Membrane Insertion of Cytochrome P450 1A2 Promoted by Anionic Phospholipids†

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ABSTRACT: The role of phospholipids in the membrane binding and subsequent insertion of the microsomal protein rabbit cytochrome P450 (P450) 1A2 into phospholipid bilayers was investigated. The insertion of P450 1A2 into phospholipid bilayers was measured by the quenching of Trp fluorescence of P450 1A2 by pyrene and brominated and doxyl-labeled phospholipids. When the phosphatidylcholine (PC) matrix was replaced with acidic phospholipids [phosphatidic acid (PA), phosphatidylserine, and phosphatidylinositol] and phosphatidylethanolamine (PE), the extent of insertion into lipid bilayers was strictly dependent on the type of acidic phospholipids. All anionic phospholipids caused the penetration of P450 1A2 into lipid bilayers, but PA was the most efficient in facilitating deep penetration of P450 1A2 into bilayers. On the other hand, binding of P450 1A2 to liposomes was increased by acidic phospholipids to the same degree regardless of the type of acidic phospholipids. PE was found to act as an inert matrix phospholipid, similar to PC, as it exerted very little effect on the insertion of P450 1A2 into lipid bilayers and the binding of P450 1A2 to membranes. It was also found that the phospholipid-dependent membrane insertion of P450 1A2 was associated with altered enzyme activity, increased α-helix content, and increased Trp fluorescence of P450 1A2. These results indicate that negative charges on the acidic phospholipids are important for the initial binding of P450 1A2 to membranes, but the penetration of P450 1A2 into lipid bilayers is regulated by the type of acidic phospholipids, and that phospholipid-dependent insertion of P450 1A2 is accompanied by a structural change of P450 1A2.

The microsomal monooxygenase system oxidizes a variety of endogenous and xenobiotic compounds (*1*). This enzyme system includes cytochrome P450[1] (also termed heme-thiolate protein P450 by the Enzyme Commission, EC 1.14.14.1) (*2*), NADPH-P450 reductase, and phospholipids. Cytochrome b_5 and NADH-cytochrome b_5 reductase may also contribute to the electron flow (*3*). P450-dependent activities can be reconstituted by mixing P450, NADPH-P450 reductase, and phospholipid (*4*, *5*). P450 and NADPH-P450 reductase seem to be distributed randomly on the plane of membranes, and they interact through lateral diffusion by forming a functional complex for the electron transfer (*6*−*8*), although the organization of constituent proteins in phospholipid membranes and their mechanism of interaction are not fully understood yet. On the other hand, P450 is present in the membrane in large excess over the reductase, the limiting component in microsomes, with the molar ratios ranging from 10:1 to 25:1 depending on treatment with inducers (*9*, *10*). Phospholipids in the immediate vicinity of P450 in liver microsomes have been reported to be highly organized as compared with those in bulk membranes (*11*), suggesting that P450 may have important interactions with the phospholipid molecules immediately surrounding the protein. It has also been proposed that the interaction of phospholipid with P450 might be necessary for maintaining an active protein conformation and its ability to interact with NADPH-P450 reductase and necessary for efficient electron transfer (*12*).

Rabbit P450 1A2 can be incorporated into preformed phospholipid vesicles (*13*). Recently, we have shown that the change of P450 1A2 activity is associated with the conformational change of P450 1A2 induced by phospholipid and salt (*14*, *15*). The roles of different phospholipids in the binding of P450 1A2 to membranes and the insertion of P450 1A2 into lipid bilayers were considered in detail. We report here that P450 1A2 binds to negatively charged phospholipids with a higher affinity in the initial binding step to the lipid surface but acidic phospholipids regulate the second insertion step of P450 1A2 into phospholipid bilayers, and phospholipid-dependent insertion of P450 1A2

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[1] Abbreviations: P450, cytochrome P450; PC, phosphatidylcholine; PE, phosphatidylethanolamime; PS, phosphatidylserine; PA, phosphatidic acid; PI, bovine heart phosphatidylinositol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; pyrene-PC, 1-palmitoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine; PyrS DHPE, *N*-(1-pyrenesulfonyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; 5-doxyl-PC, L-α-1-palmitoyl-2-(5-doxyl-stearoyl)-phosphatidylcholine; 16-doxyl-PC, L-α-1-palmitoyl-2-(16-doxyl-stearoyl)-phosphatidylcholine; 6,7-Br_2-PC, L-α-1-palmitoyl-2-(6,7-dibromostearoyl)-phosphatidylcholine; 9, 10-Br_2-PC, L-α-1-palmitoyl-2-(9, 10-dibromostearoyl)-phosphatidylcholine; SRP, signal recognition particle; LUVs, large unilamellar vesicles; ER, endoplasmic reticulum.

