Phospholipids modify substrate binding and enzyme activity of human cytochrome P450 27A1

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Abstract **Cytochrome P450 27A1 (P450 27A1) is an important metabolic enzyme involved in bile acid biosynthesis and the activation of vitamin D_3 in mammals. Recombinant P450 27A1 heterologously expressed in *Escherichia coli* was found to be copurified with phospholipids (PLs). The PL content varied in different preparations and was dependent on the purification protocol. A link between the increased amounts of PLs and deterioration of the enzyme substrate binding properties was also observed. Tandem negative ionization mass spectrometry identified phosphatidylglycerol (PG) as the major PL copurified with P450 27A1. Subsequent reconstitution of P450 into exogenous PG vesicles assessed the effect of this contamination on substrate binding and enzyme activity. Two other PLs, phosphatidylethanolamine (PE) and phosphatidylserine (PS), were also tested. PG and PE increased the K_d for 5β-cholestane-3α,7α,12α-triol and cholesterol binding, whereas PS had no effect on either substrate binding. PG and PE did not significantly alter 5β-cholestane-3α,7α,12α-triol hydroxylase activity and even stimulated cholesterol hydroxylase activity. PS inhibited 5β-cholestane-3α,7α,12α-triol hydrolyase activity and had no effect on cholesterol hydroxylase activity. Our study shows the potential for PLs to regulate the activity of P450 27A1 in vivo and alter the amount of cholesterol degraded through the "classical" and "alternative" bile acid biosynthetic pathways.—** Murtazina, D. A., U. Andersson, I-S. Hahn, I. Bjorkhem, G. A. S. Ansari, and I. A. Pikuleva. **Phospholipids modify substrate binding and enzyme activity of human cytochrome P450 27A1. *J. Lipid Res.* 2004.** 45: **2345–2353.**

Supplementary key words cytochrome P450 27A1 • heterologous expression • *Escherichia coli* • bile acid biosynthesis

Cytochromes P450 (P450s) comprise a superfamily of heme proteins that generally carry out monooxygenation reactions. In mammals, P450s metabolize a wide range of both endogenous and exogenous compounds. Endogenous substrates include steroids, fatty acids, prostaglandins, leukotrienes, bile acids, and biogenic amines, whereas exogenous substrates include environmental pollutants and pharmaceuticals. Although much has been learned from studies of relatively abundant P450s, such as P450cam from *Pseudomonas putida*, P450s 1A2 and 2B4 from rabbit liver microsomes, and P450 11A1 from bovine adrenal cortex mitochondria, the development of heterologous expression systems (1–3), especially in *Escherichia coli* (4), has provided an opportunity to characterize less abundant forms of P450s. There is broad interest in recombinant P450s because these enzymes have potential uses in the synthesis and discovery of drugs; they also can serve as biosensors and can be used in bioremediation (5). P450 27A1 (P450 27A1 or CYP27A1) is a multifunctional enzyme that catalyzes hydroxylations of cholesterol, bile acid precursors, and vitamin D_3 in mammals (6–8). Initial characterization of this P450 was carried out using preparations purified from rabbit and pig liver (6, 8, 9). Since cDNAs encoding rabbit, rat, and human enzymes were isolated (10–12) and an efficient *E. coli* expression system was developed for the human isoform (13), the majority of the structure/function research has been carried out using the heterologously expressed enzyme. Recombinant human P450 27A1 is routinely expressed in this laboratory at a 600–800 nmol/l culture level and purified to homogeneity using a three-step purification procedure (14, 15). The present investigation was initiated in an effort to understand why substrate binding properties of partially purified recombinant P450 27A1 vary from preparation to preparation. We established that contamination with *E. coli* phospholipids (PLs) is the likely reason for interpreparation variability of the enzyme substrate binding con-

Abbreviations: CAD, collisionally activated dissociation; P450 27A1, cytochrome P450 27A1; ES-MS/MS, electrospray ionization tandem mass spectrometry; MW, molecular weight; PBS, potassium phosphate buffer; PE, phosphatidylethanolamine; PL, phospholipid; PG, phosphatidylglycerol; PS, phosphatidylserine; WT, wild type.

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stants. We then tested how different PL species, added exogenously, affect substrate binding and the activity of P450 27A1. This part of the work provided insight into how "classical" and "alternative" pathways for cholesterol metabolism in mammals could be regulated in vivo.

MATERIALS AND METHODS

Materials

All bacterial culture media were purchased from Becton Dickinson Microbiology Systems (Sparks, MI). Restriction enzymes were from New England Biolabs (Beverly, MA), and *E. coli* strain DH5αF'IQ was from Invitrogen (Carlsbad, CA). δ-Aminolevulinic acid, ammonium thiocyanate, cholesterol, 2-hydroxypropyl-β-cyclodextrin, octyl-Sepharose CL-4B, and diacyl-*sn*-glycero-3-phosphoethanolamine were from Sigma-Aldrich (St. Louis, MO). Isopropyl-β-D-thiogalactopyranoside was from Research Products International Corp. (Mount Prospect, IL). Hydroxyapatite was from Bio-Rad (Richmond, CA); DE-52 cellulose was from Whatman, Inc. (Clifton, NJ); and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine] were from Avanti Polar Lipids (Alabaster, AL). Chloroform and iron (III) chloride hexahydrate were from Fisher (Fair Lawn, NJ); methanol was from EMD (Gibbstown, NJ); [^{3}H]cholesterol was from Amersham Bioscience (Piscataway, NJ); and cold and ^{3}H-labeled 5β-cholestane-3α,7α,12α-triol were synthesized as described (16). All chemicals were of American Chemical Society grade, and all organic solvents were of HPLC grade.

