Phosphatidic acid–phosphatidylethanolamine interaction and apocytochrome *c* translocation across model membranes

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The translocation of apocytochrome *c* (apocyt.c) across large unilamellar vesicles (LUVs) constructed from mixtures of anionic and zwitterionic phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), has been studied. It was shown that the import ratio of horse heart apocyt.c in LUVs composed of phosphatidic acid (PA) combined with PE and PC ($62 \pm 10\,\%$) was much higher than that in LUVs made of PE and PC plus any other acidic phospholipid species ($20 \pm 5\,\%$). This feature was shared by tuna heart and chicken heart apocyt.c. In addition, the greater efficiency of the PA/PE/PC system versus others in facilitating apocyt.c translocation was maintained using synthetic anionic phospholipids with the same acyl chains. Besides, apocyt.c induces more leakage of entrapped fluorescein sulphonate (FS) from the interior of PA/PC/PE vesicles compared with phosphatidylglycerol (PG)/PC/PE ones. By measuring the intrinsic fluorescence emission spectrum and the accessibility of the preprotein to the fluorescence quencher, acrylamide, differences could be detected in the conformational changes of apocyt.c as a consequence of its interaction with PA/PE/PC and PG/PE/PC vesicles, respectively. Particularly notable is that PE is indispensable for the PA/PE/PC system to most efficiently facilitate apocyt.c translocation across the model membranes. With the fraction of PE increasing from 0 to 30 mol%, the translocation efficiency of apocyt.c as well as its ability to induce FS efflux was significantly enhanced in PA-containing LUVs, whereas this was not observed in the case of replacement of PA by PG or phosphatidylserine. It is also interesting to note that in LUVs containing PA, dioleoyl-PE, but not dielaidoyl-PE, can exert such influences, indicative of the role of non-bilayer formation propensity. On the basis of these results it is postulated that PA might increase the bilayer-destabilizing effects of PE, and hence increase the translocation efficiency of apocyt.c and its leakage-induction ability.

Key words: large unilamellar vesicle, non-bilayer lipid, PA–PE interaction.

INTRODUCTION

Apocytochrome *c* is the haem-free precursor of cytochrome *c*, which is synthesized in the cytosol and post-translationally imported into mitochondria by following a quite unique pathway compared with other mitochondrial precursor proteins [1]. It does not possess a cleavable N-terminal presequence, and neither ATP nor a membrane potential is required for its import. Further, no proteinaceous component responsible for its import has ever been identified. Although much effort has been made to uncover the mystery of its import [2–6], a conclusive mechanism remains ambiguous.

At physiological pH, soluble apocytochrome *c* is positively charged. Therefore, in membranes, anionic phospholipids are important targets of this preprotein. De Kruijff and co-workers [7–10] studied in detail the interaction between horse heart apocytochrome *c* and phospholipids using model membranes, mainly composed of mixtures of anionic phospholipids (e.g. L-α-phosphatidyl-L-serine, PS) and the zwitterionic phospholipid L-α-phosphatidylcholine (PC). Also, the dependency of apocytochrome *c* translocation on different anionic phospholipid species was studied in large unilamellar vesicles (LUVs) composed of 50 mol% PC and 50 mol% another anionic phospholipid [11]. The efficiency of the anionic phospholipids to facilitate apocytochrome *c* translocation was found to be in the order cardiolipin (CL) > L-α-phosphatidylinositol (PI) ≈ PS. However, the differences between them were not pronounced. In the present paper we will show that the import ratio of apocytochrome *c* into LUVs composed of L-α-phosphatidic acid (PA) combined with L-α-phosphatidylethanolamine (PE) and PC is much higher than that into LUVs made of PE and PC plus any other acidic phospholipids. In order to test whether the presence of 1,2-dioleoyl-*sn*-glycerol-3-PE (DOPE), which is well known as a kind of non-bilayer-forming phospholipid [12], influences the translocation process, Rietveld et al. [11] studied the import of apocytochrome *c* in DOPE/bovine brain PS (1:1) vesicles. It was reported that the presence of DOPE didn't stimulate the translocation of apocytochrome *c* across the membranes. However, we found that, in the LUVs containing PA instead of PS or L-α-phosphatidyl-DL-glycerol (PG), PE strongly enhances apocytochrome *c* translocation across model membranes as well as its ability to induce the leakage of entrapped fluorescein sulphonate (FS). Therefore, studies on the PA–PE interaction and apocytochrome *c* translocation are carried out in this work. We postulate that PA might play a role to facilitate the formation of non-lamellar structures by PE, and hence to stimulate apocytochrome *c* translocation across the model membranes.

MATERIALS AND METHODS

Materials

All chemical reagents were of the highest purity available commercially. Egg yolk PC, bovine liver PE and 1,2-dielaidoyl-

Abbreviations used: CL, cardiolipin; DEPE, 1,2-dielaidoyl-*sn*-glycerol-3-phosphatidylethanolamine; DOPA, 1,2-dioleoyl-*sn*-glycerol-3-phosphatidic acid; DOPE, 1,2-dioleoyl-*sn*-glycerol-3-phosphatidylethanolamine; DSPA, 1,2-distearoyl-*sn*-glycerol-3-phosphatidic acid; FS, fluorescein sulphonate; AEDANS, *N*-(acetyl)-*N*′-(5-sulpho-1-naphthyl)ethylenediamine; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulpho-1-naphthyl)ethylenediamine; LUV, large unilamellar vesicle; PC, L-α-phosphatidylcholine; PA, L-α-phosphatidic acid; PE, L-α-phosphatidylethanolamine; PG, L-α-phosphatidyl-DL-glycerol; PI, L-α-phosphatidylinositol; PS, L-α-phosphatidyl-L-serine.

