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PHOSPHOLIPID INTERACTIONS WITH CYTOCHROME *P*-450 IN RECONSTITUTED VESICLES

PREFERENCE FOR NEGATIVELY-CHARGED PHOSPHATIDIC ACID

B. BÖSTERLING, J.R. TRUDELL and H.J. GALLA *

Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305 (U.S.A.)

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Summary

Cytochrome *P*-450 LM2 was reconstituted by the cholate-dialysis method into vesicles containing a mixture of either phosphatidylcholine or phosphatidylethanolamine with up to 50 mol% of phosphatidic acid. Phase transition curves in the presence or absence of cytochrome *P*-450 were obtained from electron paramagnetic resonance experiments by measuring the partitioning of 2,2,6,6-tetramethylpiperidine-1-oxyl. Protein-free phospholipid vesicles exhibit a phase separation into domains of gel phase enriched in phosphatidic acid in a surrounding fluid matrix containing mainly phosphatidylcholine. The phase transition of the phosphatidic acid domains disappeared following incorporation of cytochrome *P*-450 into the bilayers. In contrast, in vesicles containing mixtures of egg-phosphatidic acid and dimyristoyl phosphatidylcholine, the phase transition of the domains enriched in dimyristoyl phosphatidylcholine was less sharp than in the corresponding vesicles containing cytochrome *P*-450. The results of both of these experiments could be explained by a redistribution of the mol fraction of the two phospholipids in the gel phase due to preferential binding of the egg-phosphatidic acid to the cytochrome *P*-450. For comparison, incorporation of cytochrome *P*-450 into uncharged vesicles of dimyristoyl phosphatidylcholine and egg-phosphatidylethanolamine did not alter the

* Present address: Department of Biophysics, University of Ulm, Oberer Eselberg, D-7900 Ulm, F.R.G.
Abbreviations: DMPC, dimyristoyl phosphatidylcholine; DMPA, dimyristoyl phosphatidic acid; egg-PC, phosphatidylcholine extracted from egg; egg-PE, phosphatidylethanolamine extracted from egg; egg-PA, phosphatidic acid prepared from egg-phosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

single symmetric phase transition of the dimyristoyl phosphatidylcholine. In a second set of experiments the effect of reconstituting cytochrome *P*-450 into egg-phosphatidylcholine/egg-phosphatidylethanolamine was studied by measuring the order parameter of spin-labeled egg-phosphatidic acid or egg-phosphatidylcholine. Incorporation of the protein increased the order parameter of the 5-doxyl spin label from 0.800 to 0.830 ± 0.005, indicating a reduction of fluidity. However, in the case of 16-doxyl phosphatidic acid spin label, an additional immobilized component was observed in the spectrum. These results suggest strong interactions between phospholipids and cytochrome *P*-450 with some additional interaction with phosphatidic acid due to its negatively-charged headgroup.

Introduction

The importance of lipid-protein interactions for the monooxygenase system NADPH cytochrome *P*-450 reductase and cytochrome *P*-450 was manifest after the first purifications [1,2]. Early attempts to restore drug metabolism activity [3] with a phosphatidylcholine-containing lipid fraction led to general use of a sodium cholate micellar reconstituted system containing dilauryl phosphatidylcholine [4—6]. High activity was obtained and the phospholipid did not react with substrates or products in studies of drug metabolism. However, in these detergent micelles it was difficult to study phospholipid-protein interactions which may be important in the function of the endoplasmic reticulum.

Cytochrome *P*-450 activity was recently demonstrated in a reconstituted vesicle system whose only lipid is egg-phosphatidylcholine [7,8]. A vesicle reconstitution with a mixture of egg-phosphatidylcholine and egg-phosphatidylethanolamine was found to accommodate a higher mole ratio of proteins in the membrane and to have higher enzymatic activity [9]. ^{31}P-NMR studies showed that this reconstituted system as well as microsomes have an isotropic phospholipid phase present in addition to the 'normal' bilayer structure [10]. In the reconstituted system, the appearance of the isotropic phase was dependent on the presence of cytochrome *P*-450. This is an example of the complex phospholipid-protein interactions in the endoplasmic reticulum [11].