Expression in *E. coli* and partial purification

Wild-type (WT) and mutant P450 27A1 were transformed into *E. coli* strain DH5αF'IQ and expressed (13, 14). Initially, partial purification of P450 27A1 included solubilization from the *E. coli* membranes with 0.8% sodium cholate followed by chromatography steps on octyl-Sepharose and hydroxyapatite columns and overnight dialysis against 100 volumes of 40 mM potassium phosphate buffer (PBS), pH 7.4, containing 20% glycerol and 1 mM EDTA (13). Later, after we discovered that significant amounts of PLs (up to 745 μg/nmol P450) are still present in preparations of WT and mutant P450s, we introduced the additional purification step, chromatography on DEAE cellulose. NaCl and sodium cholate were added to the enzyme to a final concentration of 50 mM and 0.5%, respectively, and the solution was applied to a DEAE-cellulose column equilibrated with 50 mM PBS, pH 7.4, containing 20% glycerol, 50 mM NaCl, 0.5% Na cholate, and 1 mM EDTA. A flow-through fraction was collected and dialyzed as described above to yield enzyme preparation.

Electrospray ionization tandem mass spectrometry

Electrospray ionization tandem mass spectrometry (ES-MS/MS) analyses of the chloroform-methanol extracts of P450 preparations were performed on a Q-TOF 2™ mass spectrometer with a Z-spray™ electrospray source (Micromass, Manchester, UK) using MassLynx version 3.5 software. Analytes were dissolved in acetonitrile and water (1:1, v/v) at a final concentration of 100 μg/ml. The solutions were introduced into the ES source through an electrospray capillary using a model 11 syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 1 μl/min. The ES source potentials in electrospray-negative mode (ES−) were 3.2 kV for capillary, 18 V for cone, and 1 V for extractor. The quadrupole mass filter to the TOF analyzer was set with low mass (LM) and high mass (HM) resolution of 5.0 (arbitrary number), which is equivalent to a 1.0 Da mass window for transmission of precursor ions. The source block and desolvation temperatures were set at 50°C. All ES-MS spectra were obtained by scanning the TOF analyzer. Collisionally activated dissociation (CAD) of selected mass ions for ES-MS/MS experiments was performed in a radio frequency (RF)-only quadrupole collision cell pressurized with Ar (40 pounds per square inch). The collision energy for CAD spectra (MS/MS) was set at 35 eV. The optimum collision energy of 35 eV was chosen after MS/MS product ion scans on $[M-H]^-$ over a range of energies between 10 and 40 eV. Ion beam detection was performed with a reflector, multichannel plate detector, and time-to-digital converter. Mass calibration was carried out with a standard polyaniline solution from *m/z* 150 to 2,000. The acquisition mass range was *m/z* 50 to 1,000, with a scan duration of 1.0 s and an interscan time of 0.1 s. Single analyzer profiles for ES-MS spectra were accumulated over a period of at least 60 s. For CAD experiments, mass spectra were accumulated over at least 15 min. The calculated monoisotopic mass/charge ratio of the precursor ion was set as the lock mass in each product ion mass spectrum.

Quantitative determination of PLs

PL contents in preparations of WT and mutant P450 27A1 were estimated as described (17). Lipids were extracted with chloroform-methanol using the Bligh and Dyer method (18). Typically, 0.8 ml of 8 μM P450 in 40 mM PBS, pH 7.4, containing 20% glycerol and 1 mM EDTA, was mixed with 2 ml of methanol, vortexed for 10 s, and then 1 ml of chloroform was added, followed by a 30 s vortexing and addition of 1 ml each of water and chloroform. After vortexing for another 30 s and centrifugation at 2,000 *g* for 10 min, the lower organic phase was removed, placed in a glass tube, and evaporated to dryness under nitrogen. The dried extract was redissolved in 2 ml of chloroform and treated with 1 ml of thiocyanate reagent (obtained by dissolving 27 g of $FeCl_3 \cdot 6H_2O$ and 30 g of NH_4SCN in 1 liter of water). After vortexing for 1 min and centrifugation at 2,000 *g* for 10 min, the red lower phase was removed and its optical density was measured at 488 nm and compared with that of the calibration curve obtained using known amounts of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-1(1-glycerol)].

Substrate binding assays

The assays were performed in 1 ml of 50 mM PBS, pH 7.4, containing 20% glycerol as described (19). WT and mutant P450 27A1 (0.3 μM) were titrated with 5β-cholestane-3α,7α,12α-triol dissolved in ethanol and cholesterol dissolved in 45% aqueous 2-hydroxypropyl-β-cyclodextrin. After each experiment, the P450 content was quantified by the reduced CO-difference spectrum (20) to confirm that there was no enzyme denaturation during titration. Apparent binding constants (K_d) were calculated by fitting data into the quadratic equation

$$\Delta A = 0.5\Delta A_{max}(K_d + [E] + [S] - \sqrt{(K_d + [E] + [S])^2 - 4[E][S]}) \quad \textit{(Eq. 1)}$$

using a nonlinear regression of Graph-Pad Prism software, where ΔA is a spectral change at different substrate concentrations [S], ΔA_{max} is the maximal amplitude of the spectral response, and [E] is the enzyme concentration. The mode of PL inhibition of substrate binding was determined from the analysis of the intersection of double-reciprocal lines (Lineweaver-Burk plots) of the substrate-induced spectral change versus the concentration of free substrate at several PL concentrations. The concentration of free substrate was calculated using the following equation: $[substrate]_{free} = [substrate]_{total} - (\Delta A/\Delta A_{max})[P450]$. The K_i values were determined from the *x* intercept of a plot of the slope of the double-reciprocal lines as a function of PL.