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sn-glycerol-3-PE (DEPE) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). The other phospholipids as follows were products of Sigma (St. Louis, MO, U.S.A.): natural phospholipids, including egg yolk PA and PG, bovine liver PI, bovine heart CL and bovine brain PS; and synthetic phospholipids, including 1,2-dimyristoyl-*sn*-glycerol-3-phosphatidic acid, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidic acid, 1,2-dioleoyl-*sn*-glycerol-3-phosphatidic acid (DOPA), 1,2-distearoyl-*sn*-glycerol-3-phosphatidic acid (DSPA), 1,2-dimyristoyl-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol)], 1,2-dipalmitoyl-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol)], 1,2-dioleoyl-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol)] and 1,2-distearoyl-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol)]. *N*-(Iodoacetyl)-*N*′-(5-sulpho-1-naphthyl)ethylenediamine (IAEDANS) and FS were obtained from Molecular Probes (Junction City, OR, U.S.A.). Acrylamide, cytochrome *c* (horse heart type VI, tuna heart and chicken heart), trypsin and soya bean trypsin inhibitor were also purchased from Sigma.

A PSE buffer (10 mM Pipes, pH 7.0/50 mM NaCl/0.2 mM EDTA) was prepared from double-distilled water. Apocytochrome *c* was prepared from cytochrome *c* by removal of the haem moiety and subjection to a renaturation procedure as described previously [13]. Protein concentration was determined using a molar absorption coefficient of $10\,580\ M^{-1}\cdot cm^{-1}$ at 277 nm [14]. Renatured protein in PSE buffer containing 0.01 % β-mercaptoethanol was frozen in liquid nitrogen and stored in aliquots at −40 °C, and always thawed just before use.

Preparation of vesicles

Desired amounts of lipids were mixed in chloroform at the ratios indicated for the specific experiments and taken to dryness under a stream of nitrogen, and the lipids lyophilized overnight. Liposomes with large internal aqueous spaces and high capture were prepared by reverse-phase evaporation according to the method of Szoka and Papahadjopoulos [15], which was described by Rietveld et al. [11]. The final vesicle pellet was suspended in about 200 μl of buffer, and aliquots were drawn for determination of phosphorus [16] and the trapped volume. The final vesicle yield ranged from 10 to 30 %, and vesicles composed of the different lipid mixtures used showed an internal volume of 20–30 μl/μmol of P_i.

Small unilamellar vesicles used in fluorescence experiments were prepared by sonication with a bath sonicator, Bransonic 12. A dry lipid film of phospholipids was hydrated and vortexed to form multilamellar vesicles in PSE buffer, following sonication for 45–60 min in ice-cold water to clarity. The sonicated vesicles were then placed at 0 °C (above T_c, the transition temperature from the gel to the liquid-crystalline phase) for 2 h. Undispensed lipids and multilamellar vesicles were pelletted and removed by centrifugation (20 min, 100 000 ***g***, 4 °C).

Translocation assay and *N*-(acetyl)-*N*′-(5-sulpho-1-naphthyl)ethylenediamine (AEDANS) labelling

Apocytochrome *c* translocation across the lipid bilayer of the LUVs containing trypsin was assayed essentially as described by Tong et al. [13].

Apocytochrome *c* labelled with IAEDANS was prepared as described previously [17]. The stoichiometry of AEDANS groups in the modified protein was 1.8–2.0 AEDANS groups/protein molecule as determined spectrophotometrically.

Fluorescence experiments

All fluorescence measurements were performed at 30 ± 0.1 °C with a Hitachi F-4010 spectrofluorimeter equipped with a thermostatically controlled cell block and polarization accessory. Fluorescence spectra were recorded by the spectrofluorimeter operating in spectrum correction and CAT (computing for averaging transient, repeating four times) modes with spectral bandwidths of 5 nm for both excitation and emission. The absorbances at the excitation wavelength of the protein samples used were never more than 0.05, and the Raman scatter contribution was removed by subtraction of a buffer blank. Wavelengths at which maximum emission occurred (E_{max}) were determined from the first differential of the corrected emission spectrum.

After incubation of apocytochrome *c* with liposomes for 30 min at 30 °C, acrylamide quenching experiments were carried out at an excitation wavelength of 295 nm. Acrylamide aliquots were added from a 8 M stock solution; fluorescence was monitored at 340 nm. The values obtained were corrected for dilution, the scatter contribution and the absorptive screening by acrylamide. Data were analysed according to the Stern–Volmer equation: $F_0/F = 1 + K\text{sv}[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of quencher, [Q] is the molar concentration of quencher and *K*sv is the apparent Stern–Volmer quenching constant for considering the static quenching by acrylamide. For practical reasons, it was decided to use the slope of a linear fit of the Stern–Volmer plot for concentrations up to 60 mM as a criterion for the apoprotein accessibility to acrylamide [18].

After incubation of AEDANS-labelled samples with liposomes for 30 min at 30 °C, and equilibrium with stirring for 15 min, steady-state polarization measurements were carried out on AEDANS-labelled apocytochrome *c*. The intensities of the horizontal and vertical components of the emitted light ($I_{0,0}$ and $I_{0,90}$) were measured. Thus polarization (P) was calculated according to:

$$G = \frac{I_{90,0}}{I_{90,90}}$$

and

$$P = \frac{(I_{0,0} - G \times I_{0,90})}{(I_{0,0} + G \times I_{0,90})}$$

where G is the grating correction factor. Each value is the mean of 10 measurements.