In microsomes there is more protein than lipid (w/w) and up to 20% of the phospholipids are negatively-charged, therefore phospholipid-protein interactions are expected to be of special importance in this membrane. A specific interaction of particular phospholipids with cytochrome *P*-450, at the low lipid to protein ratio in microsomes, could strongly influence enzyme-substrate or protein-protein interactions.

Interactions of cytochrome *P*-450 with different lipids would have implications for drug metabolism if the lipid environment determines the function of cytochrome *P*-450. Phosphatidylcholine is sufficient for reconstitution of metabolic activity; the question arises whether other lipids have a modifying effect on activity or metabolic pathway. Recently the existence of weakly-perturbed regions outside of the first phospholipid shell surrounding a protein was shown in reconstituted cytochrome oxidase complexes [12] and in rod

outer segments [13] by electron paramegnetic resonance (EPR) experiments using spin-labeled lipids.

In this paper we described lipid-protein interactions of cytochrome *P*-450 reconstituted in phospholipid vesicles containing a mixture of either phosphatidylcholine or phosphatidylethanolamine with up to 50 mol% phosphatidic acid. Phosphatidic acid has been used in previous studies of lipid-protein interactions in model membranes [14,15]. The advantage of using a synthetic lipid is the existence of a well-defined lipid phase transition which can be investigated by spectroscopic methods. Mixtures of charged and uncharged phospholipids were prepared such that, at a critical temperature, both fluid and solid phase domains coexist. The mol fraction of each phospholipid in the two domains is different. Large changes in the cooperativity and temperature of the phase separation may be caused by preferential interaction of one of the phospholipids with cytochromes *P*-450 because a change in the mol fraction of the two phospholipids in the gel phase domain will result.

Materials and Methods

Materials. Dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidic acid (disodium salt) (DMPA) were obtained from Fluka. Phosphatidic acid (egg-PA) prepared by phospholipase treatment of phosphatidylcholine extracted from eggs was purchased from Sigma Chemicals. Egg-phosphatidylcholine (egg-PC) was purified from egg yolks [16]. Spin-labeled phosphatidylcholines were prepared from egg phosphatidylcholine and stearic acid spin label carrying the nitroxide group at either C-5 or C-16 of the fatty acid chain [17]. The spin-labeled fatty acids were purchased from Syva. Spin-labeled phosphatidylethanolamine and spin-labeled phosphatidic acid were made by enzymatic head group exchange or phospholipase D cleavage of the spin-labeled phosphatidylcholine [18]. Chromatographic purification was carried out by preparative thin-layer chromatography on silicic acid plates (2 mm thick, 20 × 20 cm) developed in chloroform/methanol/water (65 : 35 : 4, v/v).

Purification of cytochrome P-450 and reconstitution in phospholipid vesicles. Cytochrome *P*-450 LM2 was purified from phenobarbital-treated rabbits [9] to a specific content of 18 nmol/mg protein. Reconstitution in phospholipid vesicles was achieved by modification of the slow cholate dialysis technique that was developed for reconstitution of cytochrome *P*-450 LM2 in phospholipid vesicles consisting of egg-phosphatidylcholine or mixtures of egg-phosphatidylcholine and egg-phosphatidylethanolamine [9]. Phospholipids and cytochrome *P*-450 were solubilized by sodium cholate in 0.3 M potassium phosphate buffer pH 7.5 with 20% glycerol. Sodium cholate was then removed by dialysis against eight changes of 400 ml 0.02 M potassium phosphate buffer pH 7.9 containing 20% glycerol during 3 days under a N_2 atmosphere. The preparations were then dialyzed against 0.1 M potassium phosphate buffer pH 7.0 containing 20% glycerol and pelleted by centrifugation overnight at 45 000 × *g* at the same temperature that the dialysis was carried out.

