Involvement of Nonlamellar-Prone Lipids in the Stability Increase of Human Cytochrome P450 1A2 in Reconstituted Membranes†

Taeho Ahn,*,‡ Chul-Ho Yun,§ and Doo-Byoung Oh||

Department of Biochemistry, College of Veterinary Medicine, and Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Korea, and Metabolic Engineering Laboratory, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-333, Korea

Received January 10, 2005; Revised Manuscript Received March 31, 2005

ABSTRACT: The effect of nonlamellar-prone lipids, diacylglycerol (DG) and phosphatidylethanolamine (PE), on the stability of human cytochrome P450 1A2 (CYP1A2) was examined. When 100% phosphatidylcholine (PC) in standard vesicles was gradually replaced with either DG or PE, the stability of CYP1A2 increased; the incubation time-dependent destruction of spectrally detectable P450, decrease of catalytic activity, reduction of intrinsic fluorescence, and increased sensitivity to trypsin digestion were significantly alleviated. The ternary system of PC/PE/DG increased the stability of CYP1A2 more, even at lower concentrations of each nonlamellar-prone lipid, than that of the binary lipid mixture (PC/nonlamellar lipid). By incorporating the nonlamellar-prone lipids, the CYP1A2-induced increase of the surface pressure of the lipid monolayer was much higher compared to that for 100% PC. Increased surface pressure indicates a deep insertion of the protein into lipid monolayers. Nonlamellar lipids also increased the transition temperature of CYP1A2 in thermal unfolding and reduced the incubation time-dependent detachment of membrane-bound CYP1A2 from vesicles. Taken together, these results suggest that nonlamellar lipids per se and/or the phase properties of the membrane containing these lipids are important in the enhanced stability of CYP1A2 and the concomitant maintenance of catalytic activity of the protein.

Cytochromes P450 (CYP or P450)[1] are the major enzymes involved in the oxidation of a variety of xenobiotic chemicals and endogenous substrates (*1*), and multiple forms of CYPs are present in mammals (*2*). All microsomal CYPs are present in membranes, and at least some CYP enzymes are considered to interact with the specific lipid components of membranes. This notion is supported by the fact that phospholipids in the vicinity of CYPs in liver microsomes are more highly organized than those in bulk membranes (*3*). It has also been proposed that the interaction of phospholipids with CYP might be necessary for maintaining an active conformation and for efficient electron transfer (*4*).

Human CYP1A2, located predominantly in the liver, participates in the metabolism of a variety of compounds, including the activation of potentially carcinogenic aryl and heterocyclic amines (*5*). In terms of the specific interaction of CYP1A2 with membranes, it was found that rabbit CYP1A2 could be incorporated into preformed phospholipid vesicles composed of phosphatidylcholine (*6*). We have shown that rabbit CYP1A2 activity is associated with the conformational change of the protein induced by phospholipids (*7*) and that anionic phospholipids stimulate the catalytic activity of the CYP1A2 by enhancing membrane binding and incorporation (*8*).

The nonlamellar structure of membranes has been shown to play an important role in various cell functions (for review, see refs *9*, *10*). Regarding the functional regulation of CYP by nonlamellar lipids, it has been suggested that nonlamellar lipids such as branched phosphatidylcholines and cardiolipin might have a significant effect on the stimulation of mitochondrial $P450_{SCC}$ (CYP11A1) (*11*, *12*).

PE, the second most abundant phospholipid of cell membranes, is known to be a nonlamellar-prone lipid. This lipid destabilizes bilayer structures and lowers lamellar to the hexagonal II phase transition ($L-H_{II}$ transition) temperature of membranes (*13*). DG, another potent nonlamellar lipid, is a neutral lipid that can also lower the $L-H_{II}$ transition temperature of PE significantly (*14*, *15*). Furthermore, DG stabilizes the H_{II} structure of PE even at low concentrations (2−3 mol %) (*16*).

In this report, we extended our previous study on the effect of phospholipids on the function of CYP1A2 by studying

† This work was supported by Grant No. R05-2003-000-10504-0 from the Korea Science and Engineering Foundation to T.A.

* Address correspondence to this author at Department of Biochemistry, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea. Tel: +82-62-530-2823. Fax: +82-62-530-2809. E-mail: thahn@chonnam.ac.kr.

‡ Department of Biochemistry, College of Veterinary Medicine, Chonnam National University.

§ Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University.

|| Korea Research Institute of Bioscience and Biotechnology.

[1] Abbreviations: CYP or P450, cytochrome P450; DG, diacylglycerol; DOG, 1,2-dioleoyl-*sn*-glycerol; DOFP, 1,2-dioleoyl-1-fluoro-2,3-propanediol; DOGA, 1,2-dioleoyl-D-propanediol; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; *E. coli*, *Escherichia coli*; $L-H_{II}$ transition, lamellar to the hexagonal II phase transition; LUV, large unilamellar vesicle; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PMSF, phenylmethanesulfonyl fluoride; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PS, phosphatidylserine; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

10.1021/bi050051e CCC: $30.25
Published on Web 06/02/2005

the influence of nonlamellar-prone lipids such as PE and DG on the stability of the protein. The present study shows that nonlamellar-prone lipids increase the stability of CYP1A2 and shift the partition of the protein toward membranes between the soluble and membrane-bound form of the protein. These results can be used in the design of a proper CYP-lipid mixed array system by providing useful information concerning the interaction of CYP with specific phospholipids and on the physical properties of membranes, which are the matrix for membrane-bound proteins.

MATERIALS AND METHODS

Materials. All phospholipids and DOG were from Avanti Polar Lipids (Alabaster, AL). 7-Ethoxyresorufin and cumene hydroperoxide were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Protein Purification and Enzyme Activity Assay. Recombinant human CYP1A2 containing C-terminal $(His)_5$ was expressed in *Escherichia coli* DH5α and purified as described (*17*, *18*). Purified CYP1A2 was dialyzed at 4 °C for 24 h against 50 mM Tris-HCl buffer (pH 7.4) containing 20% glycerol (v/v), 1.0 mM EDTA, and 0.1 mM dithiothreitol and stored at −70 °C until used. SDS−PAGE was used to assess final protein purity, and protein concentration was determined using a bicinchoninic acid procedure according to the manufacturer's direction (Pierce, Rockford, IL). The catalytic activity of CYP1A2 was assayed as described (*8*): the reaction volume was 500 μL. CYP1A2 (0.5 μM) and lipid vesicles (400 μM) were mixed in the presence of cumene hydroperoxide. The hydroperoxide system was used instead of NADPH-P450 reductase and NADPH in order to avoid complicating effects of NADPH-P450 reductase on the stability of CYP1A2. The reaction was started by adding 7-ethoxyresorufin as a substrate. After the sample was incubated at 30 °C for 3 min, the reaction was stopped by addition of the same volume of cold methanol. The product was estimated by measuring the fluorescence intensity at 458 nm under an excitation wavelength of 358 nm.

