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Catalytic Modulation of Human Cytochromes P450 17A1 and P450 11B2 by Phospholipid

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Abstract

Unlike most of the drug-metabolizing cytochrome P450s, microsomal P450 17A1 and mitochondrial P450 11B2 catalyze sequential multi-step reactions in steroid biosynthesis. The membrane phospholipid composition might be one parameter that modulates the efficiency and processivity of specific pathways. Here we systematically examined the effects of physiologically relevant phospholipids on the catalysis of purified P450 17A1, P450 11B2, and P450 11B1 in reconstituted assay systems. Both dioleoylphosphatidylcholine (DOPC, 18:1) and dilauroylphosphatidylcholine (DLPC, 12:0) were found to be very efficient in reconstituting 17-hydroxylase and 17,20-lyase reactions of P450 17A1. Phosphatidylethanolamine (PE) specifically enhanced 17,20-lyase activity up to 2.4-fold in the presence of phosphatidylcholine. On the other hand, P450 11B2-catalyzed production of aldosterone from 11-deoxycorticosterone was very low and from 18-hydroxycorticosterone nil, implying low processivity. DOPC or cardiolipin, which is exclusively located in the inner mitochondrial membrane, maximized aldosterone yield. In sharp contrast, reconstitution of homologous P450 11B1 with DOPC significantly decreased corticosterone formation without affecting the synthesis of 18-hydroxycorticosterone. The intrinsic fluorescence of P450 17A1 and 11B2 increased in the presence of DOPC, DLPC and PE. Acrylamide quenching studies showed that PE decreased solvent accessibility for tryptophan in P450 17A1, as did 20:4 PC or 18:2 PC for P450 11B2. A moderately positive correlation between the proportion of high-spin substrate-bound species and catalytic activity was only observed in the presence of phosphatidylcholines with low-temperature phase transition. These results demonstrate the potential for phospholipids to regulate the activity of steroidogenic P450 activities and thereby steroid hormone biosynthetic pathways.

Graphical Abstract

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Keywords

Phospholipid; P450 17A1; P450 11B2

Introduction

The biosynthesis of steroid hormones requires the coordinated expression of various cytochrome P450 enzymes and the shuttling of steroid intermediates between the mitochondria and smooth endoplasmic reticulum (ER) (1). Human P450 17A1 is primarily expressed in adrenals and gonads, and P450 17A1 exhibits two distinct catalytic activities. The 17-hydroxylase activity converts pregnenolone and progesterone to 17-hydroxypregnenolone (17Preg) and 17-hydroxyprogesterone (17OHP), respectively, and facilitates adrenal glucocorticoid synthesis; its 17,20 lyase activity further converts primarily 17Preg to the major androgen precursor dehydroepiandrosterone (DHEA) (2). On the other hand, human P450 11B1 and 11B2 are expressed in the adrenal but not the gonads, and these enzymes are responsible for the terminal steps of glucocorticoid and mineralocorticoid production, respectively (1, 3). P450 11B1 catalyzes the conversion of 11-deoxycortisol to cortisol; P450 11B2 preferentially converts 11-deoxycorticosterone (DOC) to aldosterone via corticosterone and 18-hydroxycorticosterone (4). The presence of P450 17A1 and P450 11B1 in the zona fasciculata of adrenal cortex enables cortisol synthesis, while the exclusive expression of P450 11B2 in the zona glomerulosa allows aldosterone formation. These steroidogenic P450s are embedded in lipid bilayers of two distinct compartments. P450 17A1 is anchored to the ER membrane and receives electron from P450 oxidoreductase (POR) (5); whereas P450 11B1 and P450 11B2 are located in mitochondria inner membrane and are partnered with adrenodoxin (Adx) and adrenodoxin (ferredoxin) reductase (AdR) (6). Despite the known importance of phospholipids in regulating protein structure and activity, and the potential for dysregulation in mitochondrial lipid trafficking to disrupt steroid biosynthesis (7), the influence of specific phospholipids on the catalysis and processivity of these steroidogenic P450s has been largely neglected.

Lipid bilayers in mammalian cells consist of various lipid types that modulate cell signaling and protein function (8). Membrane lipid composition changes in several disease states and varies markedly over different cellular compartments (9, 10). One central feature of a lipid bilayer is its hydrophobic thickness, defined by the lengths of the fatty acyl chains in the component lipids. Membrane proteins in a lipid bilayer adopt conformations to match that of the surrounding lipid bilayer, since mismatch with the hydrophobic thicknesses may result in distortion of the protein and loss of activity (11). Phospholipid head group structures also exert an impact on the function of P450s depending on specific interactions between the

lipid head groups and the protein. The major phospholipids in human adrenal cortex are phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with 3:1 ratio in ER, and 1:1 ratio in mitochondrial membrane. The fatty acid moiety of PC and PE is predominantly composed of saturated species (16:0, 18:0) at position 1 of the glycerol and unsaturated chains (18:1, 18:2, 20:4) at position 2 (12). In addition, ~2% phosphatidylinositol (PI) is also present in ER membrane. PI is important for intracellular signaling, but its structural functions are not clear (8). On the other hand, cardiolipin (CL) has been reported to be exclusively associated with the mitochondrial inner membrane and is involved in maintaining mitochondrial functionality and membrane integrity (13).