FS leakage determination

Changes in vesicle permeability due to protein association can be determined quantitatively by employing impermeant fluorescent markers that undergo self-quenching, as was previously described [19]. However, upon addition of apocytochrome *c*, severe aggregation of LUVs, for which fluorescence was grossly attenuated, made this method unfeasible. Here, LUVs containing FS (10 mM) were prepared by reverse-phase evaporation, and separated from extravesicular FS by washing. Following incubation of increasing amounts of apocytochrome *c* with 50 nmol of LUVs in 100 μl of PSE buffer at 30 °C for 30 min, the vesicles were sedimented by centrifugation (25 min, 35 000 ***g***, 4 °C). Half of the supernatant (S) was sampled out, to which, and to the remainder (R) in the centrifugation tube as well, 350 μl of PSE buffer containing 0.1 % Triton X-100 was added to dilute and dissolve the samples. The control followed the same procedure except for the addition of buffer instead of apocytochrome *c*. FS contained in the two parts of the solution was quantified using a spectrofluorimeter, and the fluorescence intensity corrected for self-quenching according to the standard curve of fluorescence versus [FS]. FS was excited at 490 nm and emission

at 520 nm was recorded with spectral bandwidths of 1.5 and 3 nm for excitation and emission, respectively. The percentage of FS leakage, therefore, was determined by:

$$\text{FS leakage } (\%) = \left[\frac{2 \times S}{S+R} - B \right] \times 100$$

where B is the leakage extent of the control.

RESULTS

Translocation of apocytochrome *c* across LUVs composed of various anionic phospholipids

Because apocytochrome *c* strongly interacts with negatively charged phospholipids, Rietveld et al. [11,20] investigated and compared the translocation of horse heart apocytochrome *c* across the vesicular membranes composed of equimolar amounts of PC and the negatively charged phospholipids CL, PS or PI. The lipid dependency of apocytochrome *c* translocation across vesicles containing different anionic phospholipid species was found in the order CL > PI ≈ PS. However, only small differences between them could be observed.

In the present study on the translocation of apocytochrome *c* across model membranes, PA, a quantitatively minor constituent of outer membrane of mitochondria [21], was also employed to be compared with other acidic phospholipids. Herein, a system composed of PC, PE and an acidic phospholipid species with the molar proportions 5:3:2 was used. The time-dependent digestion of apocytochrome *c* by enclosed trypsin was shown in Figure 1. Very surprisingly, the rate of horse heart apocytochrome *c* being digested in PA/PC/PE LUVs is about 2–3 times as high as that in vesicles containing any other acidic phospholipid (Figure 1). Similar results were obtained if tuna heart or chicken heart apocytochrome *c* were employed (results not shown). That is, the translocation rate of different apocytochrome *c* species tested in LUVs composed of PA/PC/PE is much higher than that in PG/PC/PE LUVs.

Furthermore, comparison of apocytochrome *c* translocation across LUVs composed of PA (Figure 2A) and PG (Figure 2B) with identical acyl-chain compositions was carried out. The components of zwitterionic phospholipids PC and PE were the same in all the systems. As shown in Figure 2, the import rates of apocytochrome *c* in PA-containing LUVs are markedly higher than those in PG-containing ones.

In the case of PA-containing LUVs, the translocation rate is sensitive to the variation in the acyl-chain length and unsaturation. Increasing chain length leads to a progressive reduction in the translocation efficiency (Figure 2A). A single *cis* double bond in the acyl chains results in an increase in lipid fluidity, and hence enhances the translocation as revealed by comparison of DOPA with DSPA.

Comparison of FS efflux from LUVs composed of PA/PC/PE and PG/PC/PE upon addition of apocytochrome *c*

To understand further the difference of apocytochrome *c* interaction with PA/PC/PE and PG/PC/PE vesicles, the entrapped-FS release experiments were carried out to investigate the effects of the apocytochrome *c* association on the barrier properties of both vesicles. FS was enclosed in the LUVs, and its efflux was measured following addition of apocytochrome *c* to

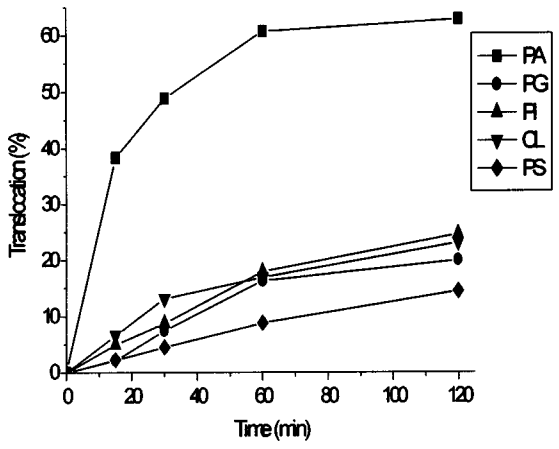

Figure 1 Comparison of apocytochrome *c* translocation across LUVs containing different acidic phospholipids

Trypsin-containing vesicles (108 nmol of P_i) of 50 mol% PC, 30 mol% PE and 20 mol% PA, PG, PI, CL or PS were incubated with 40 μg of horse heart apocytochrome *c*. The mean translocation from three experiments is shown (S.D. ±5–10% of the translocation percentages shown).

