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Preferential association of apocytochrome *c* with negatively charged phospholipids in mixed model membranes

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The mitochondrial precursor protein, apocytochrome *c*, binds to model membranes containing negatively charged phospholipids (Rietveld, A., Sijens, R., Verkleij, A.J. and Kruijff, B. (1983) EMBO J. 2, 907–913). In the present paper the effect of apocytochrome *c* on the lipid distribution in model membranes, consisting of neutral and acidic phospholipids, is examined. Both ESR and fluorescence energy transfer experiments show that the protein preferentially interacts with the negatively charged phospholipid in the mixed model membranes. Semi-quantitative analysis of the fluorescence energy transfer from the single tryptophan in apocytochrome *c* to the parinaric acid in phosphatidylserine or phosphatidylcholine in mixed bovine brain phosphatidylserine / egg phosphatidylcholine vesicles reveals and average donor-acceptor distance of 22–26 Å and 26–30 Å for phosphatidylserine and phosphatidylcholine, respectively. In addition, these experiments demonstrate that this preferential interaction does not induce the separation of large domains enriched in complexes of apocytochrome *c* with negatively charged phospholipids and domains enriched in neutral lipids.

Introduction

Most mitochondrial proteins are synthesized in the cytoplasm in a precursor form and next transported into the organelle [1,2]. The mechanisms for such import are unknown, but are often presumed to involve receptors in the outer mitochondrial membrane [3,4]. Although these protein type interactions certainly could be of importance for the targeting of precursor proteins, their function in the formation of a translocation pathway across the outer and/or inner mitochondrial membrane is less obvious. Since phospholipids determine to a large extent the barrier properties of a biological membrane, it is reasonable to suggest that they are involved in protein translocation. Investigations on precursor protein-lipid interactions in model membranes are one way to gain insight into this possibility. Apocytochrome *c*, which is the extramitochondrially synthesized precursor protein of the inner membrane protein, cytochrome *c*, is a unique model for studying the involvement of

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Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PS, phosphatidylserine; PC, phosphatidylcholine; *n*-PGSL, 1-acyl-2[*n*-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol; *n*-PSSL, 1-acyl-2[*n*-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoserine; *n*-PCSL, 1-acyl-2[*n*-(4′,4′-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3--phosphocholine; PnA-PC, 1-palmitoyl-2-*cis*-parinaroylphosphatidylcholine; PnA-PS, 1-palmitoyl-2-*cis*-parinaroylphosphatidylserine; DSC, differential scanning calorimetry; Pipes, 1,2-piperazinediethanesulfonic acid; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; ESR, electron spin resonance.

protein-lipid interactions in protein translocation. The conversion of apocytochrome *c* into the mature protein does not require proteolytic cleavage of a part of the polypeptide chain, as often observed for mitochondrial precursor proteins [2], but only involves covalent coupling of a heme moiety [5]. Therefore, this precursor protein can, by removing the heme group from the holoprotein [6], be produced in quantities sufficient for physicochemical analysis. In addition, this chemically prepared apocytochrome *c* has been shown to compete with the in vivo synthesized protein [7,8] with respect to binding to mitochondria.

We have reported previously that, due to its basic character, apocytochrome *c* binds specifically to negatively charged phospholipids [9]. This interaction which is primarily electrostatic is attended by a deep penetration of the protein into the lipid bilayer [10], resulting in the exposure of (a part of) the protein to the internal vesicle medium [11]. Recent binding experiments [12] of apocytochrome *c* with mixed phosphatidylcholine (PC)/phosphatidylserine (PS) vesicles demonstrated that the association constants of these lipid-protein complexes are independent of the PS fraction in the vesicles. In addition, the number of PS molecules involved in the interaction was found to be constant and electrostatically stoichiometric, strongly suggesting that apocytochrome *c* induces a lipid redistribution in these mixed lipid vesicles. In this paper we try to gain more direct insight into the mechanism of this protein-induced lipid reorganization. It was revealed by both ESR and fluorescence energy transfer experiments that apocytochrome *c* demonstrates a preferential interaction with negatively charged phospholipids in mixed lipid systems without the induction of a phase separation.

