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Interactions of mitochondrial precursor protein apocytochrome *c* with phosphatidylserine in model membranes. A monolayer study

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(1) The interaction of apocytochrome *c* with different molecular species of phosphatidylserine was studied using monolayers at constant surface area or constant surface pressure. The protein inserted readily into dioleoylphosphatidylserine monolayers up to a limiting pressure of 50 mN/m, whereas the interaction decreased with increasing molecular packing of the phosphatidylserine species, indicating the importance of the hydrophobic core of the lipid layer for the interaction. (2) The high affinity of apocytochrome *c* for dioleoylphosphatidylserine is indicated by the low K_d of 0.017 μM. There is little or no interaction with phosphatidylcholines. The importance of charge interactions is underlined by its ionic strength and pH dependency. (3) Experiments using ^{14}C-labelled apocytochrome *c* indicate that cholesterol can enhance the protein binding. (4) It was demonstrated that apocytochrome *c* monomers penetrate the monolayer whereas oligomers can be formed in an adsorbed layer and washed off without changing the surface pressure. Preincubation of apocytochrome *c* in 3 M guanidine, to obtain the monomeric form, was essential to measure the full effect of interfacial interaction. (5) The molecular area of apocytochrome *c* changed from 1200–1300 Å^2/molecule in the absence of lipid to 700–900 Å^2/molecule after penetration of dioleoylphosphatidylserine monolayers. (6) Apocytochrome *c*–dioleoylphosphatidylserine interactions are only possible when the monolayer is approached from the subphase. It is concluded that the charge interactions are required for binding and penetration of the protein.

Introduction

Apocytochrome *c* is the heme-free cytoplasmically synthesized precursor form of the inner mitochondrial membrane protein cytochrome *c*. During mitochondrial import the protein has to interact with and to translocate across the outer mitochondrial membrane. Lipids, which determine to a large extent the barrier properties of a membrane, are believed to have a function in protein translocation [1]. During or after the posttranslational translocation the heme group is covalently attached [2]. The mature protein, cytochrome *c*, is located on the inner mitochondrial membrane, where it is reversibly bound to a specific locus probably by favourable electrostatic interaction. The precursor protein has a disordered structure [3], the holocytochrome *c* is highly structured [4].

Abbreviations: PS, phosphatidylserine; DLPS, DMPS, DPPS, DOPS, POPS, DDPS, DEPS, respectively, dilauroyl, dimyristoyl, dipalmitoyl, dioleoyl, 1-palmitoyl-2-oleoyl, didocosenoyl, and dielaidoyl molecular species of PS; PC, phosphatidylcholine; DPPC, DOPC, respectively, dipalmitoyl and dioleoyl molecular species of PC.

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(Apo)cytochrome *c* is constructed of 104 amino acids and has an excess of nine positive charges at neutral pH. It has been shown before that apocytochrome *c* and cytochrome *c*, in agreement with their basic character, bind specifically to negatively charged lipids [5–9]. Apocytochrome *c* causes a strong perturbation of the lipid packing. The DSC and ESR results suggest that apocytochrome *c* prevents the formation of a gel phase and restricts the chain motion in a liquid crystalline phase, most likely due to a deep penetration of the protein in the bilayer. CD experiments of apocytochrome *c* showed that after interaction with negatively charged detergents a conformational change is introduced, resulting in an increase in α-helical structure [9]. The possible transfer of apocytochrome *c* over phospholipid model membranes has been demonstrated by the degradation of apocytochrome added to the outside of phosphatidylserine large unilamellar vesicles by trypsin trapped inside the vesicles [10–12].

In this study monolayer techniques were used to determine the different parameters involved in the interaction of apocytochrome *c* with model membranes. The involvement of charge interactions was studied by varying the charge density in mixtures of phosphatidylcholine and phosphatidylserine or by changing the ion concentration and pH. The importance of hydrophobic interactions was determined by varying the chain length and unsaturation of the fatty acid constituents of phosphatidylserines. The molecular dimensions of apocytochrome *c* were calculated using radiolabelled apocytochrome *c*.

Materials and Methods

Materials

The following molecular species of phosphatidylserine (PS) and phosphatidylcholine (PC) were synthesized according to established methods [13,14]: dilauroylphosphatidylserine (DLPS); dimyristoylphosphatidylserine (DMPS); dipalmitoylphosphatidylserine (DPPS); dioleoylphosphatidylserine (DOPS, 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS); didocosenoylphosphatidylserine (DDPS); dielaidoylphosphatidylserine (DEPS) dipalmitoylphosphatidylcholine (DPPC); dioleoylphosphatidylcholine (DOPC).