In addition to the N-terminal transmembrane anchor (14, 15), the F–G loop of most P450s also attaches to the membrane. The F–G loops of mitochondrial P450 11A1 and P450 11B2 are their major sites of insertion into the inner mitochondrial membrane (14, 16, 17). The F–G loop also regulates the entry and egress of substrates (18), and this flexible region is particularly important for the binding of hydrophobic membrane-associated substrates at this interface. Thus, the membrane composition has the potential to influence not only P450 stability, dynamic behavior, and activity but also substrate delivery, dissociation, and processivity for multi-step enzymes, as has been suggested for P450 11A1 (16).

In the present study, we have investigated the effect of changes in the fatty acid chain length of PC and major and minor phospholipid compositions on the activities of a reconstituted human P450 17A1, P450 11B2 and P450 11B1. Our results show that acyl chain length, degree of unsaturation, and phospholipid composition in the membrane all significantly alter the activities and potentially the processivity of steroidogenic cytochrome P450s.

Materials and methods

Ampicillin, 5-aminolevulinic acid, isopropyl- β -D-thiogalactopyranoside (IPTG), 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (DLPC), cardiolipin (from bovine heart), and steroid substrates were obtained from Sigma (St Louis, MO). All other PCs, PE (18:0–18:1), PI (from bovine liver) were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Protease inhibitor cocktail were from Bimake (Houston, TX). Bactotryptone and yeast extract were purchased from Difco (Detroit, MI). Molecular biology reagents, including restriction enzymes, ligases, and Phusion polymerase, were obtained from New England BioLabs (Beverly, MA).

Plasmids

The expression plasmids were generous gifts obtained from the following investigators: modified human CYP17A1-G3H6 in pCW, N-27-human POR-G3H6 in pET22, cDNA of human Adx, CYP11B1, CYP11B2, and AdR from Professor Walter L. Miller (University of California, San Francisco, CA); human cytochrome *b*₅ in pLW01-b5H4 from Professor Lucy Waskell (University of Michigan).

Expression and purification of recombinant proteins in *E. coli*

Modified human P450 17A1 was expressed with GroEL/ES chaperones (pGro7 plasmid) in *E. coli* JM109 cells as described (19, 20). Modified human POR was expressed in *E. coli*

C41(DE3) cells (OverExpress, Lucigen, Middleton, WI) and purified according to a previously published procedure (21, 22). The protocol for expression and reconstitution of recombinant human CYB5A was based on the procedure of Mulrooney and Waskell (23). Human Adx was expressed in *E coli* strain C41(DE3) and purified as described (24). Human AdR (25), P450 11B1, and P450 11B2 were expressed in C41(DE3) with pGro7 and purified as described (26).

Phospholipid preparation

Liposomes of various phospholipids were prepared by sonication (27). Large multilamellar vesicles (LMVs) were formed by hydration of the lipids in water. The 1 mg/mL suspension of LMVs was sonicated on ice for 2–3 min by using a Model 100 Sonic Dismembrator (Fisher Scientific) that was set at an intensity of 8 upon which the turbid solution became translucent, indicating the formation of small unilamellar vesicles (SUVs). Stock solutions were stored at 4 °C and used within two weeks.

Reconstituted enzyme assays

Human P450 17A1 (10 pmol, final concentration 0.05 μ M) was reconstituted with 1 molar equivalent of POR with or without 1 molar equivalent of cytochrome *b*₅ and 100 molar equivalents phospholipid. The mixture was set at room temperature for 5 min and diluted to 0.2 mL with 50 mM potassium phosphate buffer (pH 7.4) and substrate. The resulting mixture was mixed gently and set at 37 °C for 3 min. NADPH (1 mM) was added, and the incubation was continued at 37 °C for 20 min before extracting with 1 mL dichloromethane. For incubations with 17Preg substrate, the dried incubation extracts were dissolved in methanol (70 μ L) and suspended in the same 0.1 M potassium phosphate buffer (100 μ L). Water (100 μ L) and cholesterol oxidase (5 μ L, 100 units/mL) were added, and the resulting solution was shaken at 30 °C at 200 rpm for 6 h before extracting with 1 mL dichloromethane. The organic extracts were dried under a nitrogen flow. Human P450 11B2 or P450 11B1 (40 pmol, final concentration 0.2 μ M) was mixed with 60 molar equivalents of Adx, 1 molar equivalent of AdR, and 200 molar equivalents of phospholipid in <20 μ L volume and incubated at room temperature for 5 min. The reaction mixture was then diluted to 0.2 mL with 50 mM potassium phosphate buffer (pH 7.4), and DOC substrate (200 μ M). The resulting mixture was pre-incubated at 37 °C for 3 min before adding NADPH (1 mM) and incubating at 37 °C for another 30 min. The reaction mixture was extracted with 1 mL dichloromethane, and the organic phase was dried under a nitrogen flow. Because the yield of aldosterone was so low and activity not necessarily linear with time, the reactions were allowed to go to complete substrate consumption, and activity was expressed as yields of each product.