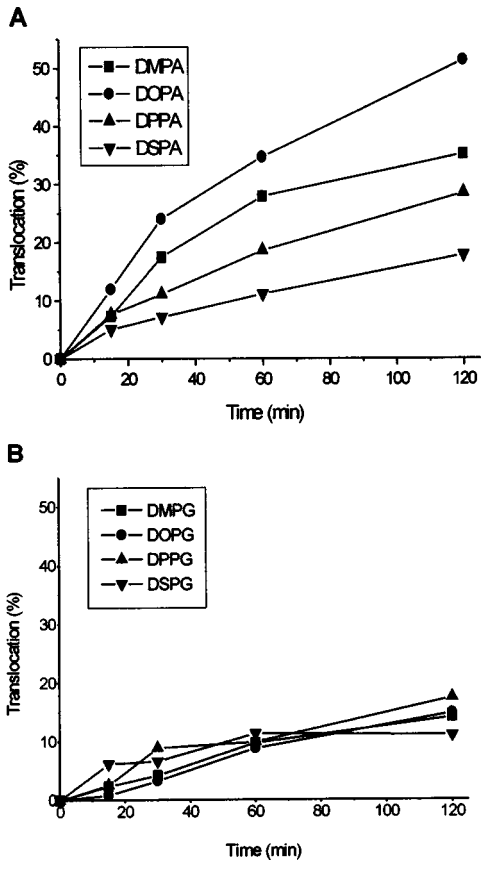

Figure 2 Comparison of apocytochrome *c* translocation across model membranes with different acyl-chain length and unsaturation of PA (A) or PG (B)

Digestion of apocytochrome *c* was measured in incubation mixtures of trypsin-containing vesicles (108 nmol of P_i) composed of 50 mol% PC, 30 mol% PE and 20 mol% PA or PG bearing different acyl chains, incubated with 40 μg of horse heart apocytochrome *c*. DMPA, 1,2-dimyristoyl-*sn*-glycerol-3-phosphatidic acid; DPPA, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphatidic acid; DMPG, 1,2-dimyristoyl-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol)]; DPPG, 1,2-dipalmitoyl-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol)]; DOPG, 1,2-dioleoyl-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol)]; DSPG, 1,2-distearoyl-*sn*-glycerol-3-[phospho-*rac*-(1-glycerol)].

the vesicle suspensions with a spectrofluorimeter. As shown in Figure 3, the difference in the extent of dye leakage from the PA/PC/PE and PG/PC/PE vesicles is pronounced and in agreement with that in the apocytochrome *c* translocation assay. In order to test whether vesicle fusion takes place after apocytochrome *c* addition, experiments were performed by using lipid-mixing assays. The results showed that in both PA/PC/PE and PG/PC/PE vesicles, apocytochrome *c* translocation and induced leakage were not related to the occurrence of vesicle fusion (results not shown).

Comparison of conformational changes following the interaction of apocytochrome *c* with PA/PC/PE and PG/PC/PE LUVs

It is generally suggested that due to the interaction of apocytochrome *c* with the phospholipids in model membranes, which is initially electrostatic [11,20], and secondarily hydrophobic [11,22], the protein conformation has been changed remarkably. Such changes in the protein conformation are thought to be the driving force for apocytochrome *c* transfer from an aqueous compartment into membranes [11,22]. Thus it can be questioned whether there is any difference in the conformational changes resulting from the interactions of apocytochrome *c* with the PA/PC/PE and PG/PC/PE vesicles. Three methods, as follows, were applied to measure the differences in the conformational changes.

Intrinsic fluorescence determination

Apocytochrome *c* from most species has one tryptophan residue at position 59, which dominates the fluorescence spectrum. Association of apocytochrome *c* with membranes will result in a blue shift of the peak fluorescence intensity (λ_{max}) and an enhancement of the emission intensity (I). Using an excitation wavelength of 295 nm, the fluorescence emission spectrum is dominated by the Trp fluorescence, whereas tyrosine and phenylalanine residues make small contributions to the overall intrinsic protein fluorescence. Tuna heart apocytochrome *c* has two tryptophan residues (at positions 59 and 33), which assist in observing the changes in λ_{max} and I with relatively less interference from apocytochrome *c*-induced vesicle aggregation. Fluorescence emission spectra of tuna heart apocytochrome *c* following interaction with PA/PC/PE or PG/PC/PE vesicles are shown in Figure 4, and the emission spectrum of apocytochrome *c* in solution served as a control. The results obtained demonstrate that Trp residues are located in a more hydrophobic environment in PA/PE/PC LUVs.

Quenching of the intrinsic fluorescence of apocytochrome *c* by acrylamide

In order to study and compare the membrane-integrated state of apocytochrome *c* following the interaction with PA/PC/PE and PG/PC/PE LUVs, the quenching of the intrinsic fluorescence of apocytochrome *c* by acrylamide was measured. The results shown in Figure 5 indicate clearly that the quenching efficiency of intrinsic fluorescence of apocytochrome *c* was lower in the PA/PC/PE vesicles than that in PG/PC/PE ones. This may mean that the preprotein penetrated more efficiently into the hydrophobic core of PA/PC/PE bilayers.