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coincides with the changes in conformation and enzymatic activity of the protein. The possible role of phospholipids in the membrane insertion and conformational change of proteins is discussed.

EXPERIMENTAL PROCEDURES

Chemicals. 7-Ethoxycoumarin and cumene hydroperoxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Most of the phospholipids (POPC, POPE, POPS, PI, POPA, 5-doxyl-PC, 16-doxyl-PC, 6,7-Br_2-PC, and 9,10-Br_2-PC) were from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Pyrene-labeled phospholipids were purchased from Molecular Probes (Eugene, OR). The unlabeled phospholipids, except PI, used here are synthetic phospholipids with acyl chains of palmitic and oleic acids. Other chemicals were of the highest grade commercially available.

Protein Purification and Enzyme Activity Assay. P450 1A2 was purified from liver microsomes of 5,6-benzoflavone-treated rabbits as described (*16*). P450 1A2 was electrophoretically homogeneous and had a specific content of 17 nmol of P450/mg of protein. Protein concentrations were estimated using a bicinchoninic acid procedure according to the manufacturer's directions (Pierce, Rockford, IL). P450 concentrations were determined by Fe^{2+}-CO versus Fe^{2+} difference spectroscopy (*17*). The assay of P450 1A2 activity was done in 25 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl using the method described elsewhere, with slight modification (*14*, *15*). The reaction volume was 500 μL. P450 1A2 (0.5 μM) and lipid vesicles (50 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 a complicating effect of NADPH-P450 reductase interaction. The reaction was started by adding 7-ethoxycoumarin as a substrate. After the sample was incubated at 25 °C for 10 min, the reaction was stopped by adding the same volume of cold methanol. The product was estimated by measuring the fluorescence intensity at 458 nm (358 nm excitation wavelength).

Liposome Preparation. In all experiments, POPC liposomes were used as a standard vesicle. To study penetration or binding of P450 1A2 to membranes by use of energy transfer between pyrene and the Trp in P450 1A2, 2 mol % of pyrene-PC or pyrS DHPE was incorporated into membranes. In the quencher-containing samples, POPC was replaced by 20 mol % doxyl-labeled or 30% brominated phospholipids.

After the appropriate amounts of lipids were mixed in chloroform, the solvent was evaporated under a stream of argon gas. The dry lipids were hydrated in buffer solution (25 mM Tris-HCl, pH 7.4, containing 100 mM NaCl) by vortex mixing and subsequent brief sonication in a bath sonicator (30 s). To obtain the homogeneous LUVs, the dispersion was frozen and thawed five times and extruded 25 times through two polycarbonate membranes (100 nm pore size). All LUVs used for this work were stable for at least 3 days as determined by <10% deviation in light-scattering values. The concentration of liposome stock solution was 0.2−1.0 mM, and a portion of the solution was diluted to study the interaction of P450 1A2 to membranes. The concentrations of nonfluorescent phospholipids were determined by phosphorus assay (*18*), and those of pyrene-containing probes were determined spectrophotometrically (ϵ_{342} = 42 000 M^{-1} cm^{-1} in ethanol) (*19*).

Fluorescence Measurements. All fluorescence experiments were performed at 25 °C, maintained using a circulating water bath. Fluorescence emission spectra were recorded with a Shimazu RF-5301 PC spectrofluorometer equipped with a thermostated cuvette compartment. To measure the emission fluorescence of Trp residues in P450 1A2, 295 and 341 nm excitation and emission wavelengths were used. For the measurement of the excimer (E) and the monomer (M) of pyrene-containing liposomes, the excitation wavelength was 342 nm and the emission wavelength was 360−500 nm. The E/M ratio of pyrene-labeled PC was calculated by fluorescence intensity at 375 nm (for monomer) and 480 nm (for excimer), respectively. The samples were incubated for 5 min before measurement. In all fluorescence experiments, 500 μL of sample solution was used in a quartz cuvette.