Reconstitution with egg-PC, egg-PC/DMPA and egg-PC/DMPE. The lipids were mixed in the desired ratios in $CHCl_3/CH_3OH$. The solvent was removed from aliquots containing 30 mg lipid by a stream of N_2 followed by 2 h of high

vacuum. One ml of 0.3 M potassium phosphate buffer at pH 7.5 with 20% glycerol and 40 mg sodium cholate were added to the phospholipids and sonicated in a bath for 1 min at 20°C. The suspension was clarified by shaking in a waterbath at 50°C under N_2 for 1 min. After cooling to 15°C the solubilized lipid was added to the protein solution in the same buffer giving final concentrations of 0.25 mg protein/ml and 1.5% sodium cholate. After an equilibration time of 1 h at 22°C dialysis was started. Dialysis and centrifugation were carried out at 16°C.

Reconstitutions with DMPC. The same procedure was used but the temperature was kept at 22°C throughout the dialysis and centrifugation in order to remain above the fluid-gel phase transition of DMPC. In some cases the preparation was tested for homogeneity before pelleting by density gradient centrifugation in linear 10—60% glycerol gradients at $10^5 \times g$ for 12 h. Absorption spectroscopy measurements showed that cytochrome *P*-450 was not degraded to *P*-420 during the described reconstitutions that and the yield of incorporated protein was 70 to 85%.

Phospholipid vesicles without protein were prepared by the same dialysis procedure as well as by hydration of a lipid film. The lipids were mixed in $CHCl_3/CH_3OH$ (1 : 1, v/v), after evaporation of the solvent, 0.1 M potassium phosphate buffer pH 7.0 containing 20% glycerol was added to a final lipid concentration of 10 mg/ml. The samples were vortexed for 10 min above the lipid phase transition temperature. The homogeneity of the mixed lipid systems were checked by ultracentrifugation as described above for samples containing cytochrome *P*-450.

Electron parametric resonance experiments. Spin-labeled phospholipids (1% of the total lipids) were added to the cholate solution before reconstitution. 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) was added to the pelleted membrane preparations to obtain a final TEMPO concentration of 0.1 mM. The lipids or lipid-protein pellets were homogenized with a Vortex mixer. EPR spectra were measured with a Varian E-104A EPR-spectrometer with on-line digitized recording and curve fitting using a DEC PDP — 11/03 computer. Temperature was maintained by a thermostated nitrogen stream and measured with a thermocouple attached to the outer wall of the sample quartz tube. Spectra of phosphatidylcholine spin labels were analyzed in terms of the order parameter (S) [19]. Phase transition curves were derived from partitioning of the spin probe TEMPO as described by McConnell et al. [20]. The amount of spin probe dissolved in the hydrophobic membrane region (H) and in the water phase (P) was obtained from the intensities of the corresponding spectral components. The partitioning coefficient $f = H/(H + P)$ derived from the EPR-spectra at a given temperature is a measure for the membrane fluidity. The presence of 20% glycerol in the aqueous phase of all samples was required for the stability of cytochrome *P*-450. It had only a small effect on the order parameter and phase transition temperature measurements.