Materials and Methods

Materials

DMPC and DPPC were synthesized as described previously [13]. Bovine brain PS used for ESR experiments was purchased from Lipid Products (South Nutfield, U.K.) and for the other experiments it was isolated and purified as described elsewhere [14]. Egg-yolk PC was isolated according to well-established procedures [15]. 1-Palmitoyllysophosphatidylcholine (lysoPC) was prepared from DPPC by treatment with phospholipase A_2 from *Crotalus adamateus* [16]. *cis*-Parinaric acid (PnA) was purchased from Molecular Probes (Junction City, OR, U.S.A.) and bovine heart cardiolipin (sodium salt) from Avanti-Polar lipids (Birmingham, AL, U.S.A.). *n*-PGSL, *n*-PCSL and *n*-PSSL were synthesized essentially as described by Marsh and Watts [17].

PnA-PC and PnA-PS were prepared as described by Somerharju et al. [18]. Briefly, this method involved acylation of 1-palmitoyl-lysophosphatidylcholine by an equimolar amount of parinaroyl anhydride [19] followed by phospholipase-D-mediated base exchange to yield PnA-PS [20]. PnA-PS was separated from 1-palmitoyl-2-*cis*-parinaroylphosphatidic acid by high-pressure liquid chromatography on a polygosil silicic acid column (0.45 × 25 cm) using chloroform/methanol/ammonia/water (50 : 50 : 2 : 1, v/v) as a solvent [20]. Pure PnA-PS was obtained in a yield of 9% as determined by measuring the absorbance of PnA-PS in ethanol, using a molar extinction coefficient of 78 000 at 305 nm [21]. The PnA/phosphorus ratio was 0.97 for PnA-PC and 0.89 for PnA-PS. The purity of both lipids was confirmed by their absorption spectra [22] and by thin-layer chromatography, using chloroform/methanol/ammonia/water (90 : 55 : 5.5 : 5.5, v/v) as a solvent. PnA-PS and PnA-PC were dissolved in ethanol at a concentration of 0.6 and 6.0 mM, respectively, in the presence of 0.01% (w/v) butylated hydroxytoluene and stored at −80°C in dark bottles under an argon atmosphere. Before use, PnA-lipids were routinely checked by measuring the absorption spectrum, which confirmed that the PnA-lipids were stable under the conditions of storage.

Apocytochrome *c* was prepared from horse heart cytochrome *c* (type VI, Sigma, St. Louis, MO, U.S.A.) as described by Fisher et al. [6], and next subjected to a renaturation procedure [23]. The protein was stored at −20°C in 100 mM NaCl/10 mM Pipes (pH 7.0), at a concentration of approx. 1.5 mg/ml. *N,N'*-Dicyclohexylcarbodiimide, butylated hydroxytoluene and 4-(dimethylamino)pyridine were purchased from Janssen Pharmaceutica (Beerse, Belgium), Polygosil silicic acid (5–20 μm) was a product of Serva (Heidelberg, F.R.G.).

ESR

Samples were prepared by hydrating a dry film of approx. 1.25 μmol lipid (containing 1% (w/w) of spin-labelled lipid) with buffer, or with apocytochrome *c* solution (2 mg/ml) in buffer. The mixtures were incubated for 30 min at 30°C. After centrifugation (20 min, 3000 × *g*) of the mixture at room temperature, the pellet was transferred to a sealed-off 100 μl capillary. The complex was further concentrated in the capillary by centrifugation at room temperature (20 min, 3000 × *g*) before ESR measurements, which were carried out at 30°C. The spectra were recorded on a Varian E-12 Century Line spectrometer with nitrogen gas-flow temperature regulation. Details of the spectroscopic measurements are described elsewhere [17,24]. The effective order parameters are defined by

$$S^{\text{eff}} = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})} \cdot \frac{a_0^1}{a_0}$$

where $A_{\parallel}$ is identified with $A_{\max}$ and $A_{\perp}$ is obtained from the inner hyperfine splitting (cf. Fig. 1), according to

$$A_{\perp}(G) = A_{\min}(G) + 1.4\left[1 - \frac{A_{\parallel} - A_{\min}}{A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})}\right]$$

The effective isotropic hyperfine splitting constant is given by: $a_0 = 1/3(A_{\parallel} + 2A_{\perp})$.