Enzyme assays

Turnover numbers for 5β-cholestane-3α,7α,12α-triol and cholesterol were determined in a reconstituted system as described (13, 19). Cholesterol 27-hydroxylase activity was assayed in 1 ml of 40 mM PBS containing 1 mM EDTA, 5% glycerol, 0.08 μM P450, 1.0 μM adrenodoxin reductase, 4.0 μM adrenodoxin, 30 μM cholesterol, [^{3}H]cholesterol (250,000 cpm), and 1 mM NADPH. The reaction time was 4 min. When 5β-cholestane-3α,7α,12α-triol was used as the substrate, the reconstituted system contained 0.014 μM P450, 0.07 μM adrenodoxin reductase, 5.0 μM adrenodoxin, 1% glycerol, 30 μM 5β-cholestane-3α,7α,12α-triol, 5β-[^{3}H]cholestane-3α,7α,12α-triol (250,000 cpm), and 1 mM NADPH, and the reaction time was 3 min. Enzymatic assays were carried out at 37°C and terminated by the addition of 2 ml of CH_2Cl_2. The CH_2Cl_2 extracts were removed, evaporated, dissolved in methanol, and analyzed by HPLC as described earlier (13).

Reconstitution with exogenous PLs

Each of the PLs tested, phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE), was suspended in 50 mM PBS, pH 7.4, containing 20% glycerol at either 0.1 mg/ml concentration (PG and PS) or 0.5 mg/ml (PE) and sonicated for 10 min at 25°C with a Branson sonifier at a setting of 2. The clarified suspension was used as a stock solution for reconstitution with 0.3 μM P450 27A1 in 2.5 ml of 50 mM PBS, pH 7.4, containing 20% glycerol. PL was added first to the buffer and the buffer was sonicated again for 10 s, then P450 was added followed by a 10 min incubation at 37°C and an overnight incubation at 4°C. The next morning, an aliquot was taken for enzyme activity measurements and the rest of the solution was used for spectral binding studies.

RESULTS

Substrate-binding properties of WT and mutant P450 27A1

Table 1 summarizes apparent binding constants for 5β-cholestane-3α,7α,12α-triol and cholesterol of four distinct WT P450 27A1 preparations, two preparations of the T110S mutant, and two preparations of the V482L mutant. All enzymes were obtained during the course of our ongoing studies aimed at identification of the substrate-contact residues in P450 27A1. WT-1, WT-3, WT-4, V482L-1a, and T110S-1a were purified using octyl-Sepharose and hydroxyapatite columns, whereas the isolation of WT-2, T110S-1b, and V482L-1b included an additional chromatography step on a DEAE-cellulose column. Four WT preparations were expressed and purified independently, whereas T110S-1a and T110S-1b and V482L-1a and V482L-1b represent the same preparation without (1a) and with (1b) additional chromatography on DEAE-cellulose. Table 1 also includes the P450 spectral purity indices (A_{418}/A_{280}) to evaluate contamination with *E. coli* proteins (a value of 1.27 corresponds to a homogeneous P450 27A1 as analyzed by SDS-PAGE) and data on PL content because PLs are known to affect substrate binding and catalytic properties of membrane-bound P450s (reviewed in Ref. 21). Of the three parameters used to characterize the enzyme, substrate binding, spectral purity index, and PL content, only the first and last appeared to be linked. WT-1 and WT-2, which had the same PL content but different spectral purity indices, had essentially the same apparent binding constants. WT-3, which contained more PLs than WT-1 and WT-2 but had the same purity index as WT-2, had an unchanged K_d for 5β-cholestane-3α,7α,12α-triol and a 2.5- to 3.1-fold increased K_d for cholesterol. More significant, 5.7- and 23-fold decreases in affinity for 5β-cholestane-3α,7α,12α-triol and cholesterol, respectively, were observed for WT-4 that contained 5.7 μg PL/nmol P450. The T110S-1a that contained 25 times more PLs than T110S-1b did not exhibit a spectral response upon titration with cholesterol and had a 9-fold higher binding constant for 5β-cholestane-3α,7α,12α-triol. Data from Table 1 suggest that PL content should not exceed 1.8 μg/nmol P450 to obtain preparations with similar substrate binding properties. Our studies also indicate that chromatography on DEAE-cellulose is an efficient method to remove contaminating PLs, as indicated by a significantly lower PL content of V482L-1b and T110S-1b compared with that of V482L-1a and T110S-1a.