Chromatography, data acquisition, and determination of kinetic constants

Reaction products were analyzed using an Agilent 1260 Infinity HPLC system with UV detector. Extracted steroid products were dissolved in 20 μ L of methanol, and 5 μ L injections were resolved with a 50 $\times$ 2.1 mm, 2.6 μ m, biphenyl 100 Å column (Phenomenex, Torrance, CA), equipped with a guard column at a flow rate of 0.4 mL/min. A methanol/water linear gradient was used: 32.5% to 88.3% methanol from 0 to 10.6 min, 88.3% to 12 min, 99.1% from 12.1 min to 14 min, and 32.5% from 14.1 min to 15 min. Products were

identified by retention times of external standards chromatographed at the beginning and ends of the experiments, and the data were processed with Laura4 software (LabLogic) and graphed with GraphPad Prism 7 (GraphPad Software, San Diego, CA).

Spectroscopic methods

Fluorescence intensity was measured on Tecan Infinite® M1000 Microplate Reader. The excitation wavelength was set to 280 nm, and emission spectra were collected from 290 to 430 nm. The P450 concentration used was 1 μ M, and the measurements were performed in 50 mM potassium phosphate buffer (pH 7.4), in the presence or absence of 100 μ M various phospholipids. Acrylamide titrations were performed by adding to a 1 μ M solution of P450 acrylamide in a 5.4 M stock to final concentrations of 0.04–0.22 M with fixed excitation of 280 nm and an emission wavelength of 312 nm for P450 17A1 or 340 nm for P450 11B2. Excitation bandwidths were set to 5 nm, and emission bandwidths were set to 5 nm for P450 17A1 and 10 nm for P450 11B2. Corrections for the inner-filter effect due to acrylamide absorption were calculated according to Calhoun (28). The values were also corrected for dilution and for the buffer blank. The fluorescence quenching data were then analyzed by Stern-Volmer and Lehrer plots as described (16). Substrate-binding spectra of P450s were also obtained on the same instrument with 1 μ M P450s and 100–200 μ M substrate with phospholipid/P450 ratio at 100:1 for P450 17A1 and 200:1 for P450 11B2 in 50 mM potassium phosphate buffer, pH 7.4. The wavelength ranged from 350 to 500 nm, and readings averaged over 3 scans were taken in 1-nm steps. Spectra were corrected for scatter by subtraction of buffer or vesicles as appropriate. All absolute spectra were normalized to the absorbance at 500 nm.

Results

Microsomal P450 17A1

PC and PE are the most abundant lipids in biological membranes. Previous work in our laboratory has demonstrated that phospholipid composition markedly influences P450 17A1-catalyzed 17,20-lyase activity, in the order PS>PE>PC at a 200:1 lipid-to-P450 ratio (29). Since then, we have examined the concentration of phospholipids on the activities of P450 17A1 reconstituted with POR and cytochrome *b*₅, and we found a linear decrease in the rate of steroid metabolism with an increasing amount of phospholipid. The optimal activity for both 17-hydroxylase and 17,20-lyase reactions of P450 17A1 was obtained when the SUVs were used for reconstitution at a 100:1 DLPC-to-P450 ratio, with equal amounts of P450 17A1 and POR (Figure 1A). Working under these conditions, we screened a number of closely related PCs differing in acyl chain length and degrees of unsaturation, plus other phospholipid species (Figure 1).

Among all the single phospholipids tested, P450 17A1 reconstituted with DLPC (12:0) or DOPC (18:1) displayed the best 17-hydroxylase (Figure 1B) and 17,20-lyase activities (Figure 1C), which ranged 10–50% better than other PCs with longer fatty acid chains or more unsaturation. The effects of PE on 17-hydroxylase and 17,20-lyase activities also varied with PC species. Addition of PE to PC at 1:1 ratio had large effects on 17,20-lyase activity, with 1.4 to 2.4-fold stimulation compare to PC only, but the enhancement of 17-

hydroxylase activity was small, only 8–34%. Comparable responses were observed with a mixture of PC, PE, and PI at a ratio of 73.5:24.5:2, which was chosen to closely resemble the proportions found in the native environment of the ER membrane.