Results and Discussion

Interaction of spin-labeled phospholipids with cytochrome P-450

EPR spectra of 15-doxyl spin-labeled phosphatidylcholine, phosphatidyl-

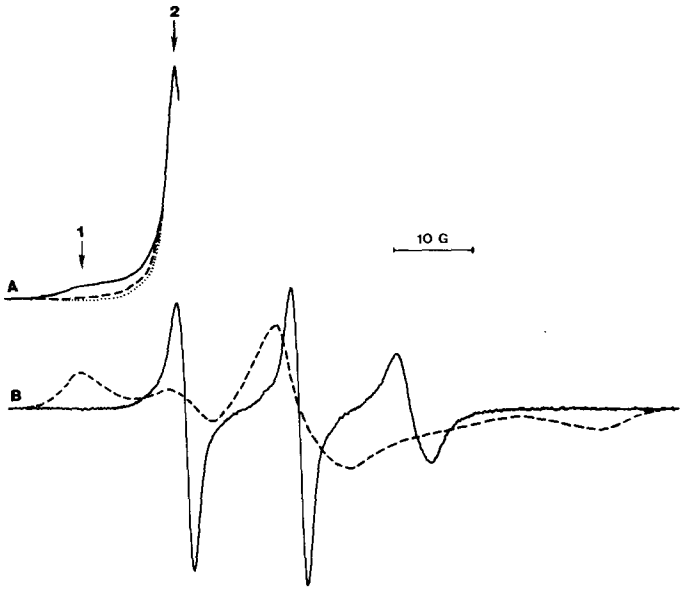

Fig. 1. (A) Low field extrema of EPR-spectra taken from 16-doxyl spin-labeled phosphatidylcholine or phosphatidylethanolamine (- - - - - -) or phosphatidic acid (———) in cytochrome *P*-450 containing egg-PC/PE/PA (16 : 8 : 1) vesicles with a lipid-to-protein ratio of 120 : 1 (mol/mol) and pure lipid vesicles (· · · · · ·). (B) Reference spectra for the multicomponent spectra in A. The dashed curve is the spectrum of $5 \cdot 10^{-6}$ M 16-doxyl spin-labeled phospholipid (phosphatidylcholine, phosphatidylethanolamine or phosphatidic acid) which is completely immobilized on delipidated cytochrome *P*-450 in solution. The spectrum of spin labeled 16-doxyl phosphatidylcholine in pure phospholipid vesicles (———) is a reference spectrum for the fluid part of the membrane (arrow 2 in A).

ethanolamine or phosphatidic acid incorporated in the membrane of reconstituted vesicles in the presence or absence of cytochrome *P*-450 were measured (Fig. 1). The vesicles contained egg-phosphatidylcholine/egg-phosphatidylethanolamine/dipalmitoyl phosphatidic acid in a ratio of 16 : 8 : 1 (w/w), a ratio similar to the lipid composition of the endoplasmic reticulum. The lipid to protein ratio was 120 : 1 mol/mol (2 : 1, w/w). Fig. 1B shows EPR reference spectra of a spin label in pure phospholipid vesicles and of a spin label ($5 \cdot 10^{-6}$ M) immobilized on delipidated cytochrome *P*-450 in solution in order to illustrate that Fig. 1A may be reconstructed as a superposition of two components of different fluidity.

An immobilized component (arrow 1) and a component of high fluidity (arrow 2) are seen in Fig. 1A which shows the low field hyperfine extrema of the spectra of spin-labeled phosphatidic acid (———), spin-labeled phosphatidylcholine or phosphatidylethanolamine (- - - - - -) in vesicles containing cytochrome *P*-450. The peak under arrow 1 relative to the one under arrow 2 is much higher in the case of phosphatidic acid spin labels than for phosphatidylcholine or phosphatidylethanolamine spin labels. There is very little immobilized component (arrow 1) in the spectra of any of the spin labels in pure lipid vesicles in the absence of the protein as demonstrated by the dotted line in Fig. 1A. Due to the broad line width of the immobilized spectrum, intensities at position 1 and 2 cannot be compared in order to get the amount of lipid in

each phase. However, qualitatively more phosphatidic acid spin label is immobilized than phosphatidylcholine or phosphatidylethanolamine spin label on the EPR time scale of 10^{-8} s.

The effect of cytochrome *P*-450 on the order parameter of 5-doxyl phosphatidylcholine spin label was measured in vesicles of egg-PC and egg-PE (2 : 1) at a lipid-to-protein ratio of 300 : 1. The lipid-protein interaction resulted in a parallel shift of the order parameters (S) versus temperature curve to higher S values. A value of S approaching one gives evidence for a rigid lipid matrix, a low value of S describes higher fluidity. The value obtained in the above bilayer in the absence of protein at 20°C is $S = 0.800 \pm 0.005$. Incorporation of cytochrome *P*-450 in a molar ratio of 1 : 300 with respect to the lipid increases the order parameter to $S = 0.830$. Qualitatively, the presence of the protein had the same effect as decreasing the temperature by 6°C. Equivalent results were obtained at temperatures between 5 and 35°C. This result suggests that the increase in order is due to some interaction of the egg-PC bilayer with cytochrome *P*-450. Possible mechanisms for the observed decrease in fluidity include short-range reduction of segmental motion of the fatty acid chains by the protein and long-range alterations in lateral pressure.