ES-MS/MS analysis of chloroform-methanol extracts from partially purified preparations of P450 27A1

ES-MS/MS was used to identify PL species that were copurified with P450 27A1. Initially, lipid extracts from the two P450 27A1 mutants, V482L-1a and T110S-1a, and two WT enzymes (WT-1 and WT-2) that had the lowest ap-

TABLE 1. Preparations of cytochrome P450 27A1 WT and mutants used in the present study

	5β-Cholestane-3α,7α,12α-Triol		Cholesterol				
Cytochrome P450	K_d[a]	ΔA_{max}[b]	K_d	ΔA_{max}	A_{418}/A_{280}[c]	Phospholipid Content	DEAE-Cellulose[d]
	μM		*μM*			*μg/nmol P450*	
WT-1	0.026 ± 0.005	0.053 ± 0.004	0.017 ± 0.006	0.018 ± 0.005	0.93	1.8	−
WT-2	0.036 ± 0.009	0.057 ± 0.004	0.014 ± 0.001	0.022 ± 0.001	0.82	1.8	+
WT-3	0.034 ± 0.005	0.045 ± 0.008	0.043 ± 0.013	0.019 ± 0.004	0.82	2.8	−
WT-4	0.15 ± 0.02	0.045 ± 0.010	0.32 ± 0.08	0.01 ± 0.002	0.72	5.7	−
V482L-1a	34.2 ± 4.5	0.043 ± 0.003	ND	ND	0.29	485	−
V482L-1b	ND	ND	ND	ND	0.31	42	+
T110S-1a	0.67 ± 0.01	0.032 ± 0.013	No spectral response	No spectral response	0.75	65	−
T110S-1b	0.067 ± 0.001	0.046 ± 0.009	0.084 ± 0.022	0.013 ± 0.003	1.0	2.6	+

The results shown are means ± SD of three to five measurements. ND, not determined; WT, wild-type.
[a] Assessed by spectral binding as described in Materials and Methods.
[b] Maximal amplitude of the substrate-induced spectral response per nanomole of P450.
[c] Spectral purity index.
[d] Indicates whether chromatography on DEAE-cellulose was a part of the purification procedure.

parent binding constants among the four WT preparations, were analyzed. Negative ion ES-MS spectra of V482L-1a and T110S-1a showed three prominent peaks at *m/z* 720, 734, and 748, which were at the noise level in the spectra of the WT P450s (**Fig. 1**). Each of the three precursor ions was then mass-selected for the MS/MS (CAD) analysis. Because the fragmentation patterns were very similar for both mutants, only those for the V482L mutant are shown in **Fig. 2**. Peaks at *m/z* 720, 734, and 748 were identified as diacylglycerophospholipids because a common fragment at *m/z* 153 [glycerophosphate-H_2O]$^-$ was seen in all three spectra in Fig. 2. These spectra also contained a common fragment at *m/z* 171, suggesting that glycerol is the polar head group in these lipids. Peaks diagnostic for PS (*m/z* 86), PE (*m/z* 140 and 196), sphingomyelin (*m/z* 168), phosphatidylcholine (*m/z* 184), and phosphatidylinositol (*m/z* 241) (22, 23) are not seen in Fig. 2. Ions at *m/z* 253, 255, 267, and 281 likely correspond to fatty acids 16:0, 16:1, 17:1 or 17:0 (cyclopropane), and 18:1, respectively. Thus, ES-MS/MS indicates that the three major lipids that contaminate partially purified preparations of P450 27A1 are PGs containing different fatty acids.

The same prominent peaks at *m/z* 720, 734, and 748 that were seen in the mass spectrum of V482L-1a (Fig. 1) were also present in the mass spectrum of V482L-1b (**Fig. 3**) that still contained significant amounts of PLs after chromatography on DEAE-cellulose (42 μg PLs/nmol P450). The peaks at *m/z* 720, 734, and 748 were absent in T110S-1b (Fig. 3) that contained only 2.6 μg PLs/nmol P450 after chromatography on DEAE-cellulose. Taken together, data on WT-1, WT-2, and T110S-1b indicate that only insignificant amounts of PGs are present in the P450 preparations with low PL contents, and these PLs are likely the low molecular weight (MW) contaminants that are responsible for the deterioration of the enzyme substrate binding properties. Thus, testing whether recombinant P450 is contaminated with *E. coli* lipids is useful to avoid subsequent problems associated with interpreparation variability. If PLs are found in the preparations of P450, chromatography on DEAE-cellulose is an efficient method to reduce the PL content and obtain enzymes with consistent properties.

Effect of exogenous PLs on substrate binding and catalytic properties of P450 27A1

To gain insight into how contamination with *E. coli* PLs affects the properties of recombinant P450 27A1, WT-2