To determine the extent of Trp quenching by KI, P450 1A2 was first incubated with the vesicles of interest for 5 min. A series of KI solutions (with the concentration ranging from 10 to 70 mM) were then added to the reaction mixtures (that had the same total concentrations of KI plus KCl, but different KI concentrations), and the fluorescence intensity at the emission wavelength of 341 nm was measured. The fluorescence data are plotted according to the Stern−Volmer equation (*20*).

Binding Assays. Binding of P450 1A2 to liposomes was measured using the precipitation method as described elsewhere (*21*). Various concentrations of liposomes containing 1 mol % biotinylated PE were incubated with 400 pmol of P450 1A2 for 5 min at 25 °C in 90 μL of reaction volume. Immobilized avidin (10 μL of a 7.5 mg/mL suspension) on 6% agarose beads (Pierce) was then added, and the samples were incubated with shaking. After 10 min, the liposomes were precipitated by microcentrifugation for 5 min at 14 000 rpm. The supernatant was immediately assayed for the protein concentration. The pellets were resuspended by adding 1% sodium dodecyl sulfate solution (w/v), and P450 1A2 was separated from the immobilized avidin liposome complex by centrifugation. The concentration of membrane-bound P450 1A2 was measured using a bicinchoninic acid procedure.

The energy transfer between Trp residue(s) in P450 1A2 and pyrS DHPE incorporated into phospholipid bilayers was also used to measure the binding of P450 1A2 to liposomes.

Circular Dichroism. CD spectra were recorded on a Jasco J700 spectropolarimeter (Japan Spectroscopic, Tokyo) at 25 °C in a thermostated cuvette. The calibration of the spectropolarimeter was performed using D-10-camphorsulfonic acid, which shows a molar ellipticity of 7800 deg cm^2/dmol at 290.5 nm in an aqueous solution. CD spectra of P450 1A2 were obtained using 1 μM protein in a 0.1-cm path length cell. Blanks (buffer with or without phospholipid) were routinely recorded and subtracted from the original spectra.

RESULTS

Phospholipid-Dependent Membrane Insertion of P450 1A2. To determine if P450 1A2 can be inserted into lipid

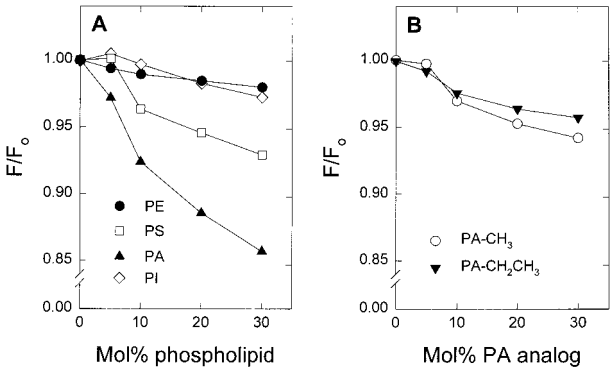

FIGURE 1: Phospholipid-dependent membrane insertion of the SA sequence of P450 1A2. The energy transfer between Trp in P450 1A2 and pyrene-PC incorporated into membranes was examined by replacing POPC with POPE and anionic phospholipids (A), and with PA analogues (B) up to 30 mol %. F/F_o represents the fluorescence intensity ratio at 340 nm for sample with (F) and without (F_o) pyrene-PC incorporated in membranes. After preparation of LUVs with or without the indicated amount of phospholipids, P450 1A2 (0.6 μM) was mixed with vesicles (80 μM) in 25 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl. After incubating the sample for 5 min at 25 °C, Trp fluorescence was measured at 341 nm (excitation 295 nm).

bilayer by adding phospholipid vesicles exogeneously, we used resonance energy transfer between P450 1A2 Trp residues and pyrene-labeled phosphatidylcholine (pyrene-PC) that had been incorporated into membranes by replacing PC with PE or acidic phospholipids such as PS, PI, or PA. The pyrene group is located at the end of the decanoyl chain at the *sn*-2 position of PC, and P450 1A2 has eight Trp residues which are spaced evenly throughtout its sequence (*22*). The phospholipids used here are the major phospholipids present in liver ER membranes, and their compositions are variable depending on physiological conditions (*23*).