Liposome Preparation. Lipid vesicles were prepared as described (*8*). In all experiments, 100 mol % of POPC (16:0−18:1-PC) liposome was used as a component for a standard vesicle. To prepare liposomes containing nonlamellar lipids in a binary mixture of PC/nonlamellar lipids, POPE (16:0−18:1-PE), DOPE (18:1−18:1-PE), or DOG (18:1−18:1-DG) was included up to 70 mol % (for PE) or 12 mol % (for DOG), respectively, at the expense of PC. Further replacements of PC with nonlamellar lipids were not undertaken due to the stability problem of lipid vesicles. In a ternary system of POPC/POPE/DOG membranes, POPE content was fixed at 30 mol % throughout all experiments and POPC was replaced with up to 5 mol % of DOG. Incorporation of DOG into membranes above 5 mol % was not used due to the same problem of stability with the binary mixture of POPC/nonlamellar lipids. All LUVs used for this work were stable for at least 2 days as determined by <10% deviation in light scattering values. The concentration of liposome stock solution was 4.0 mM, and a portion of the solution was diluted to study interaction of CYP1A2 to membranes. The concentrations of phospholipids were determined by phosphorus assay (*19*).

CO-Difference Spectroscopy. Fe^{2+}-CO versus Fe^{2+} difference spectroscopy was obtained with a Shimadzu spectrophotometer as described (*20*). After CO bubbling into the sample cuvette and baseline correction, sodium hydrosulfite was added and the difference spectrum was again measured between 400 and 500 nm at 30 °C.

Fluorescence Measurements. All fluorescence experiments to analyze conformational changes of CYP1A2 were performed at 30 °C. Fluorescence spectra were recorded with a Shimadzu RF-5301 PC spectrofluorometer equipped with a thermostated cuvette compartment. After 1 μM CYP1A2 was incubated with 800 μM lipid vesicles for each indicated time, the intrinsic fluorescence in CYP1A2 was recorded in the range of 300−450 nm with an excitation wavelength of 280 nm. The thermal unfolding of CYP1A2 was analyzed by following the decrease in fluorescence intensity at 338 nm (under an excitation wavelength of 280 nm) with a 0.5 °C/min heating rate.

Monolayer Experiments. Monolayer surface pressures were measured by the Wilhelmy plate method as described (*21*, *22*). The Teflon dish had a volume of 12 mL and a surface area of 20.3 cm^2. The subphase buffer was 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. The lipid layers were spread from a chloroform solution to give an initial surface pressure of 22 mN/m at 30 ± 0.4 °C. CYP1A2 was injected into the subphase (5 mL) in the presence or the absence of lipid monolayers, and the excess of CYP1A2 present in the subphase was washed away with 10 times the subphase volume after 30 min. The pressure changes were followed until the surface pressure reached a maximal value.

Binding Assays. Binding of CYP1A2 to model membranes was measured using precipitation by ultracentrifugation. CYP1A2 (1 μM) was incubated with 800 μM lipid vesicles for each indicated time at 30 °C. The protein-bound vesicles were centrifuged in a Beckman TLA 100.2 rotor at 70 000 rpm for 30 min, and the protein concentrations of pellets and supernatants were determined.

Limited Proteolysis. CYP1A2 (1 μM) was incubated with 800 μM lipid vesicles with various lipid compositions for 1 h at 30 °C, and a 1/100 (w/w) ratio of trypsin to CYP1A2 was added to the samples. After further incubation at 30 °C for 10 min, the reaction was stopped by the addition of 2 mM PMSF. The samples were boiled for 5 min and were analyzed by SDS−PAGE and Coomassie blue staining.

RESULTS

Effect of Nonlamellar-Prone Lipids on the Time-Dependent CO Spectra of CYP1A2. To correlate lipid compositions of vesicles with the stability of membrane-bound CYP1A2 directly, the differences in the incubation time-dependent Fe^{2+}-CO versus Fe^{2+} spectra of the protein were measured in the presence of liposomes consisting of various lipid components. As shown in Figure 1A, the soluble CYP1A2 had a typical CO spectrum when reduced by sodium hydrosulfide. The absorption maximum (λ_{max}) was at 448 nm. Increasing the incubation time to 24 h at 30 °C, the absorption intensity at 448 nm (P450) decreased gradually throughout the incubation period, indicating a destabilization of the normal orientation of CO bound to ferroporphyrin in the active site of the CYP enzyme. This spectral property was not changed upon interaction with liposomes (results not shown). We could not detect any shift of λ_{max} by adding various lipid vesicles externally. Only the intensities at

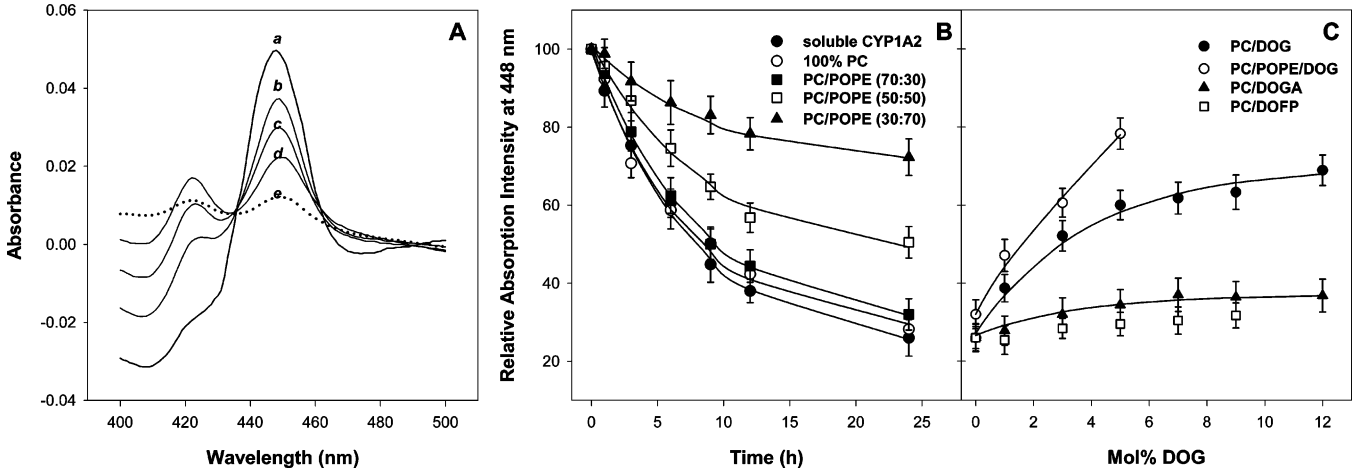

FIGURE 1: Incubation time-dependent changes of Fe^{2+}-CO versus Fe^{2+} difference spectra of CYP1A2. (A) CYP1A2 in the absence of phospholipid vesicles was incubated for (a) 0, (b) 3, (c) 6, (d) 9, and (e) 24 h at 30 °C, and then CO difference spectra were obtained as described in Materials and Methods. (B) The effects of POPE contents in a binary system of PC/PE on the time-dependent decrease of absorption intensity at 448 nm. (C) The relative intensities were recorded as a function of DOG concentration in a binary membrane of PC/DOG and in a ternary system of PC/POPE/DOG after 24 h incubation at 30 °C. In the ternary system, POPE content was fixed at 30 mol % but the PC content (initial 70%) was varied as it was replaced with upward 12% DOG. DOFP and DOGA represent 1,2-dioleoyl-1-fluoro-2,3-propanediol and 1,2-dioleoyl-D-glyceramide, respectively. In panels B and C, data points represent the mean ± SE of three independent experiments.