Steady-state fluorescence polarization measurement of IAEDANS-labelled apocytochrome *c*

The two SH groups of cysteines at positions 14 and 17 in apocytochrome *c* can be labelled with IAEDANS. Changes in the steady-state fluorescence polarization of the probe upon

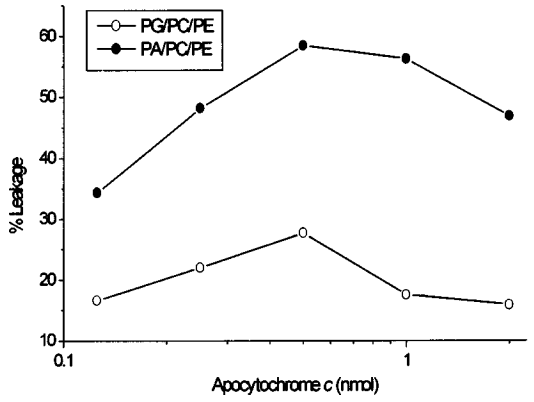

Figure 3 Comparison of leakage of entrapped FS induced by apocytochrome *c* from PA/PC/PE and PG/PC/PE LUVs

Increasing amounts of horse heart apocytochrome *c* were incubated with 500 μM of PA/PC/PE or PG/PC/PE LUVs. Each value is an average of three experiments with three different vesicle preparations of the same liposome formulation. The final extents of leakage for a given amount of apocytochrome *c* varied by no more than $\pm$6% when the extents of leakage were above 20% and by no more than $\pm$3% below 20% leakage. The experimental conditions were described in detail in the Materials and methods section.

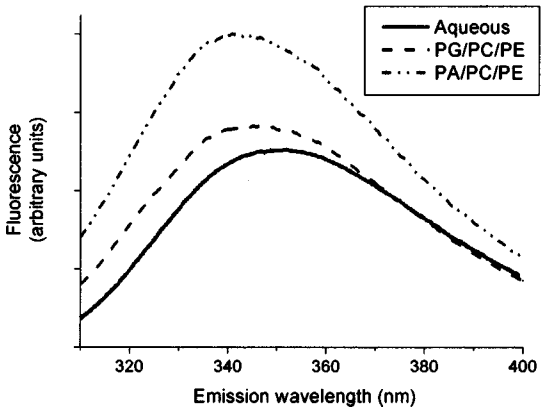

Figure 4 Difference in fluorescence emission spectra of apocytochrome *c* following addition of PA/PC/PE and PG/PC/PE small unilamellar vesicles

Tuna heart apocytochrome *c* (2 μM) was incubated with 200 μM PA/PC/PE or PG/PC/PE small unilamellar vesicles in PES buffer at 30 °C. Spectra were recorded at 30 °C, using an excitation wavelength at 295 nm. Spectral bandwidths were 5 nm for both excitation and emission. E_{max}, wavelength of maximum emission in nm, calculated by taking the first differential of the corrected, baseline-adjusted spectrum: free (aqueous), 351 nm; + PA/PC/PE, 341 nm; + PG/PC/PE, 345 nm.

addition of PA/PC/PE and PG/PC/PE vesicles were compared. Accompanying apocytochrome *c* recruitment into the lipid bilayer from the aqueous solution was an increase in AEDANS polarization from 0.042 ± 0.002 to 0.065 ± 0.002 and 0.073 ± 0.002 in PG/PC/PE and PA/PC/PE vesicles, respectively (means $\pm$ S.D.). This may indicate that the motional freedom of apocytochrome *c* tends to decrease as a consequence of interaction of the preprotein with lipids, but the difference in the PA- and PG-containing LUVs was not very significant.

The above-mentioned data indicate clearly different lipid-induced conformational changes and membrane-integrated states of the preprotein in vesicles containing PA compared with those in vesicles containing PG. On the other hand, the experiments on apocytochrome *c*-induced FS efflux demonstrated that the per-

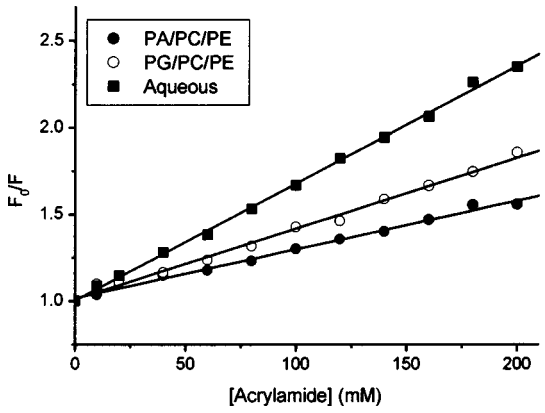

Figure 5 Stern–Volmer plots of aqueous quenching of intrinsic fluorescence of apocytochrome *c* by acrylamide in the presence of PA/PC/PE or PG/PC/PE small unilamellar vesicles

Tuna heart apocytochrome *c* (2 μM) was incubated with 200 μM PA/PC/PE or PG/PC/PE small unilamellar vesicles in PES buffer at 30 °C. Spectra were recorded at 30 °C, using an excitation wavelength at 295 nm. Spectral bandwidths were 5 nm for both excitation and emission. Apparent quenching constants, *K*sv, were determined from the slopes of Stern–Volmer plots: free (aqueous), 6.6 mM^{-1}; + PA/PC/PE, 2.8 mM^{-1}; + PG/PC/PE, 4.1 mM^{-1}. The absolute error of *K*sv in these data amounts to 0.5 mM^{-1}.

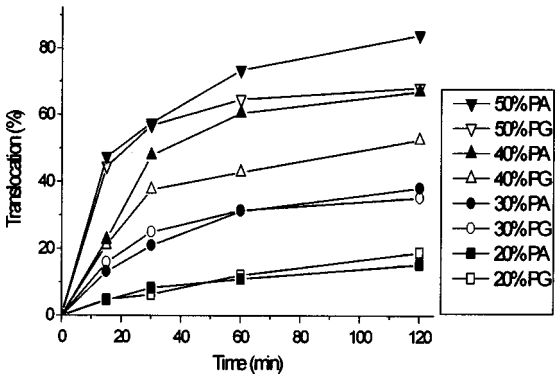

Figure 6 Comparison of apocytochrome *c* translocation across model membranes with varying PA/PC and PG/PC ratios

Digestion of apocytochrome *c* was measured in incubation mixtures of trypsin-containing vesicles (108 nmol of P_i) composed of PC and PA or PG in various ratios, incubated with 40 μg of horse heart apocytochrome *c*.

turbation of the bilayer structure by the preprotein is more obvious in PA-containing vesicles than in PG-containing vesicles. Hence the conclusion can be drawn that the bilayer structure and the conformation of apocytochrome *c* are specially altered upon incorporation of the preprotein in PA-containing vesicles, which may benefit the translocation process of the preprotein. The most efficient translocation of the preprotein in PA/PE/PC LUVs may be related to the introduction, to a much larger extent, of a less-stable bilayer structure in this system.