To further investigate the effect of phospholipids on protein conformation, we studied the intrinsic fluorescence of P450 17A1, which primarily reflects the local environments of its 8-tryptophan residues and the quenching of their fluorescence. The fluorescence emission spectrum of P450 17A1 in the absence of liposomes showed a λ_{max} of 312 nm, and the intensity increased in the presence of PC with saturated C₁₂, C₁₆ and C₁₈ acyl chains at both positions, as well as DOPC and PE, without any appreciable change in the λ_{max} values (Figure 2A and 2B). The presence of these phospholipids, therefore, does not appear to significantly modify the local environment of the fluorophores in P450 17A1, but increased intrinsic fluorescence might result from protection against quenching molecules in the solvent (30) and/or disaggregation of less fluorescent oligomers. In contrast, an incomplete spectral red-shift toward longer λ_{max} and a concomitant decrease in intensity was observed in the presence of 18:2 PC, 20:4 PC, and PI. The reasons for the larger quenching observed in the presence of these two polyunsaturated PC species seem to be more complex than for the increase with the saturated PCs, due to the flattening of the emission spectra. Fluorescence characterization of P450 17A1 in various lipid environments is summarized in Table 1. The F/F_0 ratios of P450 17A1 in the presence of phospholipids show good correlation with the catalytic activities, except for 16:0 PC and 18:0 PC, the only two species that were in a gel state due to their high phase-transition temperatures.

To further investigate the association of P450 17A1 with phospholipid membranes, we tested the ability of acrylamide to quench the intrinsic fluorescence of tryptophan residues without and with insertion into vesicle membranes. A linear Stern-Volmer plot obtained with P450 17A1 indicates a single class of fluorophores, all equally accessible to the quencher. The accessibility of tryptophans to the quencher (K_{sv}) was $12.4 \pm 0.4 \text{ M}^{-1}$ for the vesicle-free P450 17A1 (Figure 2C–D and Table 2) and was reduced slightly in the presence of PI and some PC species but was reduced the most in the presence of PE, from 7.6 to 6.7 (Figure 2D and Table 2). This result suggests that one of the quenchable tryptophan residues of P450 17A1 becomes inaccessible to acrylamide quenching following association with PE vesicles.

Next, we determined the influence of different phospholipids on the binding of substrate with P450 17A1. In the resting state, the P450 17A1 absorbance spectrum of the ferric heme is dominated by a Soret (γ) band at approximately 418 nm, which is blue-shifted to 390 nm upon substrate (pregnenolone) binding. This type I spectral change is attributed to the transition of the ferric heme iron from the resting low-spin state with a water molecule as the sixth ligand to the 5-coordinated high-spin species, and the magnitude of this change is proportional to the fractional conversion to high-spin iron (31). Pregnenolone binding to P450 17A1 elicited the greatest spectral change in the presence of DLPC and DOPC (Figure 3, Table 3), consistent with a higher fraction of bound substrate and/or more complete conversion to high-spin iron. A correlation between phospholipid-dependent variation of P450 17A1 activity and the magnitude of substrate-induced spectral changes was consistently observed. Addition of PE in the presence of various PCs did not cause any significant alteration in substrate-induced spectral changes, except with 18:0 PC.

Mitochondrial P450 11B2 and P450 11B1

In light of our findings with microsomal P450 17A1, we sought to compare our results with another multi-step steroidogenic P450 but located in mitochondria – P450 11B2. The capacity of purified P450 11B2 to produce aldosterone is poor (32), and multiple conditions have been attempted to reconstitute the low aldosterone synthase activity of P450 11B2 *in vitro*. The yield of aldosterone – both in previous studies and in our hands – is highest with DOC as the substrate, less from corticosterone, and none from 18-hydroxycorticosterone, which implies that aldosterone formation requires processivity without release of one or more intermediate products. Through systematic optimization of individual components, the molar ratio of P450:ADX:AdR:phospholipid was determined to be 1:60:1:200 (Figure 4A). DOPC, as well as brain PC and egg PC replacing DLPC during P450 11B2 reconstitution, enhanced aldosterone formation up to 3% yield after complete substrate consumption (Figure 4B). To compare against a similar mitochondrial P450 with poor apparent processivity, we also studied P450 11B1, which exhibits high 11 β -hydroxylase but very low 18-hydroxylase (and no aldosterone synthase) activities. The yield of corticosterone was best with DLPC, and DOPC significantly decreased corticosterone formation without affecting 18-hydroxycorticosterone formation during P450 11B1 reconstitution (Figure 4C). As shown for P450 17A1, we determined the effect of fatty acyl chains in PC and of other phospholipids on P450 11B2 catalysis (Figure 5). DOPC is the only single PC capable of increasing aldosterone yield (Figure 5A), while the addition of CL to DLPC at 1:1 ratio resulted in higher 18-hydroxycorticosterone and aldosterone productions concomitantly (Figure 5B). A mixture of PC:PE:CL at 2:2:1 ratio, which mimics the composition in the inner mitochondrial membrane, yielded similar results as DOPC.

P450 11B2 in the presence of various phospholipids gave very similar fluorescence spectra with emission λ_{max} values at 340 nm. The fluorescence intensity increased in the presence of 12:0 PC, 18:1 PC, and PE but decreased in the presence of 18:2 PC and 20:4 PC (Figure 6A); however, CL has no effect on intrinsic fluorescence of P450 11B2 (Figure 6B). The results of acrylamide quenching experiments with P450 11B2 in the absence or presence of various phospholipids are shown in Figure 6C–D and Table 2. The number of accessible tryptophan residues was significantly reduced in the presence of 20:4 PC and 18:2 PC, indicating that at least one of the quenchable tryptophan residues of P450 11B2 becomes inaccessible to acrylamide quenching following association with vesicles containing these polyunsaturated PCs. Spectral changes in P450 11B2 upon DOC binding (Figure 6E–6H) showed that DOPC caused a larger type I spectra shift compared to DLPC (Table 3). The addition of PE to DLPC at 1:1 ratio also significantly increased the magnitude of the spectral change (Figure 6E), and the amount of high-spin iron observed with these phospholipids correlates with the yield of 18-hydroxycorticosterone and aldosterone (data not shown).