The effect of cytochrome P-450 on the phase transition of DMPC-vesicles

Phase transition curves of reconstituted DMPC-vesicles in the absence and presence of cytochrome *P*-450 were measured using the TEMPO-partitioning method. Fig. 2 shows the temperature dependence of the fluidity parameter [20] as a function of the mol fraction of protein. Pure DMPC-vesicles without protein obtained by the dialysis preparation technique in the presence of 20% glycerol show a lipid phase transition with a midpoint at $T_m = 21$°C which is two degrees lower than in vortexed multilamellar liposomes. This is due to the smaller size and curvature of the vesicles, as compared to liposomes, and not due to residual sodium cholate. Addition of sodium cholate to vortexed multilamellar liposomes resulted in a long-tailed and broadened but not lowered phase transition.

Incorporation of cytochrome *P*-450 into a phospholipid bilayer causes two effects: the lipid phase transition temperature is increased and the width of the transitions is broadened. Moreover a flattening of the higher temperature region of the transition is observed between arrows 1 and 2 in Fig. 2. At a low lipid-to-protein ratio of 180 : 1 mol/mol (3 : 1, w/w) the temperature range of the transition is from 19 to 45°C.

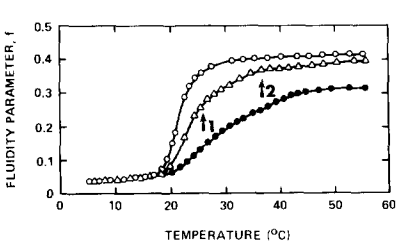

Fig. 2. Phase transition curves of DMPC-vesicles in the absence (○———○) and presence of cytochrome *P*-450 with lipid-to-protein ratio of 600 : 1 (△———△) and 180 : 1 mol/mol (●———●).

The effect of incorporated cytochrome P-450 on phase transition curves of mixed membranes containing egg-PC and DMPA

The phase transition curves of mixed membranes containing egg-PC and DMPA in different relative concentrations are shown in Fig. 3 in the absence and presence of cytochrome *P*-450. Again, phase transition curves were obtained by measuring TEMPO-partitioning as a function of temperature. No phase transition is observable in pure egg-PC membranes; incorporation of cytochrome *P*-450 leads to a decreased fluidity over the whole temperature range. This result is in agreement with measurements of phospholipid spin labels in the same system described above. Fig. 3 b—d show the phase transition curves of phospholipid mixtures containing increasing amounts of DMPA. In the absence of cytochrome *P*-450 a clearly visible phase transition is observed between 20 and 40°C. This shows that phosphatidylcholine and phosphatidic acid are not randomly distributed within the membrane but form lateral phase separations. At low temperature, gel phase domains enriched in phosphatidic acid coexist with domains containing mainly phosphatidylcholine so that the phase transition of DMPA is still measurable in the presence of egg-PC. The phosphatidic acid domains exhibit a phase transition temperature of about 35°C which is lower than pure DMPA bilayers (T_m = 47°C). This finding shows that the phosphatidic acid domains contain some egg-PC which lowers the phase transition temperature. This finding is consistent with the results obtained by Galla and Sackmann [21] in mixed phosphatidic acid/phosphatidyl-