PA had the most significant effect on the penetration of P450 1A2, in comparison with other phospholipids, as shown by the lowest F/F_o value when the concentration of PA was increased at the expense of PC (Figure 1A). PS also decreased F/F_o but was less efficient than PA. However, PE- and PI-containing vesicles had no effect on the energy transfer. This result indicates that P450 1A2 has the ability to penetrate the membrane bilayer depending on the type of phospholipids present in membrane.

To ascertain whether PA stimulates insertion of P450 1A2 into membrane, we measured the quenching of the Trp fluorescence again with the PA analogues phosphatidylmethanol and phosphatidylethanol. Interestingly, these analogues caused diminished effects on quenching compared with PA (Figure 1B). This result indicates that the headgroup of PA is an important factor in determining the membrane insertion of P450 1A2.

We also measured the excimer (E) and monomer (M) fluorescence of pyrene-PC incorporated in membranes in the absence of proteins and determined the E/M ratio to examine the distribution of pyrene probes. It was based on the observation that the E/M ratio reflects the lateral diffusion rate of the probe and its local concentration enrichment in membranes (*24*). As the E/M ratio was not changed by replacing PC with other phospholipids up to 30 mol % in all samples tested here (results not shown), it was reasonable that PC matrix was replaced with other phospholipids up to

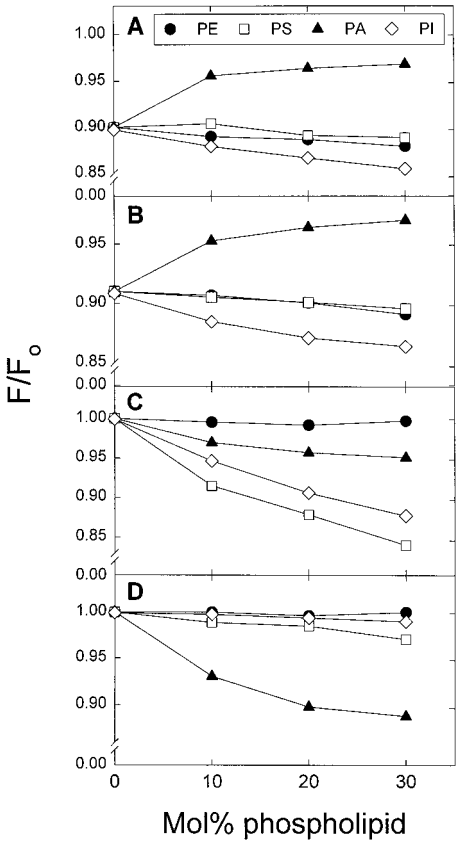

FIGURE 2: Quenching of P450 1A2 fluorescence by Br_2-PC and doxyl-PC incorporated into membrane. After mixing LUVs containing either 20% doxyl-PC or 30% Br_2-PC plus P450 1A2, the Trp fluorescence was measured at 341 nm. The quenching of Trp fluorescence was measured by 5-doxyl-PC (A), 6,7-Br_2-PC (B), 9,10-Br_2-PC (C), and 16-doxyl-PC (D), respectively. For other details see the Figure 1 legend.

30 mol % when using pyrene-PC as a quencher of Trp fluorescence.

To estimate the penetration extent of P450 1A2 into lipid bilayer by phospholipid-dependent manner in detail, brominated and spin-labeled phospholipids were used as quenching probes of the Trp fluorescence instead of pyrene-labeled PC. When 5-doxyl-PC or 6,7-Br_2-PC was used as a quencher, the fluorescence intensity of P450 1A2 was linearly decreased as increasing PI concentration compared to the case of 100% PC matrix (Figure 2, partsA and B). However, PS and PE exerted very little quenching effects and, more interestingly, the F/F_o value was augmented with increasing PA mol % at the expense of PC. In the samples containing 9,10-Br_2-PC, PS produced the lowest F/F_o value and PI and PA showed less quenching effect (Figure 2C). However, PE had no effect regardless of its concentration. In the case of 16-doxyl-PC, only PA showed a quenching effect, indicating that the Trp residue(s) of P450 1A2 can be inserted deeply up to the end of acyl chain in the presence of PA (Figure 2D). This result supports that obtained by the energy-transfer experiment between pyrene-PC and Trp in P450 1A2 described above. From these results, we speculate that phospholipids play an important role in regulating the penetration of P450 1A2 into the lipid bilayer. In particular, PA facilitates the deep insertion of the Trp residue(s) of P450 1A2 up to the middle of the lipid bilayer upon interaction with membranes.