Discussion

DLPC is commonly used as the phospholipid for many microsomal P450 enzyme reconstitutions (33, 34). Biological membranes, however, are complex multicomponent structures, which contain lipids with different head groups and alkyl chains. Here, we have identified specific phospholipids that selectively enhance the final product formation of

multistep steroidogenic enzymes, microsomal P450 17A1 and mitochondrial P450 11B2. In addition to DLPC, which is a rare component in biomembranes, DOPC with two identical oleic acids was also effective for reconstituting the activities of purified P450 17A1. Additional PE in the presence of PC appears to provide a hydrophobic environment that increases 17,20-lyase activity. On the other hand, the presence of DOPC or CL supplement increased final aldosterone production of P450 11B2 without noticeable changes in its initial 11 β -hydroxylation. In stark contrast, an apparent reduction of the 11 β -hydroxylase activity of P450 11B1 was observed with DOPC. The mechanism of the differential effect of DOPC on the 11 β -hydroxylase activity of closely related P450 11B1 and P450 11B2 is unknown; however, this influence might contribute to the regulation of corticosteroid products formed in the adrenal zones and in tumors(35).

Previous studies on P450 17A1 activity have showed that cytochrome *b*₅ stimulated 17,20-lyase activity with 2-fold higher activity when the strong cationic PC head group changed to weaker cationic PE and then to anionic PS (29). On the other hand, the major drug-metabolizing enzyme P450 3A4 was reported to require DOPC or POPC (16:0–18:1 PC) in the presence of PS to achieve maximal catalytic activity (36, 37). Addition of PE to PC has also been shown to have a stabilization effect on P450 1A2 (38). For mitochondrial P450s, CL was reported to induce activation of P450 11A1 via a decrease in K_m (39); conversely, CL inhibited P450 11B1 via an increase in K_m and a decrease in V_{max} (40). PE and CL are non-bilayer-forming phospholipids, which are characterized by more conical shapes with smaller head group diameters. When PC and PE are mixed, the conformational order of the acyl chains in PC bilayers increases markedly, because the smaller PE head group allows a tighter lateral packing of the acyl chains, and a relief of bilayer curvature stress is expected (41, 42). Moreover, both CL and PE induce membrane curvature and form a specific microdomains, which could direct selective clustering of proteins into the same specific regions to increase their interactions with each other (43). This feature might explain why PE enhanced only cytochrome *b*₅-stimulated 17,20-lyase activity of P450 17A1 and why CL increases the yield of aldosterone from P450 11B2, even though CL is not essential for aldosterone production.

Prior studies of P450 enzymes have shown that intrinsic fluorescence is strongly quenched as a function of tryptophan proximities to the heme (30), which could in part explain the increased intrinsic fluorescence of P450 17A1 and P450 11B2 in the presence of DLPC and DOPC. The λ_{max} of intrinsic fluorescence for P450 17A1 did not change during their interaction with phospholipid vesicles, but the spectra flattened in the presence of 18:2 PC and 20:4 PC. The double bonds in these fatty acid chains hinder the free rotation about C-C bonds and cause a kink in the chain, which prevents the molecules from packing closely together. The lower ratios of F/F_0 obtained with these two polyunsaturated PCs also correlate with lower turnover rates for both P450 17A1-catalyzed reactions. The fluorescence emission λ_{max} of P450 11B2 was 340 nm, indicative of more hydrophilic tryptophan environment than that of P450 17A1, where a blue shift in λ_{max} (312 nm) was observed. The intrinsic fluorescence of mitochondrial P450 11B2 is relatively insensitive to PC acyl chain length or degree of unsaturation. Aside from 20:4 PC, we found no significant difference in F/F_0 ratios with P450 11B2 in the presence of various PCs, which is consistent with the similar 11 β -hydroxylation rates with these phospholipids (Figure 5).

The physical state of the phospholipids is also an important parameter in determining the activity of most membrane-bound enzymes, and a linear correlation between activity and membrane fluidity is commonly found (44). When the surrounding lipids are in the gel phase at the assay temperature, most enzymes become inactive or at least show a dramatic loss of activity. The gel-fluid transition temperatures (T_c) of PCs used in the present study are listed in Table 1. Lipids with long acyl chains undergo this gel-to-liquid transition at a higher temperature, while unsaturation causes a significant decrease in T_c . Phospholipid species with high T_c such as 16:0 PC and 18:0 PC are more rigid and less mobile than other phospholipids, which might explain why the 17-hydroxylase and 17,20-lyase activities of P450 17A1 when reconstituted with these phospholipids is not higher, despite high F/F_0 ratios. Consistent with studies of P450 24A1 (45), we have also observed a decrease in catalytic activities of P450 17A1 with increasing phospholipid (Fig. 1A), because the effective concentration of substrate in the vesicle decreases as phospholipid is increased; however, we did not find such correlation with P450 11B2 (Fig. 4A).