λ_{max} and 420 nm changed depending upon incubation time. Therefore, we could use this spectral feature of CYP1A2 to trace possible changes in the stability of the protein as a function of incubation time upon interaction with lipid bilayers.

When 100 mol % POPC vesicles were added to soluble CYP1A2 externally and the P450 level was measured, the rate of P450 destruction was similar to that of the enzyme in the absence of vesicles (Figure 1B). On the contrary, with increasing POPE concentrations at the expense of PC, the degree of P450 destruction decreased significantly and only about 20% of P450 was lost, with vesicles containing 70 mol % PE after 24 h incubation (74% and 70% lost in the presence of 100 mol % PC or in the absence of lipid component, respectively). These results show that when CYP1A2 interacts with vesicles containing PE, the stability of the hemoprotein increases with PE in a concentration-dependent manner. However, with up to a 30 mol % replacement of PC with POPE, the dependency of P450 destruction on incubation time was very similar to that of the protein bound to 100 mol % PC vesicles. When additional amounts of PE were included in the vesicles, PE appeared to have a stabilizing effect on the enzymes. These results remind us of our previous observation that the formation of lipid domains in PC/PE membranes occurs above a certain concentration of PE (*23*) and may correlate with the phase separation in PC/PE membranes.

When DOG, a potent promoter of nonlamellar phases in various phospholipid systems (*15*, *24*), was substituted for PE up to 12 mol %, the incubation time-dependent destruction of CYP1A2 was also decreased, indicating a possible stabilizing effect of DOG on CYP1A2 (Figure 1C). A similar protection pattern against P450 destruction with the PC/DOG system was also shown even at a lower concentration of DOG in POPC/POPE/DOG (65:30:5 by molar ratio) membranes, suggesting that DG and PE exert a synergistic effect on the stabilization of the enzyme. This result seems to be related to previous reports that DG lowers the lamellar to hexagonal II phase transition temperature of PE (*14*) and that DG stabilizes the H_{II} structure of PE (*16*). As a control experiment, the effect of DOG analogues (dioleoyl-1-fluoro-2,3-propanediol and dioleoyl-D-glyceramide) on the stabilization of CYP1A2 was also examined in the POPC/DOG analogue system instead of DOG itself. These analogues had little effect on protection against P450 decreases, which suggests the importance of DOG and/or DOG-induced membrane properties in the stabilization of CYP1A2 and the specific interaction of the protein with membranes containing DOG.

Influence of Nonlamellar Lipids on the Membrane Binding of CYP1A2. To explain the stabilizing effect of nonlamellar-prone lipids on CYP1A2, we examined the binding properties of CYP1A2 to membranes. Figure 2A shows a titration of membrane-bound CYP1A2 by vesicles containing various lipids. The membrane binding of CYP1A2 was enhanced by increasing the lipid to protein (L/P) ratio and was saturated at a L/P ratio of about 800, regardless of lipid compositions of vesicles but with different amounts of the membrane-bound protein. Hereafter we analyzed the membrane binding of CYP1A2 at this L/P ratio. Figure 2B shows a decrease of membrane-bound amounts of the protein in all membranes tested when the incubation time is increased. The decrease indicates the detachment of membrane-bound CYP from the membrane and a shift of the partition into a soluble fraction. In the presence of POPE or DOPE, however, the time-dependent decrease of membrane-bound CYP1A2 was diminished as a function of nonlamellar lipid concentration when PC was replaced with the nonlamellar lipids. Notably, upon interaction with vesicles containing DOPE, which has a lower $L-H_{II}$ transition temperature than POPE (*25*), the membrane-bound amounts of CYP1A2 were slightly higher than those for POPE for all incubation times. This result suggests a possible correlation between the membrane association of CYP1A2 and the $L-H_{II}$ transition temperature of PE. DOG also showed a very similar effect to PE, as in both cases the incubation time-dependent detachment of membrane-bound CYP from the vesicle was alleviated with increasing concentrations of DOG in a binary mixture of

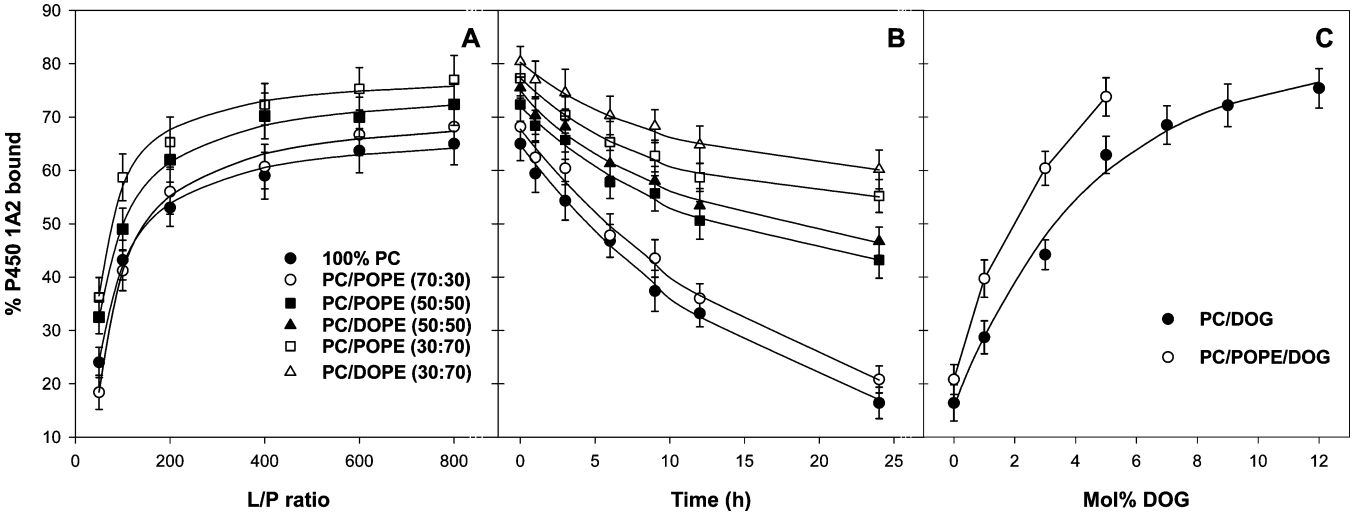

FIGURE 2: Membrane binding of CYP1A2. (A) Membrane-bound amount of CYP1A2 was determined as a function of L/P ratio. The protein content (25 μg) was fixed and the amount of lipids was increased to be each indicated L/P ratio. Membrane-bound CYP1A2 was precipitated by ultracentrifugation, and protein concentrations of the pellet and the supernatant were analyzed. (B) Effects of PE (POPE and DOPE) on the incubation time-dependent detachment of CYP1A2. (C) The quantity of membrane bound CYP1A2 was measured as a function of DOG concentration in the binary mixture of PC/DOG or in the ternary system of PC/POPE/DOG after 24 h incubation. As described in Figure 1, the POPE content in vesicles was fixed at 30 mol %. The mean ± SE values were calculated on the basis of assays with three independent experiments.