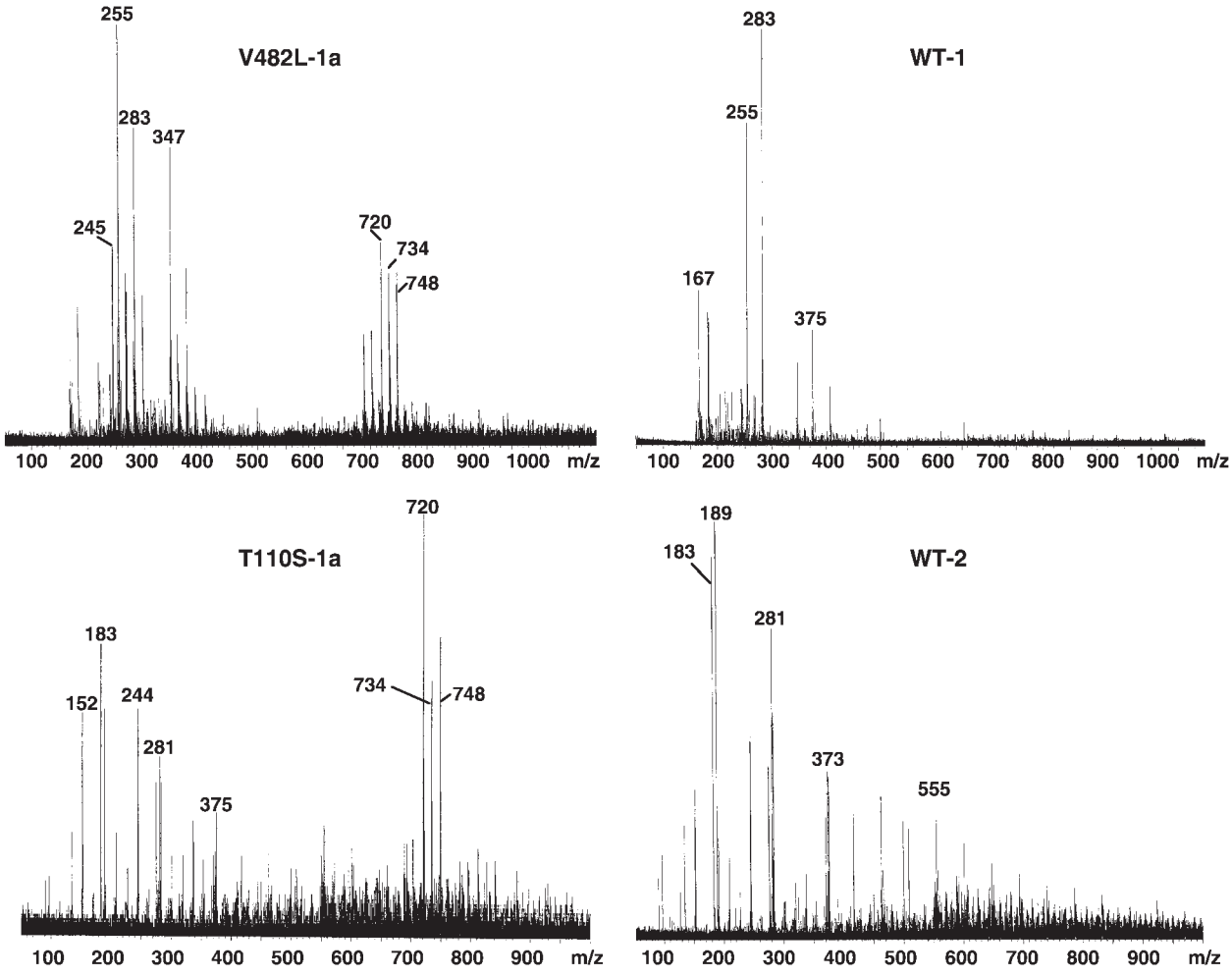

Fig. 1. Negative ion mass spectra [electrospray ionization tandem mass spectrometry (ES-MS/MS)] of chloroform-methanol extracts from different preparations of cytochrome P450 27A1 (P450 27A1) wild type (WT) and mutants.

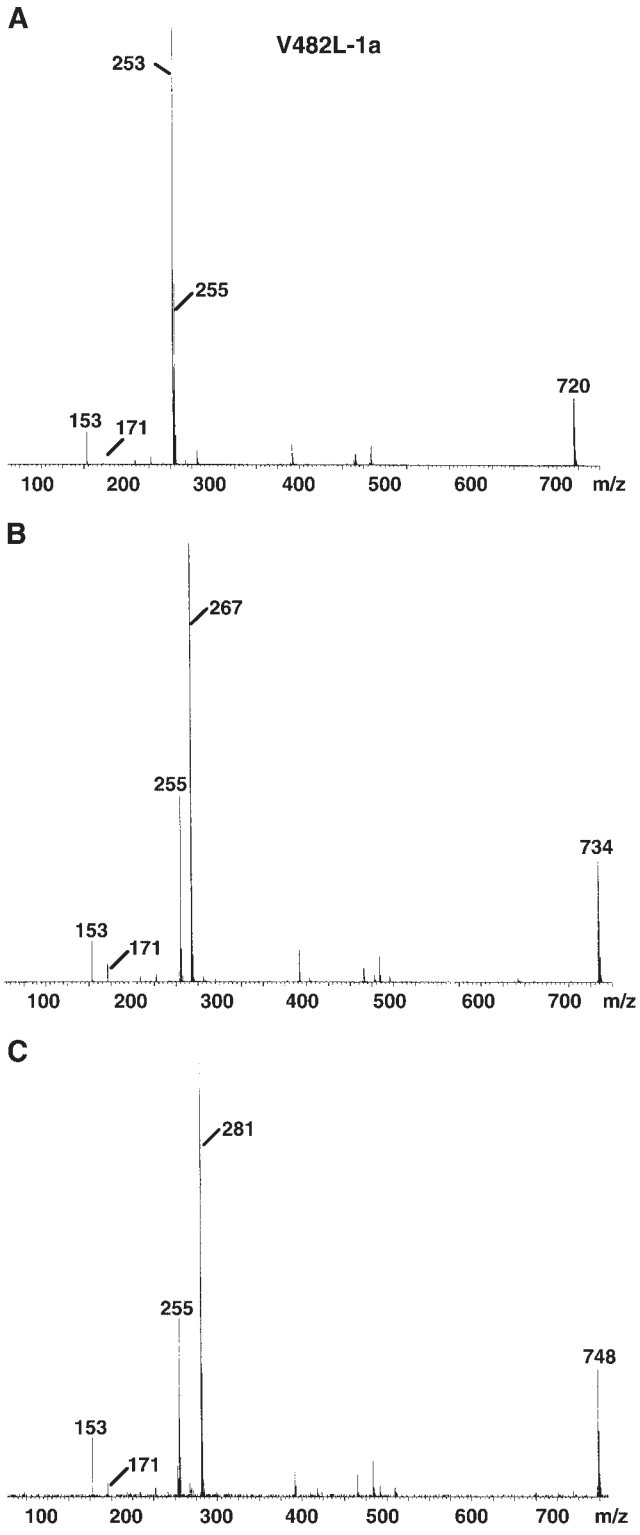

Fig. 2. Negative ion tandem mass spectra (ES-MS/MS) of the precursor ions at *m/z* 720 (A), 734 (B), and 748 (C) of the V482L-1a mutant from Fig. 1.