Prior studies have demonstrated that the F-G loop dominates the membrane binding of mitochondrial P450s (16, 17, 46), and mutations in the F-G loop of P450 11A1 and P450 27A1 all reduce the association of these proteins with intracellular membranes (17, 46). Computational studies orient the substrate-access channel of P450s in the hydrophobic domain of the membrane surface, in which lipophilic substrate partition (47–49). Furthermore, molecular dynamics simulations of membrane-bound P450 17A1 demonstrate that the rearrangement of the F-G region is coupled to the ingress of substrates from the membrane compartment via these dynamic domains embedded in the lipid layer (50). This analysis suggests that the interaction of the F-G loop with the membrane not only supports P450 stability (51) and activity but regulates substrate trajectories, which include the binding or dissociation of intermediates. For P450 11B2, intermediates are poor substrates (32, 52), and processivity from DOC is required for aldosterone biosynthesis. We interpret the poor processivity to aldosterone and variable 18-hydroxycorticosterone formation in our reconstituted system as partitioning between product release and subsequent turnovers, which is highly dependent on phospholipid composition. The strong association of the membrane with the F-G loop of P450 11B2, therefore, might be a driving force for the processivity required for aldosterone biosynthesis. The critical lipid components to maximize aldosterone production for purified P450 11B2 remain elusive.

For microsomal P450 17A1, our acrylamide quenching experiments suggest that at least one tryptophan residue, most probably W220 in the F-G loop, is inaccessible when embedded in PE liposomes. Furthermore, the relatively large quench constants obtained with P450 17A1 ($\sim 12 \text{ M}^{-1}$) compared to the typical quench constants of P450 11B2 ($\sim 2 \text{ M}^{-1}$, Table 2) and P450 11A1 (16) suggests that the P450 17A1 protein structure is more flexible and/or less deeply embedded in the phospholipid vesicle than mitochondrial P450s, which allows high solvent access and fluorescence quenching (53). We interpret these results with some caution, as the phospholipid interactions of reconstituted P450 17A1 might not precisely match the native protein in the microsomal membrane, particularly for the N-terminus (16). Nevertheless, the P450 17A1 activities and substrate-induced spectral responses remain dependent on the specific phospholipid composition, favoring 12:0 and 18:1 PC and PE. In particular, PE significantly increases 17,20-lyase activity, even when 17Preg is provided as

substrate and DHEA formation is not processive from pregnenolone. Thus, the phospholipid regulation of mitochondrial P450 11B2 and microsomal P450 17A1 share some similarities yet feature glaring contrasts, reflective of their different biophysical properties and catalytic mechanisms.

Lipid-P450 interactions have been suggested to maintain the active conformation of the protein (54), to affect the binding of P450 to POR for efficient electron transfer (55), and to facilitate the binding of hydrophobic substrates (56). Moreover, the membrane environment can also modify the redox potentials of membrane proteins, generally a shift to more positive values (57). Furthermore, the interactions between P450, substrate, and phospholipids might not only change the activity of an enzyme but also its processivity, possibly due to changes in access channel dynamics and substrate trajectories, which regulate the partitioning between dissociation and retention for another catalytic cycle. Several questions remain, concerning the mechanisms of how some lipids better support enzyme activities and how intramolecular interactions between PC and PE or CL influence enzyme processivity. These questions are particularly relevant to several multi-step P450s, which operate at key junctures of human steroidogenesis.

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Abbreviations

CYP17A1	cytochrome P450 17A1
CYP11B1	cytochrome P450 11B1
CYP11B2	cytochrome P450 11B2
POR	cytochrome P450-oxidoreductase
12:0 PC (DLPC)	1,2-dioleoyl-sn-glycero-3-phosphocholine
14:0 PC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
16:0 PC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
18:0 PC	1,2-distearoyl-sn-glycero-phosphocholine
18:1 PC (DOPC)	1,2-dioleoyl-sn-glycero-3-phosphocholine
18:2 PC	1,2-dilinoleoyl-sn-glycero-3-phosphocholine
20:4 PC	1,2-diarachidonoyl-sn-glycero-3-phosphocholine
16:0–18:1 PC (POPC)	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
CL	cardiolipin

PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
HPLC	high-performance liquid chromatography
IPTG	isopropyl- β -D-thiogalactopyranoside
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
UV	ultraviolet light.
SUVs	small unilamellar vesicles
LMVs	large multilamellar vesicles
ER	endoplasmic reticulum
DOC	11-deoxycorticosterone
T_c	gel liquid transition temperature

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Highlights

- The activity of human P450 17A1 is highest in DLPC and DOPC
- Addition of PE specifically enhances the 17,20-lyase activity of P450 17A1
- The percent high-spin P450 17A1 in various phospholipids correlates with activity
- The aldosterone synthesis of human P450 11B2 is highest in DOPC
- The activity of human P450 11B1 is highest in DLPC

Figure 1.

