of the mutants. The position of the Trp residues in each mutant is indicated. [b] The specific content of the purified samples was determined by measuring total protein concentration and the reduced CO-difference spectra to determine the concentration of P450. [c] The enzymatic activity of the P450 samples reconstituted with phospholipids was determined using the P450 Glo Assay (Promega Corp.) with luciferin 6′methyl ether as substrate and is expressed a percent of wild-type (WT) activity. [d] Standard error of the mean, $n = 3$.

Protein Expression and Purification. The expression of 2C2 mutant proteins in insect cells was carried out as described previously (*20*) according to the manufacturer (Invitrogen Corp.). Briefly, to produce recombinant baculovirus, DH10Bac *Escherichia coli* were transfected with the pFB1 vectors containing the P450 cDNA sequences, and recombinant bacmid DNA was isolated from the bacteria. *Spodoptera frugiperda* (Sf9) cells were transfected with the bacmid DNA with Cellfectin Reagent (Invitrogen Corp.), and the medium containing recombinant baculovirus was collected after incubation at 30 °C for 48 h. To obtain high-titer stocks of the recombinant virus, Sf9 cells were infected with the recombinant baculovirus twice and medium was collected after 4 days. For protein expression, *Trichoplusia ni* (TN5) cells were grown in 10 cm plates in Express Five medium, supplemented with 10% fetal bovine serum, 100 μM δ-aminolevulinic acid, and 100 μM ferric citrate, and were infected with 100 μL of high-titer baculovirus. Three to four days after infection, cells were harvested, washed twice with cold glycerol buffer (0.1 M potassium phosphate buffer, pH 7.4, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 20% glycerol), and homogenized with 15−20 strokes with the tight pestle in a Dounce homogenizer in glycerol buffer. The cell lysates were centrifuged at 1440*g* for 2 min, and the supernatant was centrifuged at 100 000*g* for 90 min in a Beckman 70.1 rotor to pellet the membrane fraction. The pellet was resuspended in microsomal buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, and 20% glycerol), and then NP-40 was added to a final concentration of 0.3%. After incubation at 4 °C for 2 h with mixing to solubilize the membrane proteins, the sample was centrifuged at 100 000*g* for 30 min, and the supernatant was applied to a Ni−NTA column, which had been equilibrated with microsomal buffer containing 0.3% NP-40. The bound proteins were eluted with microsomal buffer containing 500 mM imidazole and 5 mM CHAPS, and the eluted sample was dialyzed against glycerol buffer overnight. Protein concentrations were determined using the Micro BCA reagent (Pierce Chemical Co.) with bovine serum albumin as standard. The purified proteins were characterized by SDS−PAGE and Coomassie blue staining. P450 content was measured by reduced CO-difference spectra at 450 nm. The proteins were at least 80% pure based on the Coomassie-stained SDS−PAGE gels, and the specific contents of the P450s ranged from 10 to 16 nmol P450/mg protein (Table 1).

Enzymatic Assays. Activity of P450 2C2 was assayed using the P450-Glo Assay with the substrate luciferin 6′ methyl ether (Promega Corp.) Luciferin 6′ methyl ether is marketed as a P450 2C8 substrate (Promega Corp.) but is metabolized by P450 2C2 (unpublished results). Assays were performed in a reconstituted system in a final volume of 50 μL containing 5 pmol of P450 reductase, 10 pmol of P450 2C2, 5 μL of 5 mM POPC/PA liposomes, and 150 μM luciferin 6′ methyl ether in potassium phosphate buffer, pH 7.4. Dried phospholipids were resuspended by sonication, and the proteins and substrate were added. The reactions were started by adding 1.3 mM NADPH and incubated for 45 min at room temperature. The reaction was linear with time for 60 min (not shown). The reaction was stopped by addition of Luciferin Detection Reagent (Promega Corp.). After incubation for 20 min at room temperature, luminescence was measured in a Berthold Lumat 9507 luminometer.