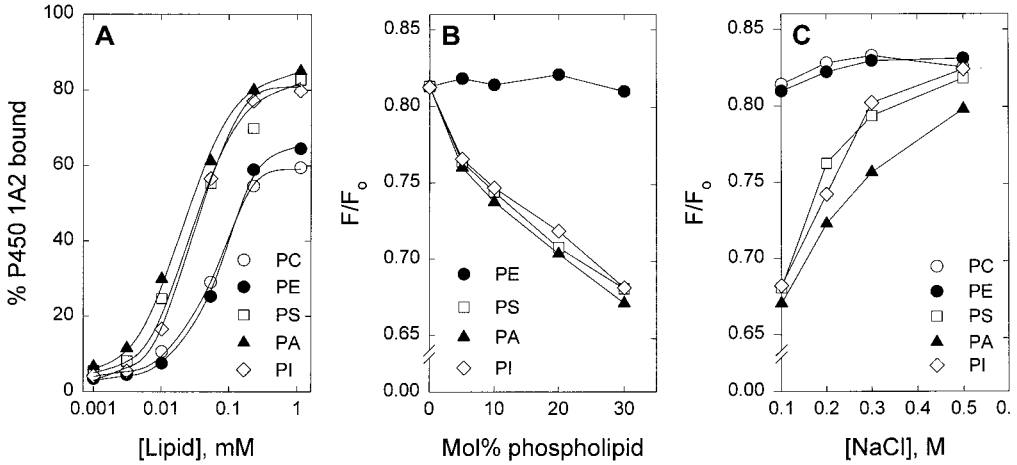

FIGURE 3: Phospholipid-dependent binding of P450 1A2 to membranes. (A) Binding of P450 1A2 to liposomes was measured by the precipitation of liposomes as described under Experimental Procedures. (B) The energy transfer between Trp in P450 1A2 and pyrS DHPE incorporated into lipid bilayers was used to examine the effect of POPE and anionic phospholipids on the amount of P450 1A2 binding to membranes by replacing POPC with indicated phospholipids. (C) The effect of NaCl on the P450 1A2 binding to lipid bilayers was also measured with the same method for (B) at 30 mol % of indicated phospholipids. All other conditions and procedures for the measurement of Trp fluorescence of P450 1A2 are the same as described for Figure 1.

Effects of Phospholipids on Binding of P450 1A2 to Membrane. To determine whether the binding of P450 1A2 to membrane also depends on the type of phospholipids comprising membranes, we analyzed the amounts of P450 1A2 bound to membranes with increasing concentrations of liposomes. All vesicles containing 30 mol % acidic phospholipids increased the binding of P450 1A2 to membranes by about 20% with similar efficiency compared to the case of 100% PC (about 60% binding) (Figure 3A). However, PE-containing liposomes had no effect on the increase of binding. To ascertain this result, the effect of acidic phospholipids on the binding of P450 1A2 to model membranes was investigated by energy transfer between Trp in P450 1A2 and pyrS DHPE incorporated into membranes in which pyrene is attached at the ethanolamine group of PE. All acidic phospholipids induced the decreased F/F_o value to the same degree, which indicates the increase of P450 1A2 binding to membrane regardless of the type of acidic phospholipids (Figure 3B). However, in the presence of PE instead of acidic phospholipids, the F/F_o ratio was not changed compared to the ratio for 100% PC. This observation coincided well with the results obtained from the precipitation method (Figure 3A).