PC/DOG and a ternary system of PC/POPE/DOG (Figure 2C). Here again, the ternary mixture showed a more significant protecting effect against the release of membrane-bound CYP1A2 than that of the binary system. These results indicate that nonlamellar lipids have a stronger interaction with CYP1A2 than with 100% PC. It should also be noted that initially, at zero hour, the amount of membrane-bound CYP1A2 was higher in the presence of nonlamellar lipids than in 100% PC with a nonlamellar lipid-dependent manner. The present binding experiment does not demonstrate a direct correlation of the nonlamellar lipid-induced change of CYP1A2 stability with lipid composition-dependent partitioning of the protein between soluble and membrane fractions. From these results, however, we can predict that a nonlamellar lipid-induced increase of CYP1A2 stability is, at least in part, correlated to the membrane-binding properties of the protein. Considering our previous results that anionic phospholipids such as PA, PS, and PI stimulate membrane binding and the insertion of rabbit CYP1A2 into phospholipid bilayers (*8*), it is interesting that these anionic phospholipids did not show a similar effect to nonlamellar lipids on the incubation time-dependent detachment of the CYP1A2 (result not shown).

Interaction of CYP1A2 with Lipid Monolayers. In order to obtain more insight into the interaction of CYP1A2 with membranes and to correlate protein stability with lipid compositions of membranes, we examined changes in surface pressure after injecting the protein into the subphase of lipid monolayers. The increased surface pressure represents the "insertion" of a protein into the lipid monolayers as it was established that agents interacting only with a lipid headgroup did not induce a surface pressure increase in monolayers (*26*).

Figure 3A shows that the monolayer surface pressure increases as a function of the amount of CYP1A2 injected into the subphase with 100% PC membrane, which was saturated at about 40 μg of protein under an initial surface pressure of 22 mN/m. In the presence of 50 mol % of POPE, the pressures were higher than those of 100% PC at all tested concentrations of CYP1A2. This result indicates that a greater amount of the protein was inserted into monolayers containing PE. Figure 3B shows the increase of the surface pressure as a function of POPE or DOPE. Regardless of the acyl chain species of PE, the pressure was enhanced with increasing concentrations of PE. Furthermore, it was found that DOPE exerts a more significant effect on the pressure increase than does POPE, suggesting that the membrane insertion of CYP1A2 might be related to how easily the $L-H_{II}$ transition occurs. Here again, the pressure increase was not remarkable until POPE contents exceeded 30 mol %. Therefore, it appears that the membrane binding and insertion of CYP1A2 is related to the stability of the protein through membrane property changes induced by nonlamellar lipids as well as by the lipids themselves. DOG also increased the surface pressure as a function of its concentration in the binary mixture of PC/DOG and in the ternary system of POPC/ POPE/ DOG (Figure 3C).

More insight into the lipid specificity of a CYP1A2 insertion into a monolayer can be gained by varying the initial surface pressure of the lipid monolayer. CYP1A2 itself is surface-active in the absence of lipid monolayers as it gives rise to a surface pressure increase of 16.7 mN/m. Therefore, the initial surface pressure was maintained above 20 mN/m throughout the experiments presented here. Figure 4 shows that the CYP1A2-induced surface pressure change was linearly reduced as increasing the initial surface pressure in the all monolayer systems tested, indicating the limitation of CYP1A2 insertion. However, the limiting insertion pressure, defined as the maximal surface pressure allowing the insertion, was significantly higher when nonbilayer lipids were included in the binary system as well as in the ternary system as compared to 100% PC. Taken into account together, the result also suggests that the enhanced insertion of CYP1A2 into a monolayer containing nonlamellar lipids as compared to 100% PC was not dependent on the initial surface pressure.

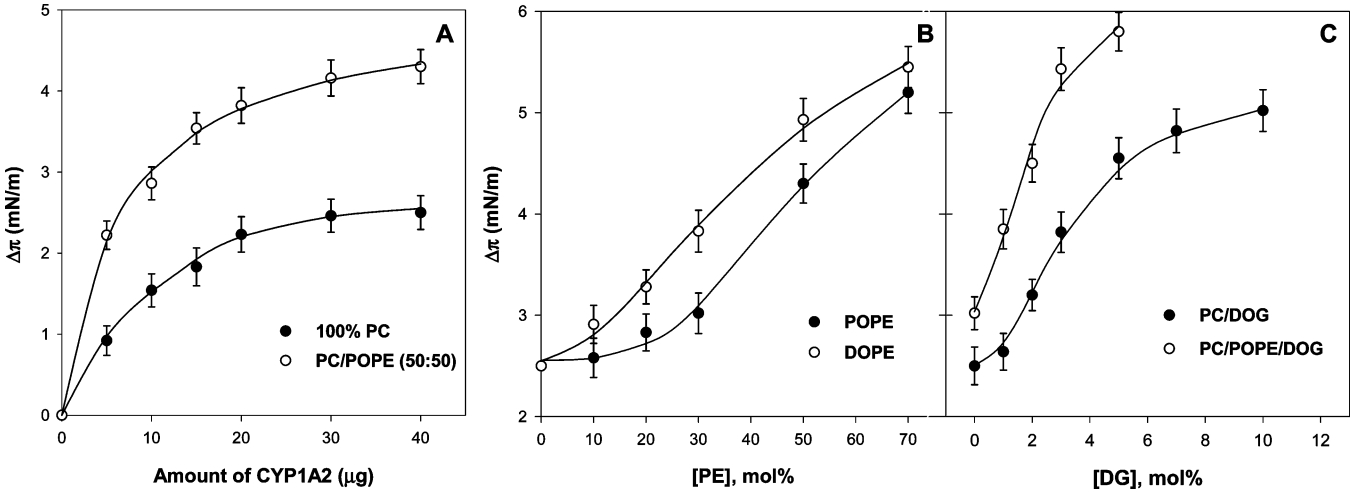

FIGURE 3: Insertion of CYP1A2 into the monolayer as analyzed by monolayer surface pressure. (A) Increase of the pressure as a function of the amount of CYP1A2 injected in the subphase. Initial pressure was 22 mN/m for POPC and POPC/POPE (50:50) monolayer. (B, C) Effects of nonlamellar lipids on the insertion of CYP1A2 were measured with increasing concentrations of PE (POPE and DOPE) (B) and of DOG in the binary mixture (PC/DOG) and in the ternary system (PC/POPE/DOG) with a fixed amount of 30 mol % PE (C). Data points represent the mean ± SE of three independent experiments.