Liposome Preparation and Binding Assay. Liposomes were prepared by the extrusion method to form large unilamellar vesicles according to the manufacturer's instructions (Avanti Polar Lipids, Inc.). POPC and PA dissolved in chloroform were mixed in a molar ratio of 4 to 1 in a glass vial. The nitroxide spin-labeled phospholipids, TEMPO, 5-Doxyl, or 12-Doxyl, were added to a concentration of 10 mol % as indicated. The chloroform suspensions of phospholipid mixtures were dried under nitrogen, desiccated overnight, and reconstituted by vortexing to resuspend the dried lipid films in 25 mM Tris, pH 7.4, and 100 mM NaCl. Large unilamellar vesicles were prepared by sonication for 15 min followed by extrusion through a 100 nm pore size polycarbonate filter in a mini-extruder as described by the manufacturer (Avanti Polar Lipids, Inc.). To incorporate P450 into the liposomes, liposomes and P450 were added in a ratio of 2500:1 to a final concentration of P450 of 0.2 μM in a volume of 250 μL. The sample was mixed and incubated for 5 min at room temperature before fluorescence was analyzed.

Tryptophan Fluorescence Measurements. Fluorescence measurements were performed in 250 μL of glycerol buffer, using a FluoroMax2 spectrofluorimeter at room temperature. Trp was excited at 295 nm (bandwidth 5 nm), and the emission spectra were measured between 300 and 400 nm (bandwidth 5 nm). The fluorescence data were corrected by subtracting the background for liposomes without protein added.

RESULTS

Characterization of P450−Liposome Complexes. P450s were incorporated into preformed liposomes containing POPC and PA in a ratio of 4:1 which was the optimum ratio of these phospholipids for insertion of P450 1A2 into the membrane (*21*). In preliminary studies, P450 2C2 incorporated into preformed liposomes by this method floated to the same density position on a Nycodenz gradient and was

as resistant to alkali extraction as P450 in proteoliposomes formed by mixing proteins and phospholipids in the presence of detergent and dialysis overnight against glycerol buffer (data not shown).

Selection of Positions for Trp Scanning. At the time these studies were initiated, the structure of a modified P450 2C5, P450 2C5/3LVdH (*13*) was the only structure of a mammalian P450 available. On the basis of trypsin sensitivity and antibody epitope accessibility of membrane-bound P450s, and the distribution of hydrophobicity on the surface of the P450 2C5/3LVdH molecule, a model for the interaction of the catalytic domain with the membrane was proposed (*13*) (Figure 1A). Selection of regions of P450 2C2 for substitution of Trp was based on this model. Four of the selected positions in P450 2C2, 80, 189, 347, and 239, were predicted to be distant from the membrane and four, 36, 69, 225, and 380, to be near or embedded in the membrane. Residue 36 is in the hydrophobic proline-rich region following the signal anchor sequence prior to the A-helix; residue 69 follows the A-helix and is near the proline-rich region in the three-dimensional structure; residue 380 is in the β2-2 strand of the two-stranded β2 sheet, and 225 is in the F−G loop.

Substitution for Natural Trp in P450 2C. There are three naturally occurring Trp residues in P450 2C2, residue 120 in the C-helix, residue 212 in the F′-helix, and residue 20 in the hydrophobic signal anchor sequence. For the Trp scanning, we attempted to construct a Trp-free P450 2C2. We had shown that aromatic substitutions for Trp-120 did not substantially reduce activity of P450 2C2 (*22*) and single substitutions of Leu for Trp-20 and Phe for Trp-212 also did not substantially reduce the activity of P450 2C2 expressed in COS-1 cells (Table 1). The double mutant W20L/W212F was also active when expressed in COS-1 cells, but the triple mutant, when Trp-120 was also mutated, was inactive (data not shown). Trp-120 is located adjacent to the heme in P450s and would not be expected to be inserted into the membrane, so that W20L/W212/F, which contains a Trp only at residue 120, was used as the parent protein for the Trp scanning.

An increase in fluorescence intensity of wild-type P450 2C2 and W212F (Trp-120, Trp-20) was observed when these proteins were incorporated into the liposomes (Figure 2A,C). Since Trp-20 is in the signal anchor transmembrane domain, such an increased intensity would be expected. Consistent with Trp-20 mediating the increased fluorescence, insertion of the Trp-20 mutant (W20L) into the membrane resulted in little change in fluorescence (Figure 2B). Similarly, mutation of both Trp-20 and Trp-212 did not result in an increase in fluorescence and either showed a decrease (Figure 2D) or little change (not shown) in different experiments. These results are most consistent with the conclusion that Trp-20 was predominantly responsible for increased fluorescence intensity when wild-type P450 2C2 was inserted into the membrane and that Trp-120 and Trp-212 were not inserted into the lipid layer.