These results suggest that charge−charge interactions between acidic phospholipids and P450 1A2 are involved in protein binding to the membranes. To test this hypothesis, the energy-transfer experiment was repeated with increasing concentrations of NaCl at 30 mol % of PE or acidic phospholipids. In the cases of PC and PE, there was no effect of salt on the quenching. However, in the samples containing acidic phospholipids, all F/F_o values were increased with increasing salt concentration and approached the value for 100% PC (Figure 3C). This result indicates that P450 1A2 binding to the membrane is inhibited by a high concentration of salt in the presence of acidic phospholipids. We also measured the E/M ratio of the pyrene probe as a measure of change of membrane structure in the absence of proteins; there was no change of the ratio (results not shown).

Taken together, these results demonstrate that all acidic phospholipids tested in our experiments (PS, PI, and PA) increased P450 1A2 binding to the lipid bilayer to the same degree, but the extent of penetration into the membrane was strictly dependent on the type of acidic phospholipids. Among the acidic phospholipids, PA was the most efficient in facilitating the insertion of P450 1A2 into lipid bilayers. PE may act as an inert matrix phospholipid, similar to PC, in the interaction of P450 1A2 with membranes.

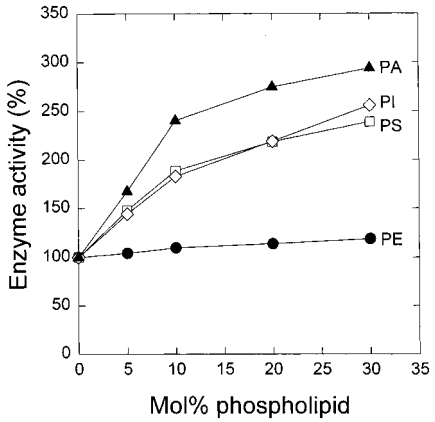

FIGURE 4: Effects of phospholipids on the catalytic activity of P450 1A2. The 100% activity value represents the normalized activity (0.18 nmol of product/min/nmol of P450) for the sample containing 100% PC as a lipid component. For other details, see Experimental Procedures.

Effects of Phospholipids on the Catalytic Activity of P450 1A2. To determine the effect of membrane penetration of P450 1A2 on its catalytic activity, we measured P450 1A2-catalyzed reactions in the presence of phospholipid bilayers. The enzyme activity of P450 1A2 was quantified by measuring its ability to catalyze the O-deethylation of 7-ethoxycoumarin in the presence of cumene hydroperoxide, in place of NADPH-P450 reductase and NADPH, to avoid the complicating effect of another membrane protein (NADPH-P450 reductase) (*14*, *15*). The activity was increased by about 3-fold compared to that in 100% PC membrane as the PA concentration was increased to 30 mol % (Figure 4). PI and PS also increased P450 1A2 catalytic activity but to a lesser extent (about 2.5-fold) than in the case of PA. With PE there was no apparent increase of enzymatic activity. This

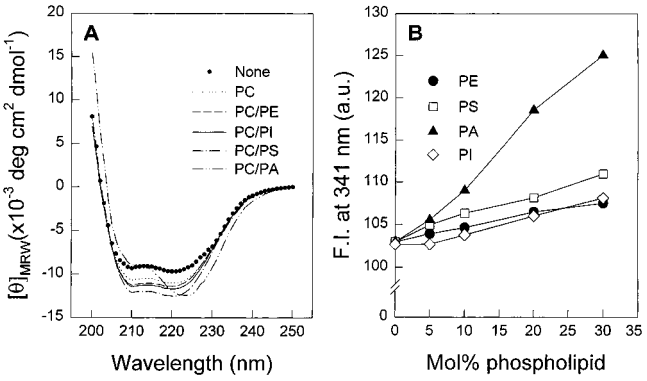

FIGURE 5: Effect of phospholipids on the CD (A) and Trp fluorescence (B) spectra of P450 1A2. Spectra of 1.0 μM P450 1A2 in 25 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl were recorded in the presence of indicated phospholipid vesicles.