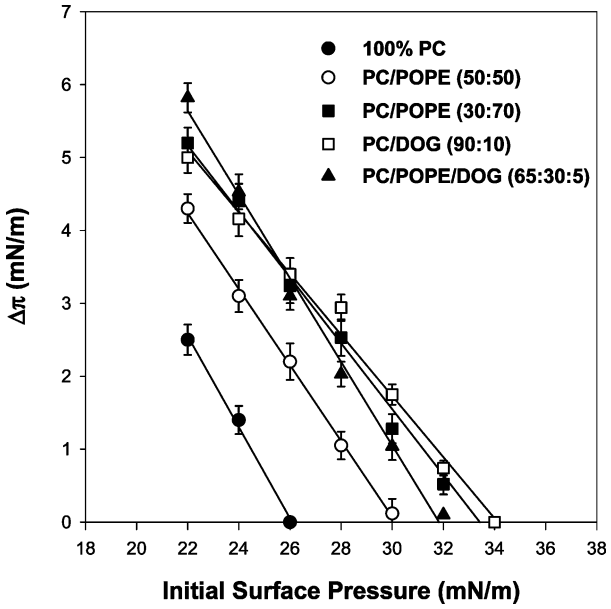

FIGURE 4: Insertion of CYP1A2 into the monolayer as a function of the initial surface pressure. Monolayer of 100% POPC (●), POPC/POPE (50:50, by molar ratio) (○), POPC/POPE (30:70) (■), POPC/DOG (90:10) (□), and POPC/POPE/DOG (65:30:5) (▲).

Effect of Nonlamellar Lipids on the Catalytic Activity of CYP1A2. To correlate the nonlamellar lipid-induced stability changes of CYP1A2 to its catalytic activity, we measured CYP1A2-catalyzed reactions in the presence of lipid bilayers. Enzyme activity was quantified by measuring its ability to catalyze the O-deethylation of 7-ethoxyresorufin in the presence of cumene hydroperoxide, in place of NADPH-P450 reductase and NADPH, to avoid the complicating effect of NADPH-P450 reductase, another membrane protein (*7*). It was found that the activity was decreased as a function of incubation time and after 24 h about 20% of initial catalytic activity remained for the case of 100% PC (Figure 5A). With increasing concentrations of nonlamellar lipids in the binary mixture of PC/nonlamellar lipids and in the ternary system of PC/PE/DOG, however, the time-dependent decrease of activity was remarkably alleviated with a different degree dependent on lipid composition. It should be noted that the relative catalytic activity in the case of the ternary system at zero hour was approximately 1.5−2-fold higher than that of 100% PC. The ternary PC/POPE/DOG system showed the most significant effect and was followed by the PC/DOG system and the PC/POPE system. It is clear, therefore, that the propensity of H_{II} formation is essential to maintain the catalytic activity of CYP1A2.

In order to represent the effect of nonbilayer lipids on the incubation time-dependent decrease in the CYP1A2 activity more clearly, the data points in Figure 5A was replotted by the ratio of relative activity at each incubation time to that of zero time (Figure 5B). In this normalized figure, nonbilayer lipid-induced alleviation of the decrease in the CYP1A2 activity was clarified in the binary system in PE concentration-dependent manner as well as the ternary system.

Conformational Changes of Membrane-Bound CYP1A2. The effect of lipid compositions on the incubation time-dependent stability of CYP1A2 was studied through conformational changes of the protein using intrinsic fluorescence and limited proteolysis. Figure 6 shows that the intrinsic fluorescence intensity of CYP1A2 decreased regardless of lipid compositions with increasing incubation time at 30 °C, indicating that a conformational change of CYP1A2 is dependent upon incubation time. In the presence of 100% POPC vesicles, the relative emission intensity was reduced by about 85% after 24 h. However, upon increasing the concentration of POPE at the expense of PC, the decrease of intrinsic fluorescence was alleviated and, at 70% PE, the emission intensity was decreased by only 35% after 24 h of CYP1A2 incubation. A similar protecting effect of PE was observed when PC was replaced with DOG in the binary mixture of PC/DOG and in the ternary system of PC/POPE/DOG.

The influence of nonlamellar lipids on the conformation and stability of CYP1A2 was also analyzed by limited proteolysis using trypsin in the presence or absence of nonlamellar lipids. As shown in Figure 7, several fragments were detected by trypsin digestion of CYP1A2 interacting

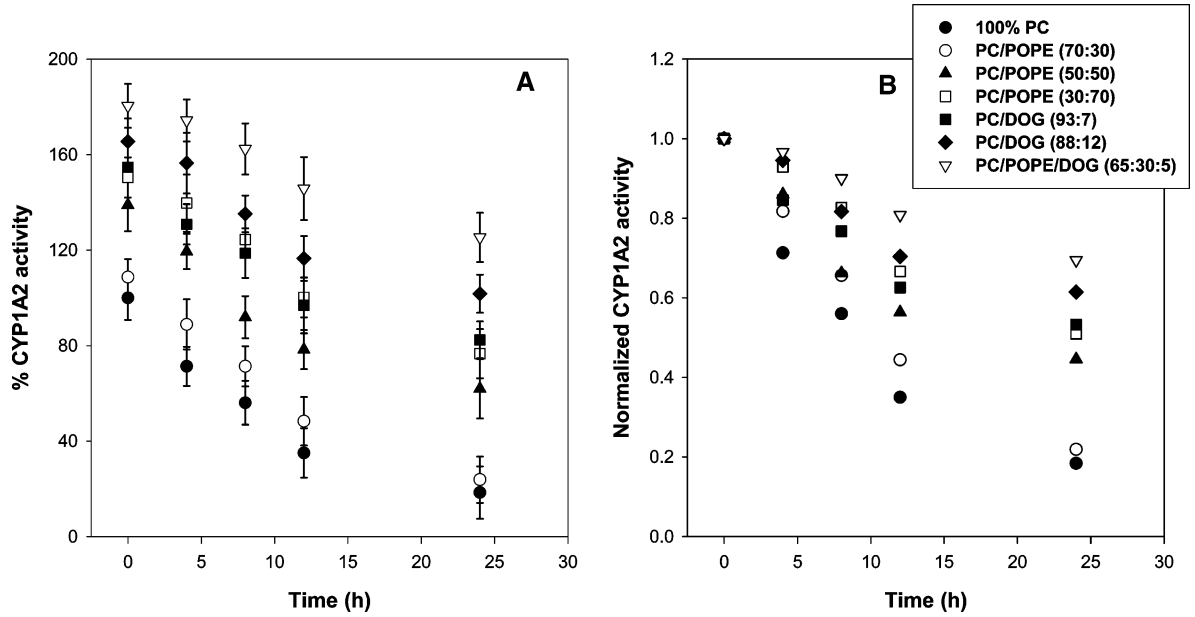

FIGURE 5: Influence of nonlamellar lipids on the catalytic activity of CYP1A2. The O-deethylation of 7-ethoxyresorufin was measured after each indicated incubation time. (A) The activity in the presence of 100 mol % PC membranes at the initial time was set to 100%. (B) The activity change of CYP1A2 was normalized by the ratio of the relative activity at each incubation time to that of zero time. For details, see Materials and Methods.