Characterization of Trp Mutants. Trp was substituted at positions that contained an aromatic residue, either in P450 2C2 or another closely related 2C subfamily member, to minimize the effect of the mutation on folding and activity of P450 2C2. Each of the mutants was expressed in insect cells, and the His-tagged proteins were purified by affinity

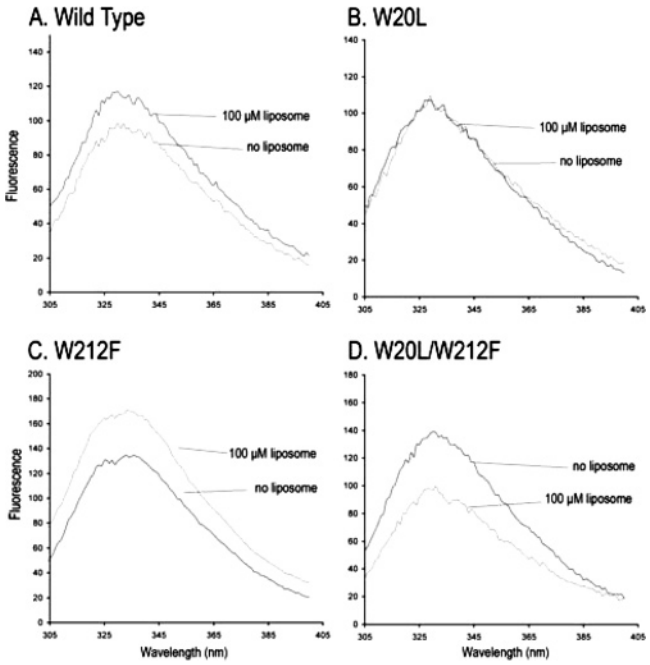

FIGURE 2: Trp fluorescence changes associated with incorporation into liposomes of P450 2C2 and its mutants with substitutions for Trp. Mutant proteins were expressed in insect cells, purified, and incorporated into liposomes as described in Materials and Methods. The fluorescence intensity of Trp was determined from 305 to 405 nm either in the absence of liposomes or after incorporation into liposomes. (A) Wild-type P450 2C2 (Trp-20, -120, -212); (B) W20L (Trp-120, -212); (C) W212F (Trp-20, -120); (D) W20L/W212F (Trp-120).

chromatography on nickel columns. Each of the purified mutants was expressed and correctly folded as determined by CO-difference spectra (not shown) and was purified to a specific content of 10−16 nmol of P450/mg protein (Table 1) and to a purity of about 80% estimated by Coomassie staining of SDS−PAGE gels (not shown). Each of the mutants was enzymatically active as determined by expression of the proteins in COS-1 cells (not shown) and by the activity of the purified proteins in a reconstituted system (Table 1). Solubilized P450 2C2 and the mutants had an emission maximum of 337−339 nm as expected for Trp in a relatively nonpolar environment.

Tryptophan Fluorescence of Membrane-Associated 2C2 Mutants. To determine whether Trp in the P450 2C2 mutants was inserted into the lipid bilayer of the liposomes and to estimate the depth of insertion, quenching of the Trp fluorescence by nitroxide spin labels attached to the phospholipid headgroups or to different carbons on the fatty acid tails of the phospholipids was measured. The spin labels quench fluorescence within a 1.0−1.1 nm distance (*23*), so that only Trp residues within the lipid bilayer proximate to the spin labels will be quenched. For these experiments, liposomes were prepared that contained POPC/PA (Control) or that contained POPC/PA with 10 mol % of either TEMPO, 5-Doxyl, or 12-Doxyl. TEMPO, attached to the choline headgroup, is on the surface of the membrane about 2.4 nm from the center of the lipid bilayer, and Doxyl-5 and Doxyl-12, attached to carbons 5 and 12, respectively, of the stearoyl fatty acid tail, are about 1.2 and 0.6 nm from the center (*24*). The fluorescence intensity of the P450s incorporated into liposomes with the spin labels (*F*) was compared to the fluorescence intensity of the P450s incorporated into control