PE is essential for the enhancing effect of PA on apocytochrome *c* translocation

As shown in Figure 1, the rate of horse heart apocytochrome *c* being digested in PA/PC/PE LUVs is about 2–3 times as high as that in vesicles containing any other acidic phospholipid species. Such significant difference was also observed in the

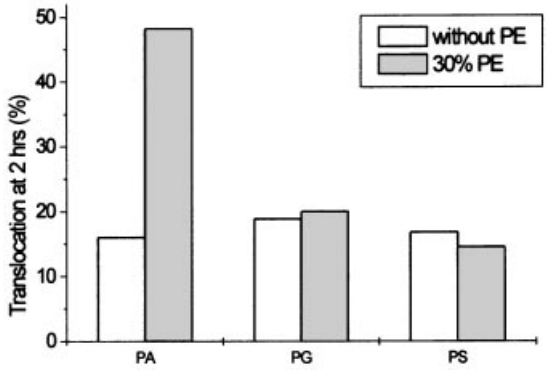

Figure 7 Comparison of apocytochrome *c* translocation across different acidic phospholipid-containing LUVs in the presence or absence of 30% PE

The fraction of the anionic phospholipid was kept constant at 20 mol%, and the mol% of PC plus PE was 80%. Trypsin-enclosed vesicles (108 nmol of P_i) were incubated with 40 μg of horse heart apocytochrome *c*. The mean translocation of three experiments is shown (S.D. $\pm$5–10% of translocation percentages shown).

comparison of apocytochrome *c*-induced FS efflux from these vesicles. Namely, apocytochrome *c* could elicit a higher extent of FS leakage from PA/PE/PC vesicles. It is interesting to note that PE is essential for these effects in the PA/PE/PC system. As shown in Figure 6, the greater efficiency of PA versus any other acidic phospholipids in facilitating apocytochrome *c* penetration into the interior of LUVs disappears in the absence of PE, unless the fraction of PA or PG is raised to above 35–40 mol%. Further, if LUVs were constructed from the acidic phospholipid (PA, PS or PG) combined with PE, the effect of PE on apocytochrome *c* translocation (Figure 7) as well as its ability to induce FS release (Figure 8) would reveal itself only in the PA-containing vesicles. Previously it was reported [11] that the presence of DOPE didn't stimulate the translocation of apocytochrome *c* in the DOPE/bovine brain PS LUVs. Taken together,

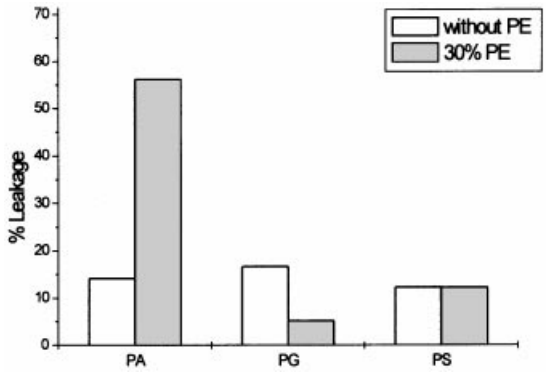

Figure 8 Comparison of the release of entrapped FS induced by apocytochrome *c* from different acidic phospholipid-containing LUVs in the presence or absence of 30% PE

The fraction of acidic phospholipids was kept constant at 20 mol%, and the mol% of PC plus PE was 80%. Horse heart apocytochrome *c* (5 μM) was incubated with 500 μM LUV. Each value is the mean from three experiments with three different vesicle preparations of the same liposome formulation. The final extents of leakage at amount of apocytochrome *c* varied by no more than $\pm$6% when the extents of leakage were above 20% and by no more than $\pm$3% below 20% leakage. The experimental conditions were described in detail in the Materials and methods section.

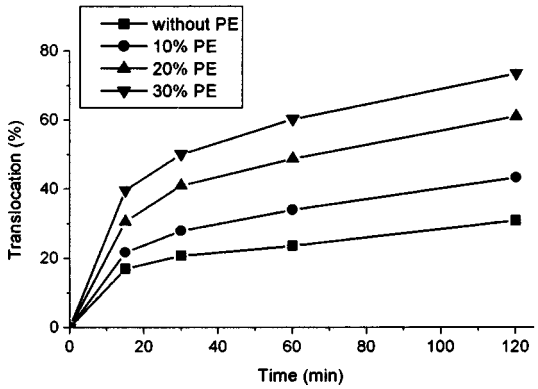

Figure 9 Apocytochrome *c* translocation across PA/PC/PE LUVs with varying PE content

The fraction of PA was kept constant at 20 mol%, and the mol% of PC plus PE was 80%. Trypsin-enclosed vesicles (108 nmol of P_i) were incubated with 40 μg of apocytochrome *c*. The mean translocation of three experiments is shown (S.D., $\pm$5–10% of translocation percentages shown).