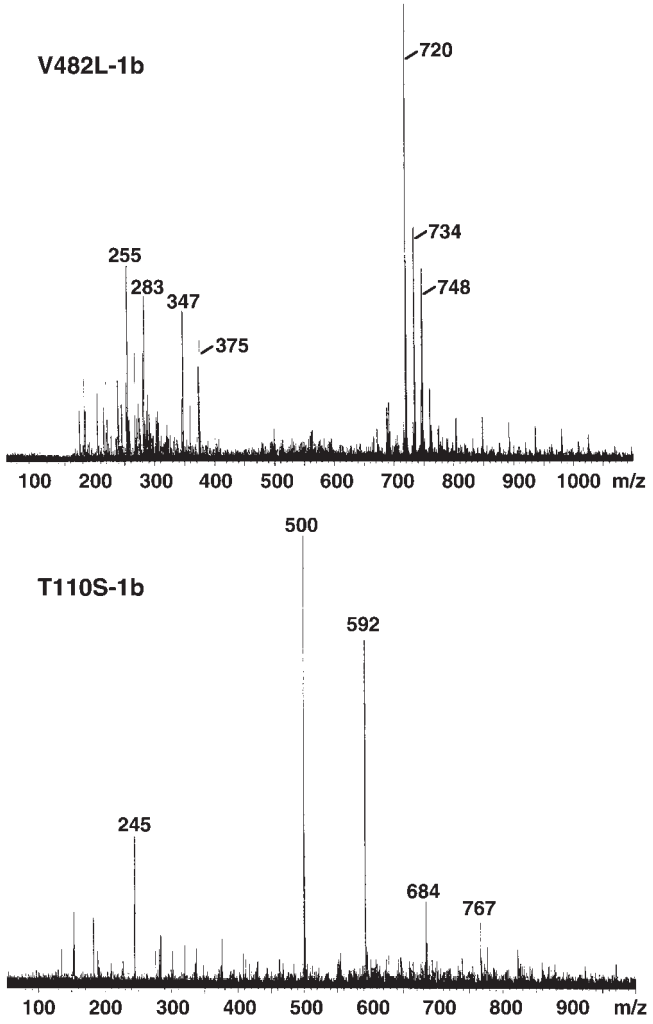

Fig. 3. Negative ion mass spectra (ES-MS) of chloroform-methanol extracts from the V482L and T110S P450 mutants after they were purified on a DEAE-cellulose column.

was added to the solution containing preformed PL vesicles and after an overnight incubation was tested for the ability to bind and hydroxylate 5β-cholestane-3α,7α,12α-triol and cholesterol. Three types of PLs were used for reconstitution: PG because it is a major contaminating PL of heterologously expressed P450 27A1; PS because a small peak at *m/z* 86, which is characteristic for the PS head group, was present in one of the ES-MS/MS spectra of the T110S-1a mutant (data not shown); and PE because it is the major anionic lipid in the inner membrane of prokaryotes (24) and one of the major PLs in the inner mitochondrial membrane of the liver in eukaryotes (25). As seen in **Fig. 4**, apparent K_d values for both of the substrates were increased when increasing amounts of either PG or PE were used for reconstitution. Binding of 5β-cholestane-3α,7α,12α-triol, however, appeared to be much less sensitive to inhibition with exogenous PLs than binding of cholesterol: 50 μg/nmol P450 of either PG or PE was required to cause a 4-fold increase in the K_d for 5β-cholestane-3α,7α,12α-triol, whereas only 4 μg PG/nmol P450 or 12 μg PE/nmol P450 was needed to cause a similar increase in the K_d for cholesterol. PS had no effect on K_d for 5β-cholestane-3α,7α,12α-triol at 12–100 μg/nmol P450 and resulted in a slightly tighter cholesterol binding at 12 μg/nmol P450. The inhibitory effect of exogenous PG on the interaction of P450 27A1 with cholesterol was

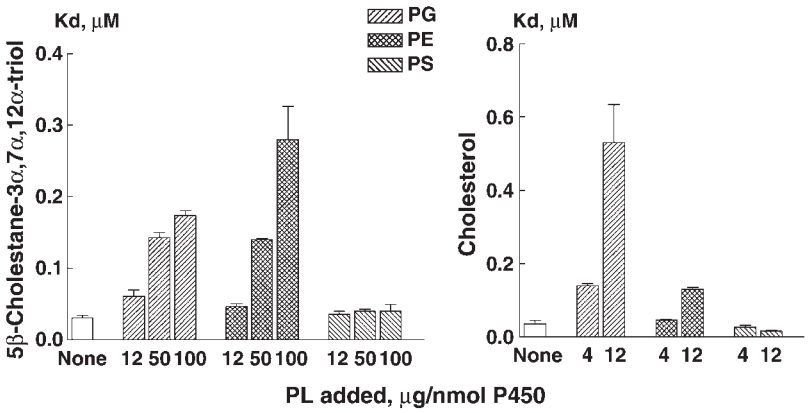

Fig. 4. Effect of exogenous phospholipids (PLs) on substrate binding properties of WT-2 (1.8 μg *E. coli* PL/nmol P450). Reconstitution with exogenous PLs was carried out as described in Materials and Methods. The results shown are means ± SD of three to five measurements. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine.