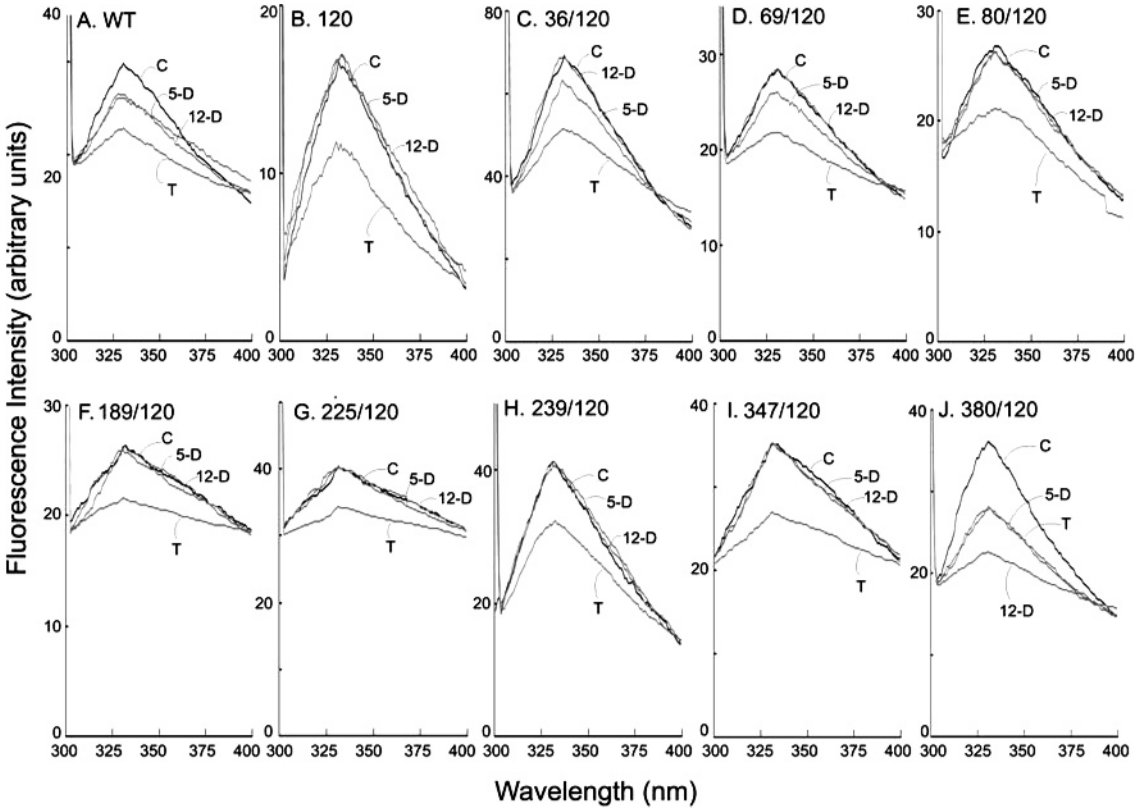

FIGURE 3: Quenching of Trp fluorescence of P450 2C2 and Trp mutants incorporated into liposomes. Wild-type (WT) P450 2C2 and its Trp mutants were expressed in insect cells, purified, and incorporated into liposomes as described in Materials and Methods. About 0.2 μM P450 protein was incorporated into PA/POPC liposomes at a molar ratio of P450 to phospholipids of 2500:1 in a volume of 250 μL. In control liposomes (C), no nitroxide spin-labeled phospholipids were added. For liposomes containing spin labels, 10 mol % of phospholipids with either TEMPO (T), 5-Doxyl (5-D), or 12-Doxyl (12-D) nitroxide spin labels was incorporated into the liposomes. Fluorescence was determined from 300 to 400 nm, and baseline fluorescence of liposomes without protein added was subtracted in each case. For each mutant, the spectra from two or three independent experiments for each liposome preparation were averaged with the maximum intensity of each curve normalized to the curve with the highest fluorescence. Wild-type P450 2C2 (WT) and W20L/W212F, which contains Trp at position 120 (120), are shown in panels A and B, respectively. W20L/W212F is the parent protein for the other mutants shown in panels C−J, and the positions of the two Trp residues in each mutant are indicated.