Fluorescence experiments

In order to prepare vesicles containing 10 and 50 mol% PnA-lipids, these lipids were mixed with appropriate amounts of bovine brain PS and egg-PC in chloroform. The solvent was removed by a stream of argon and the lipid film (0.2–1.0 μmol lipid phosphate) was suspended in 0.5 ml argon-saturated buffer. The suspension was sonicated with a Branson B-12 tip sonifier under continuous flow of argon, at 0°C for 2–5 min. The vesicle suspension was centrifuged at 27000 × *g* for 20 min in order to remove titanium and multilamellar structures. The recovery of the vesicle phospholipid was 80–100%, as determined by measuring the PnA absorbance. The PnA-phosphorus ratio was between 0.8 and 0.95 (mol/mol), indicating that the chemical stability of the vesicles was satisfactory during the vesicle preparation procedure. Energy transfer from the single tryptophan of apocytochrome *c* to PnA lipids was measured at 30°C on a Perkin-Elmer LS-5 fluorimeter equipped with a thermostatically controlled cuvette holder. The concentration of apocytochrome *c* was 50 μg/ml in argon-saturated buffer. Excitation of tryptophan (slit width 10 nm) was at 255 nm, to avoid direct excitation of the PnA chromophore. Since the line shape of the tryptophanyl emission spectrum was very similar for excitation at 255 and 290 nm it can be concluded that the four tyrosine residues [25] in the protein do not significantly contribute to the fluorescence spectrum.

Tryptophanyl and parinaroyl fluorescence was recorded between 300 and 420 nm (slit width 10 nm). The protein was titrated by stepwise addition of a PnA-lipid containing vesicle solution (0.4–2.0 mM P_i). The spectra were corrected (combined corrections were less than 10% of the total fluorescence signal) for the effects of scattering [10], inner and outer filtering effects, and changes of quantum yield of the tryptophan upon binding of apocytochrome to vesicles. For this, apocytochrome *c* (50 μg/ml) was titrated with a 1 : 1 (mol/mol) mixture of two vesicle populations. One vesicle population consisted of either PC/PS (1 : 1, mol/mol) or PS vesicles in order to mimic the fluorescence increase resulting from binding of apocytochrome *c* to the vesicles, respectively. The second vesicle population consisted of PC vesicles containing 10 or 50 mol% PnA-PC, to which apocytochrome *c* does not bind [9]. This represents a control to measure the changes of fluorescence of the protein tryptophan upon addition of vesicles containing 10 or 50 mol% PnA lipids, respectively, due to light absorption by the PnA chromophore. The quantum yield of PnA-PS and PnA-PC was similar in the mixed vesicles and the PS vesicles.

The fluorescence data were analyzed according to Förster's theory [26]. The energy transfer efficiency, *T*, from one donor to an acceptor is

$$T = \frac{R_{\text{da}}^{-6}}{R_{\text{da}}^{-6} + R_0^{-6}} \qquad (1)$$

R_{da} (in Å) is the distance between donor and acceptor and R_0 the distance at which the energy

transfer efficiency is 50% ($T = 0.5$). R_0 can be obtained from:

$$R_0 = \left(J_{da} \cdot K^2 \cdot Q_d \cdot n^{-4}\right)^{1/6} \cdot 9.7 \cdot 10^3 \text{ Å} \quad (2)$$

in which J_{da} is the spectral overlap between donor (d) and acceptor (a), Q_d the quantum yield of the donor, n the refractive index of the medium surrounding the donor and acceptor and K^2 the dipole orientation factor. The R_0 value for the particular donor-acceptor pair of apocytochrome *c* tryptophan and the parinaroylphospholipids can be calculated from the following data. The quantum yield, Q_d, was determined by:

$$Q_d = Q_0 \frac{F_a A_0}{F_0 A_a} \quad (3)$$

where Q_0 is the quantum yield of tryptophan in water (30°C) and has a value of 0.144 [27]. F_a and F_0 are tryptophanyl fluorescence integrals and A_a and A_0 the absorbances at the wavelength of excitation (255 nm) of vesicle-bound apocytochrome *c* and free tryptophan, respectively. From this, Q_d was calculated to be 0.06. From the emission maximum at 346 nm of the protein tryptophan a value of $95 \cdot 10^{-16}$ cm$^3 \cdot$ M^{-1} was estimated for the spectral overlap, J [28]. The value of the refractive index $n = 1.45$ was obtained from the absorption spectrum of PnA lipids in vesicles [22]. Substituting these values in Eqn. 2 yields R_0, the Förster distance, to be 20 Å for $K^2 = 2/3$, the value that assumes random orientation of the donor and acceptor during the excited-state lifetimes [29]. The energy transfer efficiency, T, can be measured from the quantum yield of the donor in the presence (F) and in the absence (F_0) of the acceptor according to:

$$T = 1 - F/F_0 \quad (4)$$

Fusion experiments, based on lipid mixing, have been performed according to a fluorimetric method [30] which measures the resonance energy transfer between the energy donor, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine, and an energy acceptor, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine. The vesicles were prepared as described for the fluorescence experiments. Fusion was measured at 30°C by monitoring the fluorescence intensity at 580 nm ($\lambda_{ex} = 450$ nm) after addition of apocytochrome *c* (2.0 mg/ml) or 0.5 M $CaCl_2$ (final concentration in the cuvette respectively 50 μg protein/ml and 12.5 mM Ca^{2+}) to a mixture of non-fluorescent PS/PC vesicles (100 μM) and PS/PC vesicles (5 μM) containing 1 mol% of both fluorescent probes.