essentially unaffected by the mode of PL delivery, through copurification or reconstitution, as indicated by the 0.32 μM K_d of WT-4 copurified with 5.7 μg *E. coli* PL/nmol P450 (Table 1) and the 0.14 μM K_d of WT-2 reconstituted with 4 μg exogenous PG/nmol (Fig. 4). In contrast, inhibition of 5β-cholestane-3α,7α,12α-triol binding was much more efficient when PL was copurified with P450 rather than reconstituted: 50 μg exogenous PG/nmol P450 was required to increase the K_d value of WT-2 to a level (0.14 μM; Fig. 4) similar to that of WT-4 (0.15 μM; Table 1). To determine the modality of PG and PE inhibition, Lineweaver-Burk plots of 5β-cholestane-3α,7α,12α-triol and cholesterol binding at several PL concentrations were constructed. A pattern of the intersection of these plots was similar for both PLs (shown for PG as representative; **Fig. 5**), indicating that PG and PE are noncompetitive inhibitors. The K_i values of PG for 5β-cholestane-3α,7α,12α-triol and cholesterol binding were calculated to be 59.9 and 1.6 μg/nmol P450, respectively, and those for PE were 18.7 and 2.3 μg/nmol P450.

In parallel with substrate binding measurements, assays of 5β-cholestane-3α,7α,12α-triol and cholesterol hydroxylase activities of WT-2 reconstituted into PL vesicles were also performed (**Fig. 6**). The concentration of reconstituting PL was 100 μg/nmol P450 when 5β-cholestane-3α,7α,12α-triol was used as a substrate and 12 μg/nmol P450 with cholesterol as a substrate. Despite the inhibition of substrate binding, PG and PE had no significant effect on 5β-cholestane-3α,7α,12α-triol hydroxylase activity and even stimulated cholesterol hydroxylase activity. PS decreased 5β-cholestane-3α,7α,12α-triol hydroxylase activity almost 4-fold and had no effect on the 27-hydroxylation of cholesterol.

DISCUSSION

Heterologous expression in *E. coli* offers a number of advantages because of the ease of manipulation, the low cost of culture, and the large quantities of P450 that can be obtained. However, as the results of the present study indicate, caution should be exercised when working with recombinant P450. Partially purified preparations of P450 27A1 expressed in *E. coli* were found to contain bacterial PLs and to have significantly different substrate binding constants. The K_d values were much higher in the preparations that contained more PLs. Using mass spectrometric analyses, the major molecular PL species contaminating recombinant P450 27A1 were identified as PGs. It was also established that chromatography of P450 27A1 on DEAE-cellulose results in a significant reduction of the PL

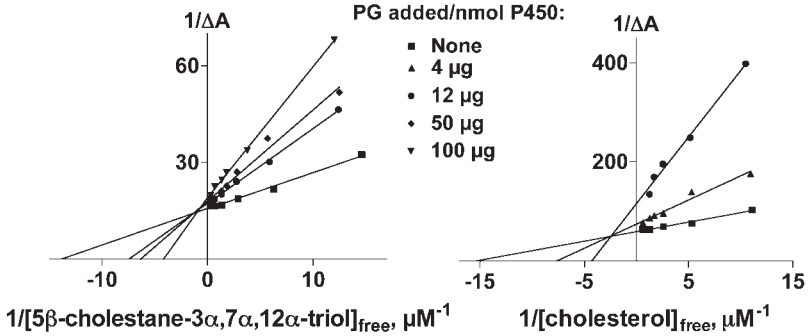

Fig. 5. Lineweaver-Burk plots of 5β-cholestane-3α,7α,12α-triol and cholesterol binding to WT-2 at different concentrations of PG.

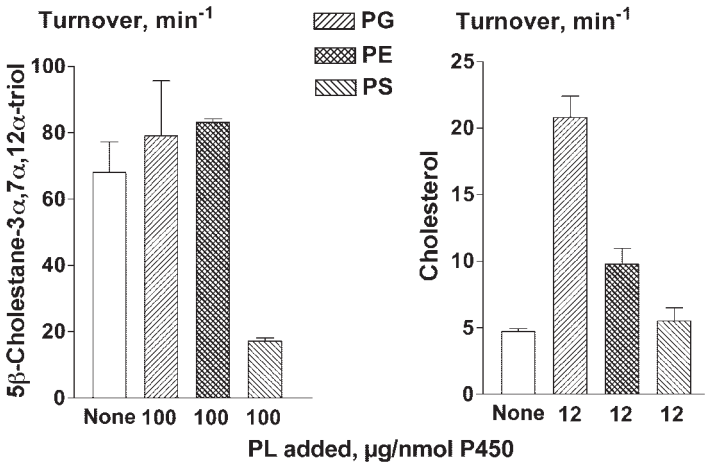

Fig. 6. Effect of exogenous PLs on 5β-cholestane-3α,7α,12α-triol and cholesterol hydroxylase activities of WT-2. Enzyme assays were carried out as described in Materials and Methods. The results shown are means ± SD of three to five measurements.

content and an improvement in substrate binding. Subsequent reconstitution experiments using exogenous PG showed a dose-dependent increase in apparent binding constants of P450 27A1 for the two physiological substrates tested, 5β-cholestane-3α,7α,12α-triol and cholesterol, and supported our initial observation suggesting that interpreparation variability of P450 27A1 substrate binding constants is, in large part, attributable to contamination with *E. coli* PLs. PG was found to be a noncompetitive inhibitor for both substrates, although the K_i value for 5β-cholestane-3α,7α,12α-triol was ~38 times higher than the K_i value for cholesterol (59.9 μg/nmol P450 versus 1.6 μg/nmol P450).