liposomes without spin labels (F_0). The spectra for the mutants are shown in Figure 3, and the degree of quenching by the nitroxide groups are presented in Table 2. Some of the spectra shown in Figure 3 are distorted compared to normal Trp emission spectra, primarily by anomalous scattering or emission at the higher wavelengths from 350 to 400 nm, the nature of which is unknown. For a given mutant protein preparation, the shapes of the spectral curves were consistent for the different liposome preparations, so that the relative quenching by the different lipid spin labels is a valid measurement for each mutant protein, but the absolute fluorescent intensities and percent quenching is not directly comparable between mutant proteins because the contribution of the apparent anomalous emission differs for each.

Fluorescence of the parent protein, W20L/W212F(Trp-120), was quenched about 27% by the surface TEMPO spin label but not by the 12-Doxyl and 5-Doxyl labels (Table 2), which is consistent with the expected location of Trp-120 outside the membrane near the phospholipid headgroups (Figure 1). For all the Trp mutants tested, quenching by TEMPO of 14−27% was observed. Because of the strong TEMPO quenching of Trp-120 fluorescence, which was present in all mutants, the quenching of fluorescence from the additional Trp by TEMPO could not be estimated accurately and was not informative. The quantitative differences in TEMPO quenching are most likely the result of differing qualities of the protein preparations that affect Trp-120 fluorescence, as well as that of the other Trp substituted. Quenching by the 12-Doxyl and 5-Doxyl labels of the

Table 2: Relative Quenching of Trp Fluorescence in Liposomes by Phospholipids Modified with Nitroxide Spin Labels

	% quenching[c]		
mutant[a]	TEMPO (2.4 nm[b])	5-Doxyl (1.2 nm)	12-Doxyl (0.6 nm)
wild-type	24 ± 1.7[d]	13 ± 2.7	11 ± 3.2
Trp-120	27 ± 4.2	−5 ± 1.6	−4 ± 3.6
Trp-36/120	25 ± 0.3	9 ± 1.3	1 ± 0.6
Trp-69/120	24 ± 1.0	9±1.2	0 ± 1.3
Trp-80/120	19 ± 1.3	1 ± 1.6	1 ± 0.4
Trp-189/120	18 ± 0.4	1 ± 0.8	2 ± 0.9
Trp-225/120	14 ± 0.6	−2 ± 1.0	0 ± 1.7
Trp-239/120	21 ± 1.1	0 ± 1.2	0 ± 0.4
Trp-347/120	24 ± 0.2	1 ± 0.7	0 ± 0.4
Trp-380/120	23 ± 0.6	22 ± 0.1	38 ± 0.2

[a] Mutant proteins were expressed in insect cells and purified as described in Material and Methods. W20L/W212F (Trp-120) is the parent protein for the remainder of the mutants. The positions of the Trp residues in each mutant are indicated. [b] Distance of the spin label from the center of the lipid bilayer. [c] The % quenching for each spin label is the percentage decrease of fluorescence intensity at the wavelength of maximum intensity for proteins incorporated into liposomes containing the indicated spin label compared to that of proteins incorporated into control liposomes without spin labels. [d] Standard error of the mean, $n = 3$.

fluorescence of mutants with Trp at positions 80, 189, 225, 239, and 347 was less than 2%, similar to the Trp-120 parent, indicating that these Trp, like Trp-120, did not penetrate into the lipid bilayer (Table 2).