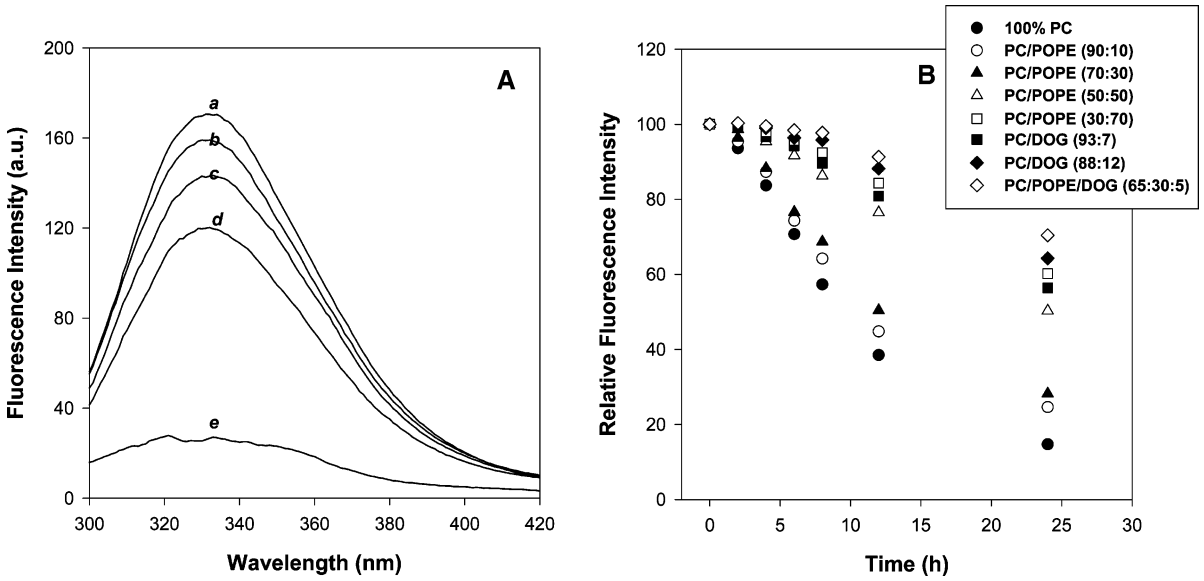

FIGURE 6: Effects of lipid bilayers on the intrinsic emission fluorescence of CYP1A2. (A) Emission spectral changes of CYP1A2 bound to 100% PC membranes with increasing incubation time: (a) 0, (b) 3, (c) 6, (d) 9, and (e) 24 h. (B) CYP1A2 (1 μM) was incubated with 800 μM membranes for each indicated time at 30 °C, and then the emission intensity at 338 nm was measured under an excitation wavelength of 280 nm.

with membranes. Upon interaction with membranes containing nonlamellar lipids, CYP1A2 became more resistant to the proteolysis than the 100% PC vesicle as higher amounts of fragments as well as more intact proteins remained. Figure 8 shows the thermal unfolding transition profile of membrane-bound CYP1A2 measured by the intrinsic fluorescence of Trp residues in the protein, which further supports that nonlamellar lipids induce the stability increase of CYP1A2: the soluble CYP1A2 had a transition temperature (T_m) of 44.2 °C. When bound to a 100% PC membrane, T_m of CYP1A2 increased slightly, up to 45.2 °C. However, the replacement of PC with POPE or DOG resulted in a significant increase in the T_m, up to 49.8 °C and 51.3 °C in the presence of 70 mol % POPE and 12 mol % DOG, respectively. This result unequivocally demonstrates that POPE and DOG stabilize CYP1A2, and it may also imply that a more compact (closed) form of the protein is achieved upon interaction with membranes containing nonlamellar lipids.

DISCUSSION

Although crystal structures of several mammalian P450 enzymes (including 2C5, 2C9, 2B4, and 3A4) have been elucidated recently (*27*−*29*), no crystal structure is currently available for the membrane-bound form of CYP1A2. Microsomal CYP1A2 is believed to be anchored to the ER membrane by a hydrophobic amino-terminal region, which is inserted into the membrane in a vectorial fashion (*8*, *30*),

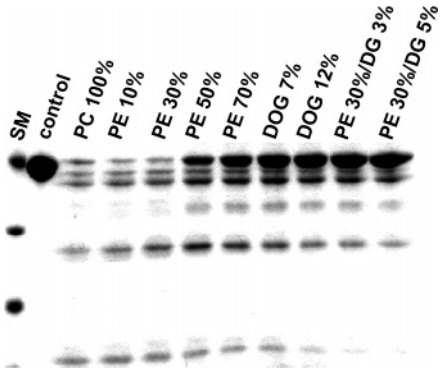

FIGURE 7: Limited proteolysis of CYP1A2 bound to membranes by trypsin. The proteolysis was conducted at the ratio of 100:1 (CYP1A2:trypsin, by weight). After incubation at 30 °C for 10 min, the trypsin digestion was stopped by the addition of an electrophoresis sample buffer containing 2 mM PMSF. Aliquots were taken from the reaction mixture and were analyzed by SDS−PAGE and Coomassie Brilliant Blue staining. SM and control represent molecular weight size marker and purified CYP1A2, respectively.

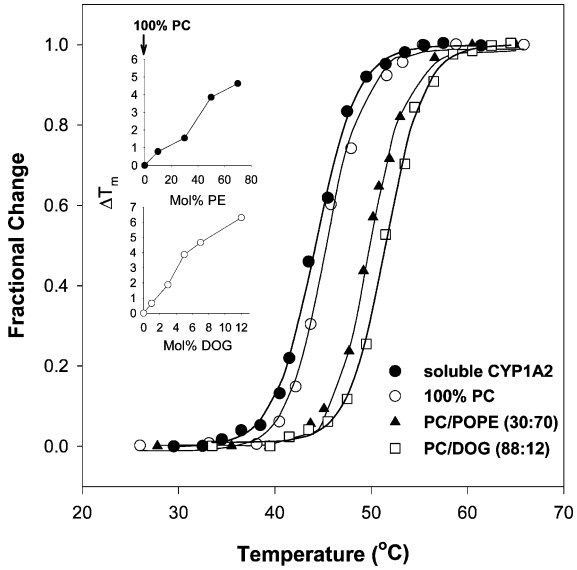

FIGURE 8: Effect of nonlamellar lipids on the thermal unfolding of membrane-bound CYP1A2. The temperature-induced unfolding of CYP1A2 was conducted in the presence of POPE or DOG. Changes in the fluorescence intensity at 338 nm were recorded as a function of temperature. The *y*-axis (fractional change) is relative fluorescence changes with the emission intensity of CYP1A2 set to zero (0) and to one (1) at the initial and final temperature, respectively. The inset represents the relative difference of T_m (ΔT_m) with increasing concentrations of POPE or DOG at the expense of PC.

and it has been suggested that the interaction with membrane is important in the proper function of the protein (*7*, *8*). Even if the structures of some microsomal CYPs have been revealed, there is no direct information to determine optimal conditions which maintain the functional activity of the protein for any desirable application.