Effect of phospholipids on P450 17A1 activities. (A) The 17- and 16 α -hydroxylase and 17,20-lyase activities of the purified P450 17A1 as a function of the DLPC/P450 ratio from 50:1 to 1000:1. Incubations were carried out with 10 μ M progesterone for 17- and 16 α -hydroxylase and 20 μ M 17Preg for 17,20-lyase activity, and the products were quantitated by HPLC analysis. All values are means of duplicate experiments. (B) Hydroxylase activities of P450 17A1 in the presence of 6 different PCs, mix of PC:PE at 1:1, or PC:PE:PI at 73.5:24.5:2. Purified P450 17A1 (10 pmol) and POR were reconstituted with P450:POR:phospholipid at a molar ratio of 1:1:100. Incubation was carried out with 10 μ M progesterone, and the 17- and 16 α -hydroxylated products were quantitated by HPLC analysis. (C) The 17,20-lyase activity of P450 17A1 in the presence of various lipids as in

(B). Incubations contained a 1:1:1:100 molar ratio of P450:POR:cytochrome *b₅*:phospholipid and 20 μ M 17Preg, and the product dehydroepiandrosterone was further converted to androstenedione as described in Materials and Methods. Error bars indicate mean $\pm$ standard deviation of triplicate assays.

Figure 2.

Phospholipid dependence of the intrinsic fluorescence spectra of P450 17A1. (A) Intrinsic fluorescence spectra of P450 17A1 (1 μM) in 50 mM potassium phosphate buffer (pH 7.4) alone and in the presence of PC (100 μM) with various acyl chains. (B) Intrinsic fluorescence spectra of P450 17A1 (1 μM) alone and in the presence of 100 μM PE (18:0/18:1), or PI from bovine liver, which contains 18:0 (46%), 20:4 (17%), and 20:3 (13%). (C) Acrylamide quenching of P450 17A1 intrinsic fluorescence in the absence and presence of various phospholipids. All values are means of duplicate experiments, and data were analyzed using a Stern-Volmer plot. (D) Lehrer plot shows change in tryptophan fluorescence versus the inverse of acrylamide concentration. The intercept on the F_0/F axis gives the inverse of the fraction of accessible tryptophan residues for P450 17A1 in the absence or presence of various phospholipids.

Figure 3.

(A–H) The absorption spectra of P450 17A1 in 50 mM potassium phosphate buffer (pH 7.4) in the absence or presence of pregnenolone (Preg) with indicated phospholipid (all at 25 °C). The final concentration of the phospholipid was 100 μ M. Spectra are representative of duplicate experiments.

Figure 4.

Effect of DOPC on P450 11B2 and P450 11B1 activities. (A) The activity of purified P450 11B2 as a function of the DOPC/P450 molar ratio from 0:1 to 600:1. Incubations were carried out with P450:AdR:Adx at 1:1:60 molar ratios and 200 μ M DOC. All values are means of duplicate experiments. (B) The 11 β -, 18-hydroxylase and 18-oxidase of P450 11B2 in the presence of 4 different phospholipids when incubated with 200 μ M DOC. Results are shown as % products after complete substrate consumption, and error bars indicate mean $\pm$ standard deviation of triplicate assays. (C) The 11 β - and 18-hydroxylase of P450 11B1 in the presence of 4 different phospholipids when incubated with 200 μ M DOC as described in Materials and Methods.

Figure 5.

Stimulation of DOPC on P450 11B2 activities. (A) 11 β -, 18-hydroxylase and 18-oxidase of P450 11B2 in the presence of 6 different PCs when incubated with 200 μ M DOC. (B) 11 β -, 18-hydroxylase and 18-oxidase of P450 11B2 in the presence of DLPC, DOPC, CL, PE, mix of PC:CL at 1:1, or PC:PE:CL at 2:2:1 when incubated with 200 μ M DOC. Results are shown as % products after complete substrate consumption, and error bars indicate mean $\pm$ standard deviation of triplicate assays.

Figure 6.

Influence of phospholipid on the intrinsic fluorescence spectra and substrate binding spectra of P450 11B2. (A) Intrinsic fluorescence spectra of P450 11B2 (1 μ M) in 50 mM potassium phosphate buffer (pH 7.4) alone and in the presence of 200 μ M PC with various acyl chains. (B) Intrinsic fluorescence spectra of P450 11B2 (1 μ M) alone and in the presence of 200 μ M PE (18:0/18:1), or cardiolipin from bovine heart, which contains 18:2 (90%), and 18:1 (5%). (C) Acrylamide quenching of P450 11B2 intrinsic fluorescence in the absence and presence of various phospholipids. All values are means of duplicate experiments, and data were

analyzed using a Stern-Volmer plot. (D) Lehrer plot shows change in tryptophan fluorescence versus the inverse of acrylamide concentration. The intercept on the F_0/F axis gives the inverse of the fraction of accessible tryptophan residues for P450 11B2 in the absence or presence of various phospholipids. (E–H) The absorption spectra of P450 11B2 in 50 mM potassium phosphate buffer (pH 7.4) in the absence or presence of DOC with indicated phospholipids at a final concentration of 200 μ M. All spectra were recorded at 25 °C and are representative of duplicate experiments.