One caveat to this conclusion is the possibility that the fluorescence of these Trp is highly quenched by heme. Heme quenching would be a major problem only if quenching by heme is nearly complete, so that additional quenching by the nitroxide spin labels would not be possible. In P450 2D6, which contains six Trp, molecular dynamics simulations indicated that Trp-128 (equivalent to Trp-120 in P450 2C2) had a much higher orientation/distance factor for energy transfer to heme than the other Trp and that energy transfer to heme from the other Trp was unimportant (*25*). Trp-120 is not completely quenched by heme, since quenching by TEMPO of fluorescence of P450 2C2 with only Trp-120 is still observed. Similarly, in P450 2D6, mutation of Trp-128 resulted in a 22% decrease in Trp fluorescence intensity for the mutant compared to wild-type, suggesting that Trp-128 contributes substantially to the overall Trp fluorescence of P450 2D6 despite quenching by heme (*25*). Since the other Trp studied in P450 2C2 are farther from the heme than Trp-120, complete quenching of fluorescence from these other Trp's by heme is unlikely.

Three of the mutants, Trp-36, Trp-69, and Trp-380, exhibited fluorescence-quenching patterns different from the parent (Table 2). The fluorescence of Trp-36 and Trp-69 was quenched about 9% in 5-Doxyl labeled liposomes, while no quenching by the 12-Doxyl label was observed. In contrast, Trp-380 fluorescence was more substantially quenched by both the 5-Doxyl and 12-Doxyl spin labels. These results suggest that Trp-36 and -69 are present in the membrane, but are near the surface, while Trp-380 is also in the membrane but is deeper in the lipid bilayer.

The depth of a fluorescence source in the membrane can be calculated from the relative quenching of its fluorescence by multiple quenchers located at different depths in the lipid bilayer by the parallax method (*26*). In the case of the P450 2C2 Trp mutants, quenching of the fluorescence of the substituted Trp by TEMPO cannot be determined because of the strong quenching of Trp-120 fluorescence by TEMPO in all the mutants. However, quenching of fluorescence by the 5-Doxyl and 12-Doxyl spin labels can be used to semiquantitatively estimate the depth of the substituted Trp in the membrane. Fluorescence of Trp-36 and Trp-69 is quenched only by the 5-Doxyl label and not by the 12-Doxyl label, so that these residues are likely located between the TEMPO label at the surface and the 5-Doxyl label 1.2 nm from the center of the lipid bilayer. Trp-380 fluorescence is quenched more by the 12-Doxyl label at 0.6 nm from the center than the 5-Doxyl label at 1.2 nm so is likely present less than 0.9 nm from the center of the lipid bilayer. If the hydrophobic part of the membrane bilayer extends 1.5 nm from the center of the lipid bilayer to the cytoplasmic surface (*27*), then Trp-36 and -69 would be located 0−0.3 nm into the bilayer, while Trp-380 would be located greater than 0.6 nm into the bilayer.

DISCUSSION

The quenching of the fluorescence of Trp residues distributed over the P450 2C2 molecule by nitroxide spin labels at different depths in the membrane largely supports the model of P450 2C5/3LVdH−membrane interactions shown in Figure 1. The observation that the fluorescence of Trp-36, Trp-69, and Trp-380 is quenched by the 5-Doxyl spin label is consistent with their predicted position in the membrane. Likewise, the lack of quenching of fluorescence by the 5-Doxyl spin label that was observed for the Trp at 120, 80, 239, 189, and 347 is consistent with their positions outside the lipid bilayer in the model.

The lack of quenching of fluorescence by the 5-Doxyl and 12-Doxyl spin labels that was observed for Trp-225, however, is not consistent with its predicted position in the lipid bilayer in the P450 2C5/3LVdH model. The modified form of P450 2C5 that was crystallized had five mutations in the 202−210 region which decreased aggregation and facilitated formation of crystals (*13*). Recently, the structures of P450 2C8 and two different P450 2C9 structures have been published (*28*−*30*) which differ in the position of 225. The 2C9 structure 1OG5 was modified with seven amino acid substitutions, K206E, I215V, C216Y, S220P, P221A, I223L, and I224L which correspond to the modified residues in CYP2C5/3LVdH. The structure in the F−G loop region of 1OG5 (not shown) was very similar to that shown for P450 2C5/3LVdH in Figure 1A. The native P450 2C9 structure, however, differed substantially in the F−G loop region with the residue 225 shifted up away from the membrane (Figure 1C). In the native P450 2C8 structure, the position of residue 225 is similar to that in P450 2C5/3LVdH (Figure 1B). The primary amino acid differences that account for the shift in the 225 position in P450 2C9 compared to P450 2C8 and P450 2C5/3LVdH are a change in Pro-220 and Leu-221 in 2C8 to Ser-220 and Pro-221 in 2C9 (Figure 1D). Pro-220 forms a tight turn in P450 2C8 and it has been proposed that the shift of the Pro to position 220 from 221 in 2C9 may increase the propensity to form an α-helical structure in the 221−223 region (*29*). P450 2C2 has a Pro at 220, as P450 2C8 does, so that it seems likely that the native structure of 2C8 is the best model for P450 2C2 in the region around position 225. With 2C8 as a model, using the orientation proposed for P450 2C5/3LVdH, positions 36, 69, and 380 are in the membrane, in good agreement with the Trp quenching data, but residue 225 is also in the membrane, which is inconsistent with the observation that Trp-225 fluorescence is not quenched.