General

All experiments were performed in 100 mM NaCl/10 mM Pipes (pH 7.0) buffer. Protein was determined according to Lowry et al. [31] with bovine serum albumin as a standard. Lipid phosphate was assayed as described by Rouser et al. [32].

Results

It has been shown earlier [9] that the basic apocytochrome *c* strongly and specifically interacts with negatively charged phospholipids. This interaction of apocytochrome *c* with model membranes exclusively composed of negatively charged phospholipids even results in a restriction of the mobility of the nitroxide-spin labels, which are attached at different positions of the *sn*-2 chain of PS or PG [33]. We wanted to extend these experiments to PS/PC mixtures in order to investigate whether the protein preferentially interacts with the negatively charged phospholipids in these mixed lipid systems. The ESR spectra of the 5-PGSL and 5-PCSL spin labels in bovine spinal cord PS/egg-PC 3:1 (w/w) mixtures both in the presence and in the absence of saturating amounts of apocytochrome *c* are given in Fig. 1. Both labels display a decrease in mobility, indicated by an increase in the outer hyperfine splitting, $2A_{\|}$, which implies that phase separation is not induced on binding of apocytochrome *c* to the lipid mixture. However, the increase in outer splitting and in the corresponding effective order parameters, S^{eff}, is greater for the 5-PGSL label than for the 5-PCSL label, indicating a preferential interaction with the negatively-charged lipid (see Table I). In the absence of protein the splittings and effective order parameters of the two labels are essentially identical. The constancy of the effective isotropic hyperfine splitting, a_0^{eff}, which is sensitive to the

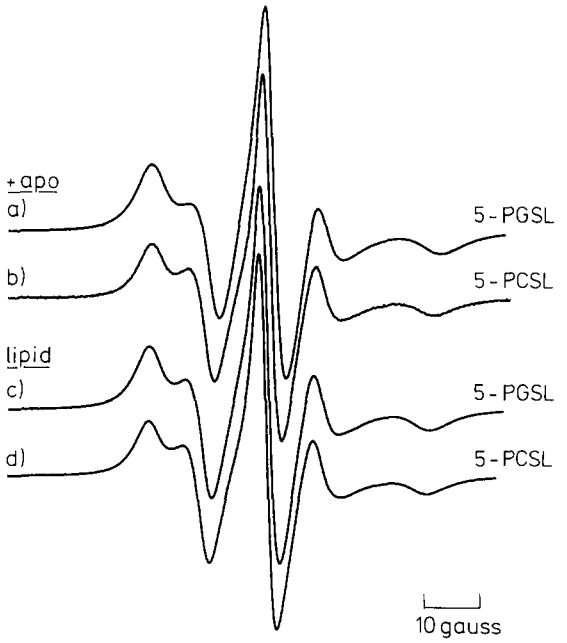

Fig. 1. ESR spectra of the 5-PGSL, phosphatidylglycerol spin label and the 5-PCSL, phosphatidylcholine spin label in bovine spinal cord phosphatidylserine/egg phosphatidylcholine (3:1, w/w) mixtures at 30°C in the presence and absence of apocytochrome *c*. (a) 5-PGSL with apocytochrome *c* (4:1 (w/w) with respect to PS). (b) 5-PCSL with apocytochrome *c* (4:1 (w/w) with respect to PS). (c) 5-PGSL in lipid alone. (d) 5-PCSL in lipid alone.

polarity of the environment, also indicates no difference in the vertical location of the label (Table I). It is also noteworthy that the increase in $A_{\parallel}$ and S^{eff} for 5-PGSL is only 40% of that observed on binding of apocytochrome to pure bovine PS (data not shown; see also Ref. 33).

In the previous study with pure PS bilayers [33] it was found that a second, more motionally restricted, spin-labelled component was induced in the spectra of the 14-PGSL and 14-PSSL spin labels on binding of apocytochrome *c*. In the 3:1 (w/w) PS/PC mixture a slight broadening was observed in the wings of the spectrum of both the negatively charged 14-PSSL and the neutral 14-PCSL label on binding apocytochrome *c* (data not shown).