Table 1

Influence of different phospholipids on the intrinsic fluorescence intensity of P450s

Experimental Conditions [*]	Transition Temperature (°C)	P450 17A1 F/F ₀	P450 11B2 F/F ₀
P450		1.00	1.00
+ 12:0 PC	−1	1.14	1.05
+ 16:0 PC	41	1.10	0.98
+ 18:0 PC	55	1.08	1.00
+ 18:1 PC	1	1.10	1.07
+ 18:2 PC	−53	0.72	0.91
+ 20:4 PC	−69	0.58	0.73
+ PE ^{**}		1.04	1.04
+ PI ^{**}		0.92	n.d.
+ CL ^{**}		n.d. ^{***}	0.99

^{*} F/F₀ represents the fluorescence intensity ratio at 312 nm for P450 17A1 and 340 nm for P450 11B2 with (F) and without (F₀) phospholipid incorporated. P450 (1 μM) was mixed with phospholipid vesicles (100 μM) in 50 mM potassium phosphate (pH 7.4). After incubation of the sample for 10 min at 25 °C, fluorescence was measured at 290–430 nm after excitation 280 nm.

^{**} Fatty acid compositions: PE contains 18:0/18:1 (>99%); PI contains 18:0 (46%), 20:4 (17%), 20:3 (13%), 18:1 (8%), and 18:2 (6%); and CL contains 18:2 (90%) and 18:1 (5%).

^{***} n.d., not determined.

Table 2

Stern-Volmer quenching constants and accessible tryptophan for P450 17A1 and 11B2 in the absence and presence of various phospholipids

Lipid**	P450 17A1*		P450 11B2*	
	Quench Constant (K_{sv})	Accessible Tryptophan	Quench Constant (K_{sv})	Accessible Tryptophan
None	12.44 ± 0.39	7.6	2.06 ± 0.11	5.2
12:0 PC	12.59 ± 0.34	7.6	3.22 ± 0.11	6.0
16:0 PC	11.49 ± 0.35	8.0	2.46 ± 0.15	4.4
18:0 PC	13.82 ± 0.67	7.4	2.49 ± 0.13	4.8
18:1 PC	8.43 ± 0.46	7.4	2.15 ± 0.13	4.9
18:2 PC	11.19 ± 0.46	7.3	2.27 ± 0.20	4.3
20:4 PC	12.87 ± 0.32	8.0	1.85 ± 0.23	2.8
PE	6.42 ± 0.46	6.7	1.97 ± 0.11	5.1
PI	8.33 ± 0.35	7.1	n.d.	n.d.
CL	n.d.***	n.d.	1.90 ± 0.11	5.0

* Aqueous or vesicle-associated P450s (1 μ M) in the presence of increasing amount of acrylamide as described in the Materials and Methods, and fluorescence was measured with excitation at 280 nm and emission wavelengths of 312 nm for P450 17A1 and 340 nm for P450 11B2.

** Fatty acid compositions: PE contains 18:0/18:1 (>99%); PI contains 18:0 (46%), 20:4 (17%), 20:3 (13%), 18:1 (8%), and 18:2 (6%); and CL contains 18:2 (90%) and 18:1 (5%).

*** not determined.

Table 3

Influence of different phospholipids on the interaction of substrate with P450s

Experimental Conditions*	P450 17A1/Preg (390 nm–420 nm)/420 nm %	P450 11B2/DOC (390 nm–420 nm)/420 nm %
No lipid	43 ± 2	53 ± 2
+ 12:0 PC	70 ± 5	70 ± 2
+ 16:0 PC	52 ± 2	89 ± 2
+ 18:0 PC	43 ± 2	94 ± 5
+ 18:1 PC	63 ± 2	86 ± 2
+ 18:2 PC	36 ± 2	67 ± 6
+ 20:4 PC	49 ± 2	72 ± 3
+ PE**	54 ± 5	113 ± 4
+ PI**	32 ± 2	n.d.
+ CL**	n.d.***	46 ± 3

* P450 (1 μM) was mixed with phospholipid vesicles in the absence or presence of substrate in 50 mM potassium phosphate (pH 7.4). The increase in the percentage high-spin iron fraction was determined from the differences of absorption between the two bands at 390 and 420 nm.

** Fatty acid compositions: PE contains 18:0/18:1 (>99%); PI contains 18:0 (46%), 20:4 (17%), 20:3 (13%), 18:1 (8%), and 18:2 (6%); and CL contains 18:2 (90%) and 18:1 (5%).

*** n.d., not determined.