Biological membranes contain significant amounts of the lipid species that form nonlamellar phases under physiological conditions (*31*). Furthermore, many lipids in membranes can be transformed from a bilayer to a nonlamellar state by changes of biological conditions such as pH value, temperature, salt concentration, and degree of hydration (*32*). It was also reported that lipid compositions of a membrane could be regulated in a "window" between being a gel and a nonlamellar structure (*33*), and that dietary eritadenine, a hypocholesterolemic factor, markedly decreases the ratio of PC to PE in liver microsomes (*34*).

The importance of nonlamellar-forming lipids in membranes on the enzyme activities and protein functions has already been suggested for many cases such as ubiquinol-cytochrome *c* reductase and mitochondrial H^+-ATPase (*35*), rhodopsin (*36*), and alamethicin conductance states (*37*). With CYP enzymes, it has also been proposed that membrane properties induced by nonlamellar phase propensity are important for regulating the catalytic activity of CYPs in a reconstituted system (*11*, *12*, *38*). These studies have mainly been undertaken in view of the stimulation of enzyme activity.

In this report, we have focused on the stability increase of CYP1A2 bound to lipids by modulating lipid compositions to optimize immobilization of CYP enzymes in the lipid monolayer. The present investigations established that DOG and PE, which are known to have the propensity to form nonlamellar structures and, generally, to destabilize membrane integrity, enhance the stability of CYP1A2 bound to membranes, and maintain its catalytic activity during incubation. Therefore, the effect of nonlamellar lipids (and/or these lipid-induced membrane properties) on biological functions was further extended to the regulation of protein stability.

When evaluating the results that nonlamellar lipids increase the binding and insertion of CYP1A2 into membranes and inhibited the incubation time-dependent detachment of the protein from the vesicles, it was seen that CYP1A2 might have a higher affinity to nonlamellar lipids per se and/or to membrane properties induced by these lipids than to those for PC. Concomitantly, the enhanced interaction of CYP1A2 with membranes containing nonlamellar lipids appears to be responsible for the increased stability of the protein. However, it should be noted that although anionic phospholipids such as PA, PS, and PI also promote the membrane binding and insertion of CYP1A2 (*8*), these acidic lipids could not show remarkable effects on the stability increase of CYP1A2 protein compared to the case with PC (results not shown). This discrepancy might be the result of the different molecular behaviors between human (in this report) and rabbit CYP1A2, although human CYP1A2 has an amino acid sequence very similar to that of rabbit CYP1A2 (*39*, *40*). We observed that nonlamellar lipids but not anionic phospholipids exert similar effects on the stability increase of rabbit CYP1A2 (results not shown). Therefore, the membrane binding and insertion of CYP1A2 would be only a partial cause for the nonlamellar lipid-induced enhancement of the protein stability, and other potential explanations have to be considered. It is conceivable that, contrary to the association of CYP1A2 with anionic phospholipids, charge−charge interactions between nonlamellar lipids and CYP1A2 are not involved in membrane binding and insertion and a different molecular mode might dominate the interaction between the protein and the membranes.

We have demonstrated previously that the mixing properties of POPC and POPE are not random, but that lipid domains of phospholipids are formed in a liquid-crystalline state (*23*). On the basis of our results, we suggest that the phase separation in the PC/PE membrane seems to be intensified above a certain critical concentration of PE. Interestingly, the present results indicate the possibility that

the interaction of CYP1A2 with membranes containing POPE might be related with the phase separation (Figures 2B, 3B, 5, 6B, and 7). The replacement of PC with up to 30 mol % of PE did not show any significant effects on the association of CYP1A2 to membranes, catalytic activity, and the conformational change of the protein. Although the current study does not provide further evidence for the nonideal mixing properties of a membrane consisting of POPC and POPE, it is tempting to speculate that the propensity of forming lipid domains increases the stability of CYP1A2. Indirectly, taking into account the previous suggestion that DG stabilizes the H_{II} structure of PE (*16*), this notion is further supported by the observation that in a ternary mixture of PC/PE/DOG (for example, 65% POPC/30% POPEE/5% DOG) experimental results were not simple arithmetic summations provoked by POPE and DOG, but synergistic effects were shown in all test cases.

Several trials with CYP enzymes have been made to confirm industrial use of the protein. Although most of the interest from pharmaceutical industries has focused on the role of CYPs in drug development, these enzymes also have potential in the discovery of other useful chemicals. Potential applications range from the use of CYPs as drug targets to their use of bioremediation (*41*). A general optimization strategy of CYP activity and stability is needed for industrial purposes, and this work may provide this kind of general strategy for the application of CYP immobilization to products such as protein chips or biocatalysts.