Table 1: Effect of Phospholipid on the Secondary Structure Contents of P450 1A2[a]

additions	α-helix (%)	β-sheet (%)	β-turn (%)	random (%)
none	34 ± 2	28 ± 3	18 ± 2	20 ± 1
phospholipids				
PC	40 ± 2	29 ± 2	8 ± 1	23 ± 2
PC/PE	39 ± 3	28 ± 2	11 ± 1	22 ± 1
PC/PI	39 ± 1	31 ± 2	8 ± 1	22 ± 2
PC/PS	43 ± 2	30 ± 1	4 ± 1	23 ± 2
PC/PA	50 ± 2	4 ± 1	19 ± 1	27 ± 2

[a] All CD spectra were recorded in 25 mM Tris-HCl buffer (pH 7.4, containing 100 mM NaCl) containing phospholipid vesicles present in the additions. Conditions were as described under Experimental Procedures. All estimates are means ± SD ($n = 3$).

result suggests that the lipid-dependent membrane binding and insertion of P450 1A2 is related to the conformational change associated with altered enzyme activity.

Conformational Changes upon Membrane Binding and Insertion of P450 1A2 into Phospholipid Vesicles. The effect of phospholipid on P450 1A2 conformation upon membrane binding and insertion into the membrane was studied by investigating structural changes of P450 1A2 using CD and Trp fluorescence spectroscopy. The effect of phospholipids on the secondary structure of the P450 1A2 was studied in the far-UV region. CD spectra of P450 1A2 in 25 mM Tris-HCl buffer (pH 7.4, containing 100 mM NaCl) are presented in the presence of various types of PC vesicles containing 30 mol % of PE, PS, PI, or PA (Figure 5A). The CD spectra were curve-fitted by the least-squares method into the reference spectra obtained form five proteins: myoglobin, lysozyme, ribonuclease A, papain, and lactate dehydrogenase (*25*). When PC, PC/PE, PC/PS, or PC/PI vesicles were added, the α-helix content generally increased by 5−9%, while the amount of β-turn decreased (Table 1, Figure 5A). On the other hand, the CD spectrum of P450 1A2 showed an increase of α-helix content by 16% and a decrease of β-sheet content by 24% in the presence of PA (Table 1, Figure 5A). Thus, upon interaction with PA, a rather dramatic change of P450 1A2 structure seems to occur. This indicates that upon interaction with phospholipid vesicles, the α-helix content of P450 1A2 increases.

The intrinsic fluorescence of P450 1A2 was measured in the presence of various types of phospholipid vesicles (Figure 5B). The fluorescence intensity generally increased with increasing concentrations of PE, PS, and PI in the PC vesicles, but there were no appreciable changes in λ_{max}. This result indicates that the change brought about by phospholipids reduces the quenching of the intrinsic fluorescence in the P450 1A2, but otherwise the overall environment of the intrinsic fluorophore appears to remain unchanged. On the other hand, the intensity of intrinsic fluorescence (not λ_{max}) obtained in the presence of PA showed a marked increase with increasing PA concentration. This and the CD results indicate that the overall conformation of P450 1A2 is changed considerably upon interaction with PA containing PC vesicles.

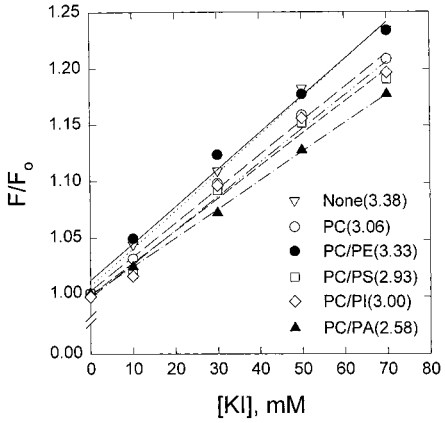

FIGURE 6: Quenching of Trp fluorescence of P450 1A2 by iodide. P450 1A2 (400 pmol) was titrated with KI in the presence and absence of phospholipid vesicles. F_o and F, respectively, are the emission intensity in the absence or in the presence of iodide. Values in the parentheses represent K_{sv}.

P450 1A2 Trp fluorescence quenching by iodide in the presence of the desired vesicles was also examined (Figure 6). The K_{sv} value estimated from the slope was decreased in the presence of PC vesicles containing several types of phospholipid in the order PE > PI > PS > PA, compared to the value of membrane-free P450 1A2. It is clear that more Trp residues in P450 1A2 are sequestered from the aqueous phase and susceptible to KI in the case of the vesicles containing acidic phospholipids. This result provides two possibilities: (i) that a large portion of P450 1A2 is incorporated into membranes, and/or (ii) that the conformational change of P450 1A2 is induced by phospholipids.