The native P450 2C8 structure contained two molecules per asymmetric unit in the crystals. The F−G loop region, which includes position 225, interacted extensively at the dimerization surface of the two molecules. Modeling of the dimer structure with the membrane based on the orientation shown in Figure 1 resulted in different orientations for each of the two monomers in the dimer relative to the membrane. However, the proximal-facing model shown in Figure 1 can be tilted a few degrees clockwise (Figure 4A), so that the resulting dimer is symmetrical relative to the membrane and the two monomers have the same membrane orientation (Figure 4B). In this rotated 2C8 molecule, residues 36, 380, and 69 remain in the membrane, but residue 225 is rotated out of the lipid bilayer and instead contributes to the dimerization interface. This model of a dimer with a F−G loop dimerization interface, thus, provides the best fit with the Trp fluorescence quenching data for P450 2C2.

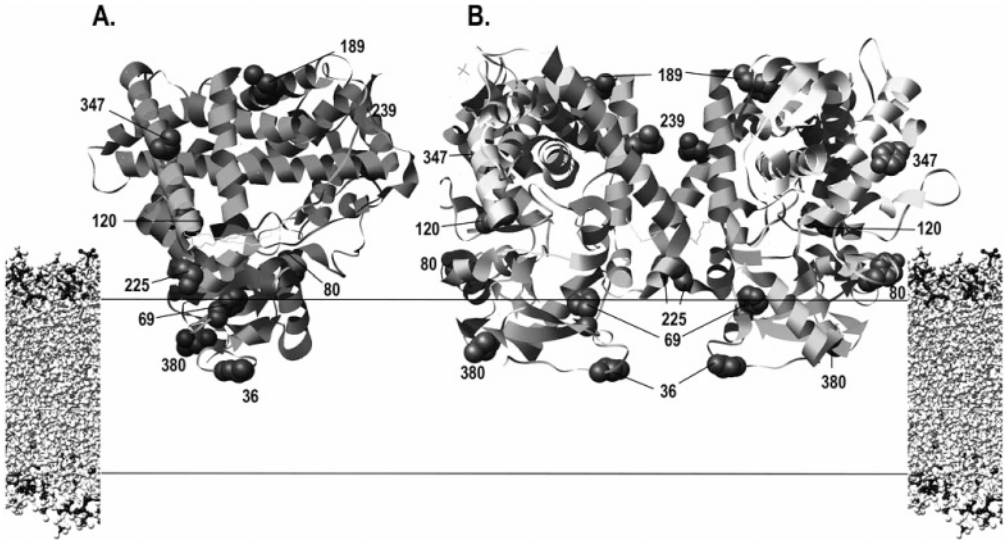

FIGURE 4: Model of P450 2C8 associated with the membrane. In panel A, the proximal view of P450 2C8 is shown with the same orientation, relative to the membrane, as the side view of the crystal structure of the dimer shown in panel B. Representation of the molecules by the Chimera program was as described in the legend to Figure 2. Shown at either side of the figure is a model of a phospholipid bilayer (*35*) shown in ball-and-stick display with the phosphate and quaternary amine groups in the phosphatidylcholine headgroups shown in black. The solid lines indicate the position of the lipid core of the phospholipid bilayer.