These experiments did not give further insight into the preferential association of apocytochrome *c* with negatively charged phospholipids. Furthermore, in the bovine brain PS/egg PC (1:1) (w/w)

TABLE I

Effective order parameters, S^{eff}, isotropic hyperfine splitting factors, a_0^{eff}, and outer hyperfine splittings, $2A_{\parallel}$, of the phosphatidylglycerol spin label, 5-PGSL, and the phosphatidylcholine spin label, 5-PCSL, in bovine spinal cord PS/PC (3:1, w/w) mixtures at 30°C in the presence (4:1 (w/w) with respect to PS) and absence of apocytochrome *c*. Values are means of at least two independent measurements. Variations in duplicates are typically for $A_{\parallel}$ about ±0.1 G, for S^{eff} about ±0.05 G and for a_0^{eff} about ±0.05 G.

System	Label	$A_{\parallel}$ (G)	S^{eff} (G)	a_0^{eff} (G)
+ Apo *c*	5-PGSL	26.6	0.611	15.1
	5-PCSL	25.8	0.572	15.1
Lipid alone	5-PGSL	25.6	0.559	15.2
	5-PCSL	25.5	0.546	15.1

system, the mobility of all spin labels tested (5-PGSL, 12-PGSL, 14-PSSL, 5-PCSL, 12-PCSL and 14-PCSL) hardly changed after apocytochrome *c* addition, again demonstrating that the PC remains mixed with the negatively-charged lipid, hence attenuating the effect of apocytochrome *c* binding.

The observed preferential interaction of apocytochrome *c* with negatively charged phospholipids was further investigated by resonance energy transfer measurements between the single tryptophan of the protein and parinaroylphospholipids. In these experiments small unilamellar vesicles (SUV) were used, for which apocytochrome *c* binding is stronger than for large unilamellar vesicles [10,12]. Sonicated vesicles consisting of PnA-PC and PS (1:1, mol/mol) or PnA-PS and PC (1:1, mol/mol) were added in increasing amounts to 2 ml buffer containing apocytochrome *c* (50 μg/ml), and the fluorescence was monitored between 300 and 420 nm. For both lipid systems, this titration resulted in a decrease of tryptophanyl fluorescence at 346 nm (Fig. 2). This is the effect of binding of apocytochrome *c* to the vesicles, resulting in a radiationless energy transfer from the apocytochrome *c* tryptophan to the PnA-lipid. The energy transfer from the tryptophan to PnA-PS (Fig. 2A) is apparently more efficient as compared to PnA-PC (Fig. 2B). The intrinsic fluorescence of the added vesicles containing 50% PnA-lipids was also measured (Figs. 2A and B), showing that the PnA fluorescence