DISCUSSION

Current models for the overall membrane topology of the microsomal P450 enzymes propose a large cytoplasmic domain anchored to the membrane by either one or two amino-terminal transmembrane segment(s) (*26*). Although no crystal structure is yet available for the membrane-bound form of the P450 1A2, the membrane topology of P450 1A2 based on prediction (*27*, *28*) and biochemical and biophysical studies (*13*, *29*) has been suggested. Microsomal P450 1A2 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 by a SRP-dependent pathway (*26*−*30*). It also has been shown that the P450 1A2 can penetrate the preformed phospholipid bilayer in liposomes (*13*). However, there is no direct evidence for membrane topology of P450 1A2, although only the N-terminal region of P450 1A2 has been proposed to penetrate the membrane (*29*). It also has been shown that human P450

1A2 (which has a very similar amino acid sequence to rabbit P450 1A2), expressed in bacteria without an N-terminal region, was still bound to the membrane, indicating that other portions of P450 1A2 may interact with the membrane (*31*). It is not possible to determine which of the Trp residue(s) among 8 residues in P450 1A2 is being inserted in this study without extensive replacement studies. The most likely candidate is Trp 25, which is a putative membrane-binding segment of the N-terminus. Although the exact Trp residues involved in the fluorescence studies have not been identified, our results show that the membrane binding and insertion of P450 1A2 are quite dependent upon the type of phospholipids.

This investigation established that the incorporation of P450 1A2 into phospholipid vesicles is markedly influenced by phospholipid composition. All acidic phospholipids (PS, PI, and PA) increased P450 1A2 binding to the lipid bilayer to the same degree, but the extent of penetration into the membrane was strictly dependent on the type of acidic phospholipid. In particular PA, among the acidic phospholipids, was the most efficient in facilitating the penetration of P450 1A2 into lipid bilayers. PE may act as an inert matrix phospholipid, similar to PC, in the interaction of P450 1A2 with membranes. The phospholipid-dependent membrane insertion of P450 1A2 coincided with the change of enzyme activity. The secondary and tertiary structures of P450 1A2 appear to be changed upon membrane binding and subsequent insertion into the phospholipid bilayer, and this conformational change is associated with the altered enzyme activity. Our results indicate that the effect of phospholipids on the membrane binding and insertion of P450 1A2 accompanying its conformational change is effective in the order PE < PS ∼ PI ≪ PA in the PC vesicles.

It has been reported that the composition of phospholipids in liver microsomes is dependent on physiological conditions (*23*, *32*). Sex differences also exist in the phospholipid composition of rat liver microsomes (*33*). The difference in phospholipids has been suggested to be related to the difference in the catalytic activity; the suggestion has been made that human liver phospholipid and P450 synthesis share common regulators and phospholipids are necessary for the maximum rate of P450 synthesis (*34*). Roles of acidic phospholipids have been implicated in vesicular systems containing other P450s (*35*, *36*). The further significance among P450s is under further consideration.

It was shown that the effect of phospholipids on P450 11A1 ($P450_{scc}$) was different from that on P450 1A2 (*37*, *38*). Only PC was very effective in inducing a strong interaction between P450 11A1 and the membrane, whereas other anionic phospholipids stimulated activity and cholesterol exchange. These results may be due to the discriminative structure of P450 11A1 located at the matrix side of the inner mitochontrial membrane, which lacks a transmembrane anchor.

Proteinaceous components (e.g., SRP and SRP receptor) are well-known to be involved in the protein insertion into the ER membrane (*39*, *40*). The role of individual lipids in the membrane insertion of proteins has not been investigated yet, although lipids have been found to contact a nascent membrane protein, as well as a nascent secretory protein, during their insertion into the ER membrane (*41*). The procedure described here may also serve as an adequate model for the study of problems involving the in vivo incorporation of proteins into biological membranes. Our results suggest that the membrane topology of proteins during the membrane insertion and translocation process can be regulated by lipids in addition to the proteinaceous components of the translocation apparatus. The lipid-dependent insertion of transmembrane protein segments might be related to the structural change of proteins induced by phospholipids, as we observed in the case of P450 1A2 in the present and previous studies (*14*, *15*).

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