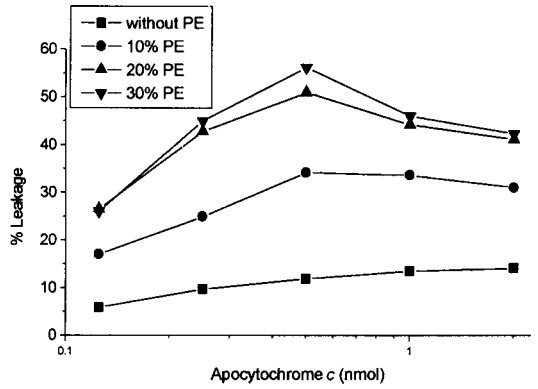

Figure 10 Leakage of entrapped FS induced by apocytochrome *c* from PA/PC/PE LUVs with various PE contents

The fraction of PA was kept constant at 20 mol%, and the mol% of PC plus PE was 80%. Increasing amounts of apocytochrome *c* were incubated with 500 μM LUV. Each value is the mean from three experiments with three different vesicle preparations of the same liposome formulation. The final extents of leakage at amount of apocytochrome *c* varied by no more than $\pm$6% when the extents of leakage were above 20% and by no more than $\pm$3% below 20% leakage. The experimental conditions were described in detail in the Materials and methods section.

these data imply that the PA–PE interaction may play an important role in the effects of PA on the behaviour of apocytochrome *c* following its interaction with the membranes. Presumably, PA may further increase the negative curvature stress induced by PE, resulting in a less-stable bilayer [23]. This may lead to an increase in apocytochrome *c* translocation and its leakage-induction ability.

In order to get more insight into the role of PA–PE interaction in the translocation of apocytochrome *c* across model membranes, LUVs composed of various PE contents were used, with the fraction of PA kept constant at 20 mol%. As shown in Figure 9, as the PE fraction increased from 0 to 30 mol% the translocation efficiency of apocytochrome *c* was also raised. Similar results were obtained with respect to the release of entrapped FS induced by apocytochrome *c*. As shown in Figure 10, the extent of FS leakage from PA-containing vesicles increased by 22 and 40% at the protein/lipid ratio of 1:100 when the fraction of PE amounted to 10 and 20 mol%, respectively. However, the difference in FS release from 20 and 30 mol% PE-containing vesicles was slight. Possibly, in this case the PA content is not sufficient, in view of the PA–PE interaction, to induce the release of more FS from the vesicles.

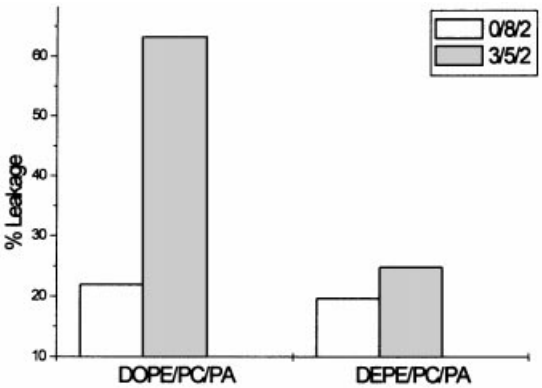

Figure 11 Comparison of leakage of entrapped FS induced by apocytochrome *c* from LUVs containing DOPE and DEPE

The fraction of acidic phospholipids was kept constant at 20 mol%, and the mol% of PC plus PE was 80%. Apocytochrome *c* (5 μM) was incubated with 500 μM LUV. Each value is a mean from three experiments with three different vesicle preparations of the same liposome formulation. The final extents of leakage at amount of apocytochrome *c* varied by no more than $\pm$6% when the extents of leakage were above 20% and by no more than $\pm$2% below 20% leakage. The experimental conditions were described in detail in the Materials and methods section.

Another approach to establish whether the increased translocation efficiency of apocytochrome *c* in PA/PE/PC LUVs was due to the presence of or ability to form inverted non-bilayer phases, DOPE- or DEPE-containing LUVs were employed to compare their ability to translocate apocytochrome *c* or to induce FS efflux from the vesicles. Both DOPE and DEPE have the same acyl-chain length, but differ in the configuration of the double bond on the side chains. This difference results in the much lower bilayer (L_α phase)-to-hexagonal II (H_{II}) phase-

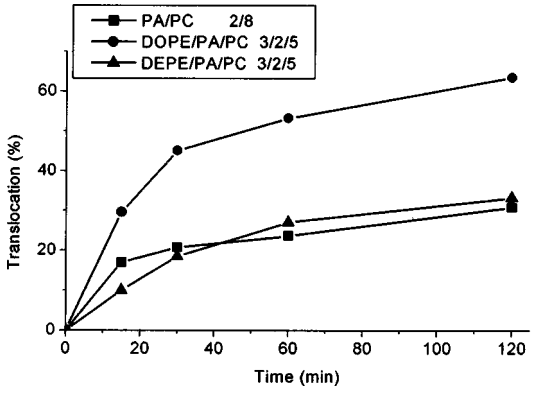

Figure 12 Comparison of apocytochrome *c* translocation across model membranes containing DOPE and DEPE

The fraction of PA was kept constant at 20 mol%, and the mol% of PC plus PE was 80%. Trypsin-enclosed vesicles (108 nmol of P_i) were incubated with 40 μg of apocytochrome *c*. The mean translocation from three experiments is shown (S.D., $\pm$5–10% of translocation percentages shown).

transition temperature for DOPE (13 °C) compared with DEPE (65 °C). Therefore, membranes containing DEPE have a much lower tendency to form non-bilayer structures at room temperature than membranes containing DOPE. Besides, the gel-to-liquid-crystalline phase transition of aqueous dispersion of DOPE occurs at a much lower temperature (−5 °C) than that of DEPE (37.1 °C). Here, the fraction of PA was kept constant at 20 mol%, and the mol% of PC plus DOPE or DEPE was 80 mol%. The results obtained showed clearly that the increases in the translocation efficiency of, and FS release induced by, apocytochrome *c* are much greater with PA/PC/DOPE than with PA/PC/DEPE LUVs (Figures 11 and 12). Therefore, this may imply that the occurrence of non-bilayer structures (even locally) is involved in the effect of the PA–PE interaction on apocytochrome *c* translocation across model membranes.