A model of the catalytic domain of P450 2C2 as a dimer is not consistent with FRET or BiFC studies of P450 2C2 in natural membranes in which oligomerization of P450 2C2 was mediated by the signal anchor sequence rather than the catalytic domain (*31*, *32*). FRET was not observed with a construct in which the P450 2C2 signal anchor was replaced with the transmembrane domain of epidermal growth factor receptor, suggesting that the F−G loop region cannot independently mediate homooligomerization of the P450 2C2 (*31*). There are several possible explanations for the discrepancy. The dimerization observed in the crystals occurred at high concentrations of protein and, thus, may be a relatively weak interaction that does not occur naturally in the membrane. Alternatively, the weak interaction might occur in the membrane cooperatively with the signal anchor sequence interactions, but is not sufficiently strong to form dimers in the absence of the signal anchor. Finally, it is also possible that P450 2C2 does not form dimers due to the differences in the sequence around position 225. In P450 2C8, position 225 is a Cys rather than Tyr, position 223 is Ile rather than Phe, and 221 is Leu rather than Ile. These changes in the size and hydrophobicity of residues could reduce the dimerization potential of P450 2C2. Further studies will be required to determine whether P450 2C2 and P450 2C8 are present as dimers in natural membranes.

It is likely that the positions of the residues within the lipid core of the bilayer will be different from the positions shown in Figure 4 which are based on structures of solubilized proteins. Insertion of P450 1A2 into POPC/PA liposomes was accompanied by a substantial change in the amount of α-helix and *β*-sheet estimated by circular dichroism measurements (*21*). Residue 380 is present in a *β*-sheet in the soluble protein structures which likely converts to an α-helix within the lipid bilayer. This would reduce the *β*-sheet content by about 20%, which is qualitatively consistent with the circular dichroism measurements. In addition, the first residue resolved in the P450 2C8 structure is Lys-28, and in the intact molecule, this residue is preceded by seven relatively hydrophilic residues, including basic amino acids, which link the N-terminal transmembrane domain to the catalytic domain. In the model shown in Figure 4, Lys-28 is deep in the membrane, but in the actual membrane, this residue and the "linker" sequence are likely to be in the region of negatively charged phospholipid headgroups. The sequence preceding residue 36 is likely, therefore, to extend up to the surface of the lipid bilayer and possibly pull residue 36 closer to the surface. This would be consistent with the quenching data that suggests that residue 36 is a similar distance from the center of the lipid bilayer as residue 69, contrary to the positions in Figure 4. Similarly, residue 380 is flanked by positively charged residues, which are conserved in both P450 2C8 and P450 2C2, at positions 375, 377, and 383. If 380 is the deepest residue in the membrane as indicated by the Trp quenching, then it is likely that the orientation of the side chains will be such that Leu-380 is pointed to the center of the lipid bilayer and the nearby basic residues will be orientated with the side chains pointing toward the surface of the lipid domain rather than as shown in Figure 4. This would not be consistent with an expected α-helix structure in this region, so the increased depth of residue 380, relative to 36 and 69, in the membrane cannot be clearly rationalized based on the structure shown.

Although there will likely be some changes in the positions of the membrane-associated residues in the native molecule compared to the solubilized forms used for crystallization, the overall orientation of the molecule relative to the membrane is likely to be the same. With the orientation shown in Figure 4, the proximal surface, which contains residues predicted to mediate interaction with P450 reductase, is available for efficient interaction with the reductase. Further, one of the substrate access channels would be oriented in the membrane so that lipophilic substrates could interact with the enzyme directly from the lipid bilayer (*13*, *14*). The interactions of the catalytic domain of P450 2C2 with the membrane, therefore, are likely to be very important functionally. In addition, the phospholipid composition of the membranes affects the incorporation of P450s into membranes and the activity of the P450s (*21*, *33*, *34*), so

that interactions in natural microsomal membranes or with liposomes of different phospholipids composition than the POPC/PA vesicles used in the present experiments may alter the depth of penetration into the bilayer. Phospholipid composition may vary depending on physiological conditions, which could result in altered bilayer penetration and activity of the P450. It will be interesting to examine whether changing the hydrophobicity of these membrane loops to alter the interaction with the lipid bilayer will have functional consequences.

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