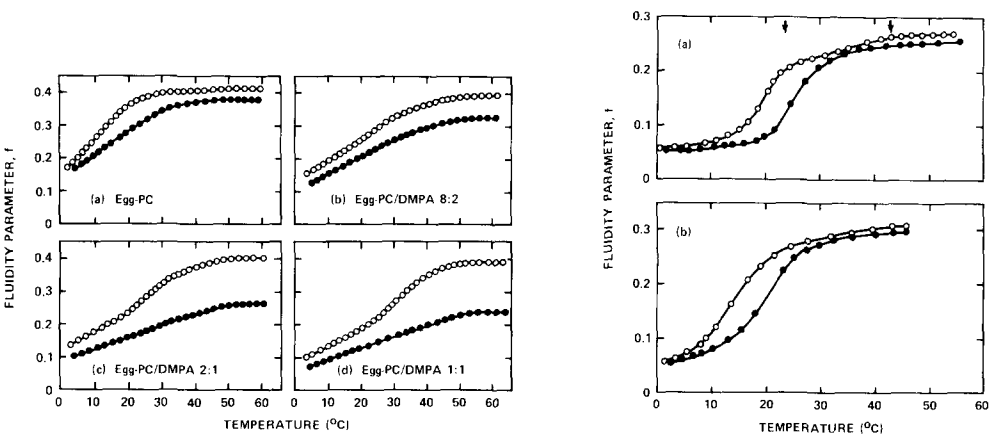

Fig. 3. Phase transition curves of mixtures of egg-PC and DMPA in different ratios. Transition curves of preparations without protein (○———○) are compared to cytochrome *P*-450 containing vesicles (●———●). (a) Pure egg-PC, lipid-to-protein ratio 300 : 1 mol/mol. (b) Mixture of egg-PC and DMPA in a 8 : 2 ratio (w/w), lipid-to-protein ratio 300 : 1 mol/mol. (c) Mixture of egg-PC and DMPA in a 2 : 1 ratio, lipid-to-protein ratio 180 : 1 mol/mol. (d) 1 : 1 mixture of egg-PC and DMPA, lipid-to-protein ratio 180 : 1 mol/mol.

Fig. 4. (a) Phase transition curve of reconstituted mixed vesicles containing DMPC and egg-PA in a 3 : 1 (w/w) ratio without (○———○) and with cytochrome *P*-450 (●———●) at a lipid-to-protein ratio of 300 : 1 mol/mol. The arrows point to the beginning and the end of the melting of a lipid phase which is only detectable if cytochrome *P*-450 is not present. (b) Phase transition curve of DMPA egg-PE vesicles (3 : 1, w/w) in the absence (○———○) and presence (●———●) of cytochrome *P*-450 in a lipid-to-protein ratio of 300 : 1. Note that the sigmoidal shape of the curve is not changed by the interaction with the cytochrome *P*-450.

choline bilayers in which they analyzed the spin exchange interaction when one lipid component was spin labeled.

In a 1 : 1 mixture of DMPA and egg-PC the phase transition of DMPA disappears in the presence of cytochrome *P*-450 (Fig. 3d). The phase transition curve in the presence of the protein resembles that of a vesicle containing little DMPA. A possible explanation of this result is that cytochrome *P*-450 interacts preferentially with the negatively-charged DMPA and thereby decreases the amount of free phosphatidic acid to the point where the phosphatidic acid concentration is too low to form a separate phase.

The effect of cytochrome P-450 on mixed DMPC/egg-PA and DMPC/egg-PE bilayers

In a mixture of DMPC/egg-PA (3 : 1 mol/mol) we observed the phase transition of DMPC due to a phase separation taking place in the membrane. Again, this phase transition is lower than that of pure DMPC. Moreover, in this lipid mixture a second domain of lipids melting between 23 and 40°C (Fig. 4a) is observed in the absence of cytochrome *P*-450. Reconstituted vesicles containing cytochrome *P*-450 in a lipid-to-protein ratio of 300 : 1 mol/mol or less do not exhibit this second higher melting phase, but instead, a sigmoidal melting curve (Fig. 4a) with a 5°C higher phase transition temperature. In accordance with the result from experiments with egg-PC and DMPA we interpret this as preferential binding of egg-PA to cytochrome *P*-450 leading to a more pure DMPC phase.