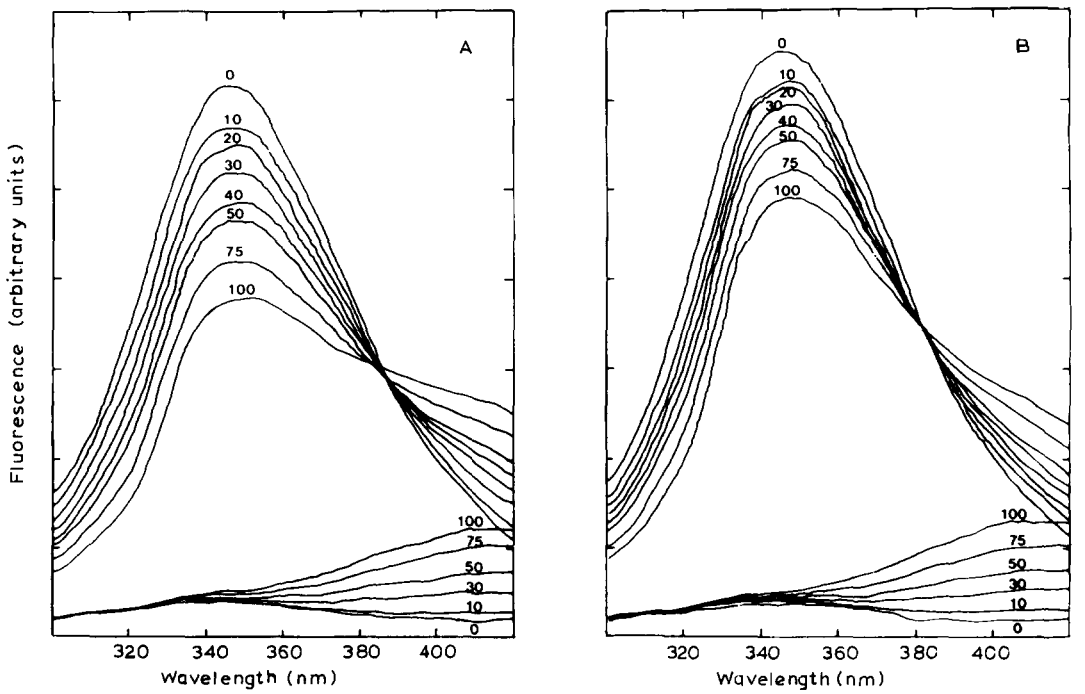

Fig. 2. Fluorescence emission spectra of apocytochrome *c* (50 μg/ml) upon titration with vesicles (0.72 mM phosphorus) containing PnA-PS/PC (A) or PnA-PC/PS (B) in a 1:1 molar ratio. Experimental details as described in the Materials and Methods section. The numbers refer to the volume of PnA-lipid vesicles added (μl). The corresponding emission spectra of the PnA-lipid vesicles in the absence of apocytochrome *c* are presented in the lower halves of (A) and (B).

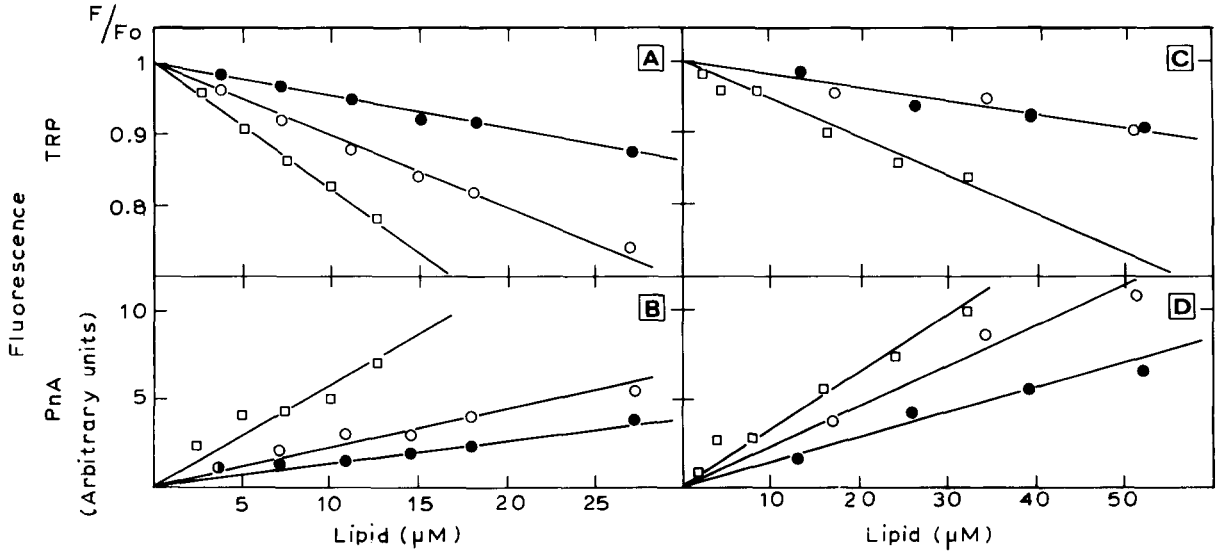

Fig. 3. Energy transfer from apocytochrome *c* tryptophan to sonicated PnA-lipid vesicles as monitored by tryptophanyl fluorescence (A, C) and parinaroyl fluorescence (B, D). Apocytochrome *c* (50 μg/ml) was titrated with vesicles (0.4–2 mM phosphorus) containing PnA-PS/PS (□), PnA-PS/PC (○), and PnA-PC/PS (●) in a 1:1 molar ratio (A, B). Similar titrations were performed with vesicles containing 10 mol% PnA-PS in PS (□), and with 10 mol% PnA-PS (○) in PC/PS (1:1 mol/mol) or 10 mol% PnA-PC (●) in PC/PS (1:1 mol/mol) (C, D). The tryptophanyl fluorescence maximum was measured between 345 and 350 nm and the parinaroyl fluorescence at 420 nm. The fluorescence was corrected as described in the Materials and Methods section.