DISCUSSION

The key result of this study is the finding that apocytochrome *c* translocation in LUVs or efflux of entrapped FS induced by apocytochrome *c* can be enhanced strongly by PA–PE interaction. This conclusion has been drawn based on the following results. (i) An enhancing effect of PA over any other anionic phospholipid on the translocation of apocytochrome *c* across the model membranes was observed only in the PE-containing vesicles. (ii) With the fraction of PE increasing from 0 to 30 mol%, the translocation rate of apocytochrome *c* as well as its ability to induce FS efflux was significantly increased in PA-containing vesicles, whereas this did not appear if PG or PS was employed instead of PA. (iii) The above-mentioned enhancing effect was only observed in LUVs containing PA combined with DOPE, but not DEPE.

Thus, the results obtained in the present paper indicate clearly that the PA–PE interaction enhances apocytochrome *c* translocation remarkably. The next question to be addressed is the role fulfilled by PA or PE in the enhancing effect. PA, as an anionic phospholipid, serves as the target for apocytochrome *c* binding by the electrostatic interaction. In addition, it is interesting to note that PA holds the potential to promote the adoption of an inverted hexagonal structure of phospholipids [24,25]. This packing property of PA will manifest itself when charge neutralization and dehydration of the headgroups occur in response to certain events, such as partitioning of amphiphiles in bilayers, introduction of Ca^{2+} and decreasing pH [26–28]. This property might affect the intrinsic curvature of the vesicles in consequence of apocytochrome *c* partitioning in bilayers, and hence favour apocytochrome *c* translocation.

PE is a typical non-lamella-forming lipid that forms inverted phases in purified form [29]. Generally, the presence of non-lamella-forming lipids results in a curvature strain, producing a 'frustrated' bilayer and facilitating some polypeptide translocation across the membranes [30]. Besides, enhanced interactions of cytoplasmic proteins and drugs with membranes containing acidic phospholipids in the presence of PE have been reported [31,32]. It seems that PE plays a more general role in the interactions of amphiphiles with membranes [23,33]. Presumably, this role of PE is also involved to facilitate the translocation of apocytochrome *c* across membranes.

Considering the special properties of PA and PE as mentioned above, two hypotheses on the mechanisms underlying enhancement of apocytochrome *c* translocation by PA–PE interaction can be conceived, as follows.

First, the combination of PA with PE might strengthen the propensity for non-bilayer phase formation, which results in pronounced negative curvature strain, and produces a less-stable bilayer. This could facilitate apocytochrome *c* translocation across the model membranes as well as FS efflux induced by the preprotein. This assumption was supported by the results shown in the present paper that, with the fraction of PE increasing from 0 to 30 mol%, the translocation efficiency of apocytochrome *c* and its leakage-induction ability are gradually enhanced. Further, the conformation difference of apocytochrome *c* following its interaction with PA/PC/PE and PG/PC/PE LUVs was observed by three different approaches of fluorescence studies. We postulate that the increase in the propensity for non-bilayer formation, strengthened by PA–PE interaction, may facilitate the appearance of a translocation-competent conformation of the apoprotein.

Second, a complex of PA and PE might be formed by attractive headgroup–headgroup interactions (such as hydrogen-bonding) between the two molecules, and then exhibit much greater spontaneous curvature than the individual lipids. Possibly, the 'coupling' between PA and PE might be even stronger when PA molecules are dehydrated due to apocytochrome *c* partitioning in the membranes. This incurs the increase of the negative curvature tendency of the membranes.

The relative simplicity of model systems based on purely lipid bilayers invites its use to study the molecular mechanism of apocytochrome *c* translocation. However, the PA concentration in the mitochondrial outer membranes is much lower ($\approx$ 10-fold) than that used in the present study. Tentatively we postulate that the distribution of phospholipids in natural membranes might be heterogeneous, therefore higher PA concentrations might be attained locally. The significant role of lipids in the translocation of apocytochrome *c* across membranes has been dwelt on in previous literature; still, more attention should be paid to it. We speculate that import of the preprotein into mitochondria might be facilitated by certain special lipid components bypassing the proteinaceous import machinery.

The detailed molecular mechanism underlying the enhancing effect of PA–PE interaction on apocytochrome *c* translocation across model membranes is still not well defined and deserves further investigation. Further, it can be questioned whether PA–PE interaction plays a more general role in the modulation of membrane functions in addition to the translocation of apocytochrome *c*. Recent evidence [34,35] suggests that PA may play an important role in vesicle-mediated trafficking in eukaryotic cells. As a cone-shaped phospholipid, PA can cause a distortion of the lipid bilayer, and thereby facilitate membrane invagination and vesicle formation. Hypothetically, PA–PE interaction is also involved in such membrane events. Related studies are still in progress.

We are grateful to Professor Richard M. Epand and Professor Ben de Kruijff for critically reading the manuscript. This work was funded by research grants from the National Natural Science Foundation of China (39730130) and the Chinese Academy of Sciences.

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Received 13 September 2000/16 November 2000; accepted 20 December 2000