The melting curve of a mixture of DMPC and egg-PE at a 3 : 1 molar ratio has been reported to show no phase separations [21]. The phase transition temperature (Fig. 4b) is lowered by about 10°C with respect to pure DMPC and is very broad. By comparison, in the egg-PA/DMPC mixture described above (Fig. 4a), the DMPC phase transition was lowered by only 3°C with respect to pure DMPC. The greater depression of T_m gives evidence for the assumption of a random mixture of the uncharged phospholipids in the case of a DMPC/egg-PE vesicle. In this case, incorporation of cytochrome *P*-450 increased the phase transition temperature but did not change the shape of the phase transition curve. This suggests that DMPC and egg-PE have about the same affinity for cytochrome *P*-450 and preferential removal of free egg-PE with the resulting formation of a DMPC gel phase is not observed.

Conclusion

The endoplasmic reticulum membrane contains high amounts of negatively-charged lipids; phosphatidylserine and phosphatidylinositol are present up to a molar content of 20%. These natural lipids are mainly unsaturated and therefore in the fluid state. Mixtures of phosphatidylcholine and phosphatidic acid were used in this study as model substances for the uncharged and charged components. An advantage of synthetic lipids is their well-known thermotropic phase transitions. Therefore in every mixture we always used one saturated component: dimyristoyl phosphatidylcholine (T_m = 23°C) or dimyristoyl phosphatidic acid (T_m = 47°C). This allowed us to monitor changes in the phase transition caused by the interaction of cytochrome *P*-450 with the lipid matrix.

The first indication for a preferential interaction of cytochrome *P*-450 with negatively-charged phosphatidic acid was obtained during our reconstitution experiments. It was not possible to obtain a homogeneous vesicle population of the mixed system in the absence of cytochrome *P*-450. Instead we obtained vesicles of phosphatidic acid as well as vesicles with phosphatidylcholine as the main component as was shown by density gradient centrifugation and phase transition curves. However, if vesicles were reconstituted in the presence of cytochrome *P*-450, only one population of vesicles was obtained. Thus, cytochrome *P*-450 mediates in the formation of mixed vesicles. The strong interaction between cytochrome *P*-450 and phosphatidic acid as compared to phosphatidylcholine and phosphatidylethanolamine was shown directly in the EPR-spectra of the corresponding spin-labeled lipids. Only with spin-labeled phosphatidic acid did we observe an immobilized component in the presence of cytochrome *P*-450.

In addition to the preferential interaction of cytochrome *P*-450 with phosphatidic acid we also demonstrated strong interaction with phosphatidylcholine and phosphatidylethanolamine. In DMPC-vesicles, for example, we observed an increase in phase transition temperature and a broadening of the phase transition. Moreover, with DMPC we observed the appearance of a second high melting phase in the presence of cytochrome *P*-450. These results can be interpreted in terms of a perturbed membrane in which the protein strongly interacts with the phospholipids in its immediate surroundings. In the mixed egg-PC/DMPA vesicles we observed the disappearance of the DMPA phase transition in the presence of cytochrome *P*-450. This provides further evidence for a preferential binding of phosphatidic acid to cytochrome *P*-450.

Most of the binary mixtures of phospholipids used in this study undergo lateral phase separations into domains of gel phase and fluid phase phospholipids. Both of these domains contain a substantial mole fraction of the second lipid component [21]. The mol fraction of the higher-melting phospholipid in the gel phase domain affects the cooperativity and temperature of the thermal phase transition. However, incorporation of cytochrome *P*-450 leads to a redistribution of the phospholipids in each domain. Charged phospholipids are concentrated in the region surrounding the proteins although they do not necessarily form a lipid halo. This preference for negatively charged lipids is an additional component of the very pronounced lipid-protein interactions in a membrane between cytochrome *P*-450 and all phospholipids such as phosphatidylcholine and phosphatidylethanolamine.

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