contribution is small under these experimental conditions. In order to allow for a semi-quantitative analysis of the data, the energy transfer from apocytochrome *c* to PnA-PS/PS (1 : 1) vesicles was also determined. As with the other vesicles tested, the tryptophanyl fluorescence decreases and the parinaroyl fluorescence increases linearly with the lipid concentration (Fig. 3A, B). This indicates that, under these conditions, binding of apocytochrome *c* to the vesicles is proportional to the lipid concentration. As can be deduced from the decrease in tryptophanyl fluorescence (Fig. 3A) and the increase in parinaroyl fluorescence (Fig. 3B), the efficiency of transfer between tryptophan donor and the parinaroyl acceptor follows the order PnA-PS/PS > PnA-PS/PC > PnA-PC/PS. This result reflects the enhanced binding of apocytochrome *c* to pure PS vesicles as compared to PC/PS (1 : 1, mol/mol) vesicles [12], and further confirms a preferential interaction of apocytochrome *c* with PS in the mixed PC/PS vesicles.

In order to check that these observations are not the result of the high PnA-lipid concentration, the experiments were repeated with vesicles of the same lipid composition, containing now only 10 mol% PnA-lipids (Fig. 3C, D). In this case, the number of PnA acceptors is 5-times less, resulting in 3-times less energy transfer from tryptophan to PnA-PS in PS (1 : 9) vesicles in comparison with PnA-PS in PS (1 : 1) vesicles (Fig. 3D). Most likely because of the intrinsically small decrease in tryptophanyl fluorescence, no significant differences in energy transfer could be detected for PnA-PS as compared to PnA-PC (10 mol%) in mixed vesicles. However, in this case, measuring the parinaroyl fluorescence increase upon energy transfer from tryptophan is a much more sensitive method, since the quantum yield of the PnA-lipids in vesicles containing 10 mol% PnA-lipids is many-fold higher as compared to 50 mol% PnA-lipids, due to the reduction in self quenching [34]. The enhancement of the parinaroyl fluorescence follows the order PnA-PS (10 mol%) in PS > PnA-PS (10 mol%), in PC/PS (1 : 1, mol/mol) > PnA-PC (10 mol%) in PC/PS (1 : 1, mol/mol; Fig. 3D), in good agreement with that obtained for the 50% PnA-lipid vesicles.

In a recent report [35], it was stated that small unilamellar PS-containing vesicles fused upon addition of apocytochrome *c*, in particular at acidic pH values. Because vesicle fusion might complicate the interpretation of the resonance energy transfer experiments, we checked, with a lipid-mixing assay, whether fusion occurred under the experimental conditions employed in these experiments. Whereas addition of Ca^{2+} to PS/PC (1 : 1, mol/mol) SUV resulted in fusion, which is in agreement with earlier observations [36], the addition of apocytochrome *c* did not result in any detectable lipid mixing (less than 5% change in fluorescence with respect to that induced by 12.5 mM Ca^{2+}).

Discussion

The present paper addresses two related questions concerning the interaction of apocytochrome *c* with model membranes, composed of both neutral and acidic phospholipids. The first question is whether apocytochrome *c* interacts preferentially with the negatively charged lipid in the mixture. Secondly, if this is the case, whether this preference will result in phase separation, e.g., the formation of domains enriched in complexes of apocytochrome *c* with negatively charged lipids separated from domains enriched in the neutral lipid.

We will first focus the discussion on the possibility of a preferential interaction of the protein with the negatively charged lipid. Direct insight into this possibility can be obtained from experiments in which the individual characteristics of the two lipids in the mixture can be determined upon interaction of the protein with the mixed model membrane. We have used two approaches: one using spin-labelled fatty acids and a second one using parinaric acid both present in either the negatively-charged or the PC phospholipid of mixed lipid vesicles. The ESR experiments demonstrate that in PS/PC (3 : 1, w/w) vesicles doped with either 1 wt% spin labelled PG or PC, the protein causes a preferential restriction in mobility of the spin label attached to the negatively-charged lipid, clearly indicating a preferential interaction with the acidic lipid. This is further supported by the larger resonance energy transfer between the tryptophan (residue number 59) of apocytochrome *c* and parinaric acid present in PS as compared to

parinaric acid present in PC, in small unilamellar vesicles composed of a 1 : 1 mixture of PS and PC. The large reduction in effect of apocytochrome *c* on the mobility of the 5-PGSL in PS vesicles after incorporation of PC in these vesicles, together with the small but significant restriction in the motion of the PC-SL and the resonance energy transfer from tryptophan to PnA-PC, suggest that the apocytochrome-*c*-binding domains contain also neutral PC molecules next to the negatively charged lipids.