REFERENCES

1. Wrighton, S. A., and Stevens, J. C. (1992) The human hepatic cytochrome P450 involved in drug metabolism, *Crit. Rev. Toxcol. 22*, 1−21.
2. Guengerich, F. P. (1995) Human cytochrome P450 enzymes, in *Cytochrome P450* (Ortiz de Montellano, P. R., Ed.) 2nd ed., pp 473−535, Plenum Press, New York.
3. Stier, A., and Sackmann, E. (1973) Spin labels as enzyme substrates. Heterogeneous lipid distribution in liver microsomal membranes, *Biochim. Biophys. Acta 311*, 400−408.
4. French, J. S., Guengerich, F. P., and Coon, M. J. (1980) Interactions of cytochrome P-450, NADPH-cytochrome P-450 reductase, phospholipid, and substrate in the reconstituted liver microsomal enzyme system, *J. Biol. Chem. 255*, 4112−4119.
5. Shimada, T., Martin, M. V., Pruess-Schwartz, D., Marnett, L. J., and Guengerich, F. P. (1989) Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(a)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons, *Cancer Res. 49*, 6304−6312.
6. Ingelman-Sundberg, M., and Glaumann, H. (1980) Incorporation of purified components of the rabbit liver microsomal hydroxylase system into phospholipid vesicles, *Biochim. Biophys. Acta 599*, 417−435.
7. Yun, C.-H., Song, M., and Kim, H. (1997) Conformational change of cytochrome P450 1A2 induced by phospholipids and detergents, *J. Biol. Chem. 272*, 19725−19730.
8. Ahn, T., Guengerich, F. P., and Yun, C.-H. (1998) Membrane insertion of cytochrome P450 1A2 promoted by anionic phospholipids, *Biochemistry 37*, 12860−12866.
9. Epand, R. M. (1998) Lipid polymorphism and protein-lipid interactions, *Biochim. Biophys. Acta 1376*, 353−368.
10. de Kruijff, B. (1997) Lipid polymorphism and biomembrane function, *Curr. Opin. Chem. Biol. 1*, 564−569.
11. Kisselev, P., Wessel, R., Pisch, S., Bornscheuer, U., Schmid, R. D., and Schwarz, D. (1998) Branched phosphatidylcholines stimulate activity of cytochrome P450SCC (CYP11A1) in phospholipid vesicles by enhancing cholesterol binding, membrane incorporation, and protein exchange, *J. Biol. Chem. 273*, 1380−1386.
12. Schwarz, D., Kisselev, P., Wessel, R., Jueptner, O., and Schmid, R. D. (1996) Alpha-branched 1,2-diacyl phosphatidylcholines as effectors of activity of cytochrome P450SCC (CYP11A1). Modeling the structure of the fatty acyl chain region of cardiolipin, *J. Biol. Chem. 271*, 12840−12846.
13. Dekker, C. J., Geurts van Kessel, W. S., Klomp, J. P., Pieters, J., and de Kruijff, B. (1983) Synthesis and polymorphic phase behaviour of polyunsaturated phosphatidylcholines and phosphatidylethanolamines, *Chem. Phys. Lipids 33*, 93−106.
14. Epand, R. M. (1985) Diacylglycerols, lysolecithin, or hydrocarbons markedly alter the bilayer to hexagonal phase transition temperature of phosphatidylethanolamines, *Biochemistry 24*, 7092−7095.
15. Das, S., and Rand, R. P. (1986) Modification by diacylglycerol of the structure and interaction of various phospholipid bilayer membranes, *Biochemistry 25*, 2882−2889.
16. Siegel, D. P., Banschbach, J., Alford, D., Ellens, H., Lis, L. J., Quinn, P. J., Yeagle, P. L. and Bentz, J. (1989) Physiological levels of diacylglycerols in phospholipid membranes induce membrane fusion and stabilize inverted phases, *Biochemistry 28*, 3703−3709.
17. Yun, C.-H., Miller, G. P., and Guengerich, F. P. (2000) Rate-determining steps in phenacetin oxidations by human cytochrome P450 1A2 and selected mutants, *Biochemistry 39*, 11319−11329.
18. Ahn, T., Yang, S. and Yun, C−H. (2004) High-level expression of human cytochrome P450 1A2 by co-expression with human molecular chaperone HDJ-1(Hsp40), *Protein Expression Purif. 36*, 48−52.
19. Vaskovsky, V. E., Kostetsky, E. Y., and Vasendin, I. M. (1975) A universal reagent for phospholipid analysis, *J. Chromatogr. 114*, 129−141.
20. Omura, T., and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature, *J. Biol. Chem. 239*, 2370−2378.
21. Demel, R. A. (1974) Monolayers-description of use and interaction, *Methods Enzymol. 32*, 539−545.
22. Breukink, E., Demel, R. A., de Korte-Kool, G., and de Kruijff, B. (1992) SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: a monolayer study, *Biochemistry 31*, 1119−1124.
23. Ahn, T., and Yun, C.-H. (1999) Phase properties of liquid-crystalline Phosphatidylcholine/Phosphatidylethanolamine bilayers revealed by fluorescent probes, *Arch. Biochem. Biophys. 369*, 288−294.
24. De Boeck, H., and Zidovetzki, R. (1989) Effects of diacylglycerols on the structure of phosphatidylcholine bilayers: a ^{2}H and ^{31}P NMR study, *Biochemistry 28*, 7439−7446.
25. Ellens, H., Bentz, J., and Szoka, F. C. (1986) Destabilization of phosphatidylethanolamine liposomes at the hexagonal phase transition temperature, *Biochemistry 25*, 285−294.
26. Demel, R. A., London, Y., Geurts van Kessel, W. S. M., Vossenberg, F. G. A., and van Deenen, L. L. M. (1973) The specific interaction of myelin basic protein with lipids at the air-water interface, *Biochim. Biophys. Acta 311*, 507−519.
27. Williams, P. A., Cosme, J., Vinkovic, D. M., Ward, A., Angove, H. C., Day, P. J., Vonrhein, C., Tickle, I. J., and Jhoti, H. (2004) Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone, *Science 305*, 683−686.
28. Scott, E. E., He, Y. A., Wester, M. R., White, M. A., Chin, C. C., Halpert, J. R., Johnson, E. F., and Stout, C. D. (2003) An open conformation of mammalian cytochrome P450 2B4 at 1.6-Å resolution, *Proc. Natl. Acad. Sci. U.S.A. 100*, 13196−13201.
29. Williams, P. A., Cosme, J., Ward, A., Angove, H. C., Matak Vinkovic, D., and Jhoti, H. (2003) Crystal structure of human cytochrome P450 2C9 with bound warfarin, *Nature 424*, 464−468.
30. Black, S. D. (1992) Membrane topology of the mammalian P450 cytochromes, *FASEB J. 6*, 680−685.
31. Goldfine, H. (1982) Lipids of prokaryotes: structure and distribution, *Curr. Top. Membr. Transp. 17*, 1−43.
32. Lindblom, G., and Rilfors, L. (1992) Nonlamellar phases formed by membrane lipids, *Adv. Colloid Interface Sci. 41*, 101−125.
33. Morein, S., Andersson, A.-S., Rilfors, L., and Lindblom, G. (1996) Wild-type *Escherichia coli* cells regulate the membrane lipid composition in a "window" between gel and non-lamellar structures, *J. Biol. Chem. 271*, 6801−6809.
34. Sugiyama, K., Yamakawa, A., Kawagishi, H., and Saeki, S. (1997) Methionine content of dietary proteins affects the molecular species composition of plasma phosphatidylcholine in rats fed a cholesterol-free diet, *J. Nutr. 127*, 600−607.

35. Li, L., Zheng, L. X., and Yang, F. Y. (1995) Effect of propensity of hexagonal II phase formation on the activity of mitochondrial ubiquinol-cytochrome c reductase and H^+-ATPase, *Chem. Phys. Lipids 76*, 135−144.
36. Brown, M. F. (1994) Modulation of rhodopsin function by properties of the membrane bilayer, *Chem. Phys. Lipids 73*, 159−180.
37. Keller, S. L, Bezrukov, S. M., Gruner, S. M., Tate, M. W., Vodyanoy, I., and Parsegian, V. A. (1993) Probability of alamethicin conductance states varies with nonlamellar tendency of bilayer phospholipids, *Biophys. J. 65*, 23−27.
38. Kisselev, P., Schwarz, D., Platt, K.-L., Schunck, W.-H., and Roots, I. (2002) Epoxidation of benzo[a]pyrene-7,8-dihydrodiol by human CYP1A1 in reconstituted membranes. Effects of charge and nonbilayer phase propensity of the membrane, *Eur. J. Biochem. 269*, 1799−1805.
39. Strom, D. K., Postlind, H., and Tukey, R. H. (1992) Characterization of the rabbit CYP1A1 and CYP1A2 genes: developmental and dioxin-inducible expression of rabbit liver P4501A1 and P4501A2, *Arch. Biochem. Biophys. 294*, 707−716.
40. Ikeya, K., Jaiswal, A. K., Owens, R. A., Jones, J. E., Nebert, D. W., and Kimura, S. (1989) Human CYP1A2: sequence, gene structure, comparison with the mouse and rat orthologous gene, and differences in liver 1A2 mRNA expression, *Mol. Endocrinol. 3*, 1399−1408.
41. Guengerich, F. P. (2002) Cytochrome P450 enzymes in the generation of commercial products, *Nat. Rev. Drug Discov. 1*, 359−366.

BI050051E