A similar effect, different sensitivity of the two substrates to the same inhibitor, was also observed in previous studies demonstrating that the immunosuppressive drug cyclosporin A is a much stronger noncompetitive inhibitor of cholesterol hydroxylation than is 5β-cholestane-3α,7α,12α-triol hydroxylation (26). Thus, two structurally and physiologically unrelated compounds, PG and cyclosporin, inhibit cholesterol binding to a much greater extent than binding of 5β-cholestane-3α,7α,12α-triol. This could be attributable to the fact that cholesterol and 5β-cholestane-3α,7α,12α-triol occupy different regions within the P450 active site. The former has a planar three-dimensional structure, whereas the latter has a bend at the A/B ring junction. Interaction with a noncompetitive inhibitor could induce a change in the conformation of the P450 active site and have more of an effect on cholesterol binding than on 5β-cholestane-3α,7α,12α-triol binding. Despite the inhibition of substrate binding by PG and PE, 5β-cholestane-3α,7α,12α-triol hydroxylase activity was essentially unaltered and cholesterol hydroxylase activity was stimulated by 2- to 5-fold. Probably some other steps, yet to be defined, in the P450 catalytic cycle were affected by PLs, allowing it to overcome the inhibition of substrate binding. Reconstitution into PL vesicle could fix P450 in an orientation that is optimal for the redox partner binding and facilitate subsequent electron transfer from the reduced redox partner to the oxidized P450. It is also possible that the catalysis was promoted because enzyme and substrate were concentrated within the PL vesicle.

As our studies with PS indicate, PLs could also have an inhibitory effect on P450 27A1 activity, likely because the structures of the head group and/or the fatty acid side chains do not favor interaction with the enzyme or the substrate. Although further research is needed to understand how PLs affect the P450 catalytic cycle, the present work clearly demonstrates for the first time that PLs could be natural modulators of P450 27A1 activity and by differential control of the rates of 5β-cholestane-3α,7α,12α-triol and cholesterol hydroxylations in vivo regulate the amount of cholesterol metabolized through the classical and alternative bile acid pathways. Our data on the effect of PE are especially pertinent because PE is one of most abundant PLs in the inner mitochondrial membrane where P450 27A1 resides: mitochondria from different mammalian cells contain 27–45% PC, 28–39% PE, 14–25% cardiolipin, 2–7% phosphatidylinositol, 1–3% lysophosphoglycerides, and 1–2% sphingomyelin (25). For comparison, the PL content of *E. coli* membranes (75% PE, 20% PG, and 5% cardiolipin) is quite different (27), although PE is the second most abundant PL in bacteria as well. The importance of the PL component for reconstituting P450-dependent activities has been known for 35 years (28, 29). Since the pioneering work of Lu, Junk, and Coon (28), most PL studies in the P450 field were focused on investigating the effects of the PL/P450 ratio and the PL head group and fatty acyl moiety on enzyme activity (30–38) as well as on the interaction of microsomal P450s with their redox partner oxidoreductase and the membrane (30, 35, 39–43). To the best of our knowledge, the novelty of the present work is that it shows for the first time that PLs that stimulate P450 activity could simultaneously inhibit substrate binding and that this inhibition is of a noncompetitive type. This is also the first report that recombinant P450 could be copurified with *E. coli* PLs.

Our data strongly suggest that contamination with PGs is the major factor contributing to the interpreparation variability of the P450 27A1 substrate binding constants; however, we cannot exclude the possibility that other unidentified low MW contaminants may also have an effect on P450 27A1 (e.g., free fatty acids). Being strain specific, lipid profiles of *E. coli* membranes nevertheless overlap

and contain common fatty acids such as dodecanoic (lauric, 12:0; MW 200), tetradecanoic (myristic, 14:0; MW 228), hexadecenoic (palmitoleic, 16:1; MW 254), hexadecanoic (palmitic, 16:0; MW 256), heptadecenoic (17:1; MW 268), cycloheptadecanoic (17:0, cyclo; MW 268), octadecenoic (vaccenic, 18:1; MW 282), octadecanoic (stearic, 18:0; MW 284), and cyclononadecanoic (19:0 cyclo; MW 296) acids (22, 44, 45). Ions corresponding to the *m/z* of several of these fatty acids were seen in the mass spectra of P450 lipid extracts (Figs. 1–3). Although some of these ions originated from the fragmentation of the PL molecules during ES, it is possible that free fatty acids may also be present in preparations of P450 27A1. Crystallographic studies of heterologously expressed P450 2C8 demonstrated that two molecules of palmitic acid were acquired from the *E. coli* host and were copurified and cocrystallized with the enzyme (46). However, these studies also showed that fatty acids were not present in the ether/hexane extracts of two other P450s, 2C9 and 2B4. Thus, not every P450 that is expressed in *E. coli* is contaminated with fatty acids, and a separate investigation is required to clarify this issue.

To summarize, *E. coli* PGs were found to be copurified with recombinant P450 and to affect its substrate-binding and catalytic properties. P450 27A1 activity was altered in a substrate-dependent manner, suggesting that PLs could play an important role in the regulation of cholesterol turnover through the classical and alternative bile acid biosynthetic pathways.

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