Information on the average distance between the tryptophan residue in apocytochrome *c* and PnA-PC and PnA-PS, respectively, can be obtained from an analysis of the fluorescence energy transfer data. From binding experiments with large unilamellar vesicles of varying PC and PS content [12], it was concluded that, in these vesicles, each apocytochrome *c* molecule interacts electrostatically with ten phosphatidylserine molecules, independent of the PS content. If it is assumed that the same stoichiometry holds for the small unilamellar vesicles employed in the present study and that PnA-PS and PS have a similar affinity for apocytochrome *c*, then to a first approximation each binding site in the PnA-PS/PS (1 : 9) vesicles contains on average one donor (tryptophan) and one acceptor (parinaric acid). With these assumptions it is possible to apply Eqn. 1 to obtain a value for R_{da} (the average distance between donor and acceptor). However, this requires knowledge of the energy transfer efficiency (T) under conditions that all apocytochrome *c* is bound. This value cannot be obtained experimentally due to the large increase in absorbance of the system when large amounts of PnA-PS-containing vesicles, required for maximal apocytochrome *c* binding, are present. A lower limit for T is estimated to be 0.16 ($F/F_0 = 0.84$, see Fig. 3A) from the highest concentration of vesicles used (32 μM). An upper limit can be estimated from the observation that the energy transfer to PnA-PS/PS (1 : 1) vesicles is 3-times higher than that to PnA-PS/PS (1 : 9) vesicles (Fig. 3A, compared at a lipid concentration of 32 μM). Since the maximal energy transfer efficiency theoretically cannot exceed 1.0, this places an upper limit of $T = 0.33$ for the PnA-PS/PS (1 : 9) vesicles. From these values of T and the value of R_0 given in the Materials and Methods section, application of Eqn. 1 results in a value of R_{da} for PS (R_{da}(PS)) between 22 and 26 Å. Unfortunately, it is not possible to obtain either R_{da}(PC) or R_{da}(PS) via a similar analysis of the 10 mol% PnA-lipid-containing vesicles, because of the very small changes in tryptophanyl fluorescence of these vesicles in the range of the titration (see Fig. 3C).

However, it is possible to use the data on the 50% PnA phospholipid-containing vesicles to obtain an estimate of R_{da}(PC). Energy transfer to PnA-PS in PnA-PS/PC (1 : 1) vesicles is about half that to PnA-PS/PS (1 : 1) vesicles. Since at a fixed phospholipid concentration the apocytochrome *c* binding to the vesicles is proportional to the PS content [12] and the PnA-PS/PC (1 : 1) vesicles contain only half the amount of PS as compared to the PnA-PS/PS (1 : 1) vesicles, the energy transfer and R_{da}(PS) are similar for the PnA-PS/PC (1 : 1) and PnA-PS/PS (1 : 1) vesicles (at 15 μM PS, $T = 0.32$ and 0.28, respectively, see Fig. 3A). For PnA-PC/PS (1 : 1) vesicles the energy transfer is about half of that for PnA-PS/PC (at 15 μM PS, $T = 0.14$). If it is assumed that the same difference also holds in 10% PnA-lipids, the calculated energy trasfer will be between 0.08 and 0.16, which yields $26 < R_{da}(PC) < 30$ Å. The values of R_{da} indicate that on the average the PS molecules are 4 Å closer to the tryptophan than are the PC molecules. The implication of these results is that, assuming the lipids in the mixed small unilamellar vesicles to be initially randomly organized, the apocytochrome *c* interaction results in lipid redistribution and selective enrichment of PS around the protein.

The foregoing analysis and the ESR data unambiguously demonstrate that the interaction of apocytochrome *c* with negatively-charged lipids does not result in the formation of an apocytochrome *c*-acidic phospholipid complex segregated from a pure neutral lipid phase. Also, NMR, DSC and freeze-fracture experiments, methods which have been successfully used before to demonstrate massive lipid phase separation [37–39], showed that the specific interaction of apocytochrome *c* with the negatively charged phospholipids in mixed systems does not result in such a phase separation [40].

In summarizing, it can be concluded that the

apocytochrome *c* – PS interaction in mixed PC/PS vesicles results in a selective enrichment of PS around the protein, but this does not result in segregation into large domains of either one of the two lipid components. The biologically intriguing hypothesis which now emerges is that the apocytochrome *c* molecule upon binding to the mixed lipid bilayer selectively attracts PS molecules which then possibly provide the protein with a translocation site enabling it to penetrate deeply into the bilayer [10], allowing (part of) it to reach the opposite aqueous compartment [11]. Whether or not apocytochrome *c* oligomer formation, which is induced by interaction with negatively charged phospholipids [12], is involved in such a mechanism is presently unknown.

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