Bovine brain phosphatidylserine was isolated and purified essentially as described [15].

Apocytochrome *c* was prepared from cytochrome *c* horse heart type VI (Sigma) by removal of the heme moiety [3] and subjecting to a renaturation procedure [16]. The protein was stored at −20 °C in 0.5-ml aliquots at a concentration of about 1.5 mg/ml in 50 mM NaCl/10 mM Pipes (pH 7.0)/ 0.01% (v/v) β-mercaptoethanol.

Apocytochrome *c* was radiolabelled by reductive alkylation with [^{14}C]formaldehyde (New England Nuclear, specific activity 10 Ci/mol) and sodium cyanoborohydride [16–18]. By this procedure the net charge of the protein is not altered. The reaction was carried out in phosphate buffer (pH 7.0) and the protein-formaldehyde ratio was 20 : 1 (w/w). Oligomeric forms of apocytochrome *c* were removed by centrifugation. 8 mol HCHO was incorporated per mol protein. The specific activity was 75.8 mCi/mmol.

All containers of the labelled protein were precoated with Surfacil (Pierce Rockford, U.S.A.). In SDS-polyacrylamide gel electrophoresis the labelled apocytochrome *c* behaved identically to the original protein. Labelled apocytochrome *c* was renatured by incubation in 3 M guanidine.

Methods, monolayer studies

Measurements at constant surface area. Interfacial measurements were performed at 30 °C in a thermostatically controled box [19]. As a subphase a buffer was used containing 50 mM NaCl/10 mM Pipes (pH 7.0). The monomolecular lipid layers were formed at the air/water interface in a Teflon trough (5.4 × 5.9 × 0.5 cm). The subphase was stirred with a magnetic bar. Apocytochrome *c* was added to the subphase through a 0.5-cm^2 hole at an extended corner of the trough; the subphase was stirred with a magnetic bar. To determine the amount of radiolabelled apocytochrome *c* incorporated in the lipid monolayer, the monolayer was collected by sucking into a counting vial [20]. Prior the subphase was flushed with a buffer solution for 12 min at a rate of 10 ml/min. This procedure did not change the surface pressure. The radioactivity in the subphase dropped to nearly background value. Surface radioactivity was

measured with a gas flow detector [21]. The interfacial radioactivity was corrected for the amount of subphase collected [22].

Measurements at constant surface pressure. The Teflon through contained two compartments (each 5.5 × 5.9 × 1 cm) connected by a narrow channel (0.5–0.3 cm). The monomolecular lipid layer is formed on the stirred subphase of one compartment. Protein was injected underneath the lipid monolayer through an injection hole. The surface pressure was kept constant by the movement of a Teflon barrier on the second compartment and the surface area increase was recorded [23].

Compression isotherms. Force-area measurements were performed at the air/water interface in a Teflon trough (32.3 cm long × 17.2 cm wide). The compression rate was 0.258 $nm^2 \cdot mol^{-1} \cdot min^{-1}$. Lipid dissolved in chloroform/methanol (4:1, v/v) was carefully released onto the air/water interface from an Agla micrometer syringe. Protein was spread by the method of Trurnit [24] along a glass rod. Monolayers collected for gel electrophoresis were spread on 50 mM ammonium acetate buffer (pH 7.0).

Results

The most readily measured parameter in lipid-protein complex formation in monolayers is the change in surface pressure at constant surface area. The surface pressure changes are interpreted as a result from the penetration of hydrophobic residues of the protein in between the lipid chains. The surface pressure increase of DOPS monolayers shows a linear increase with protein concentration up to about 0.05 μM and there is a rapid saturation at higher protein concentrations. Fig. 1 shows for phosphatidylserines of different chain length, the surface pressure increase at different initial pressures. The limiting pressure is defined as the pressure where the protein can no longer penetrate and the change in surface pressure is zero. The strongest interaction is found for DLPS, with a limiting pressure of 49 mN/m. DMPS and DPPS show a much smaller pressure increase, with limiting pressures of 38 and 31 mN/m, respectively. The penetration of apocytochrome *c* in DMPS is reduced when the temperature is reduced from 30 to 18° C (data not shown). At this lower temperature DMPS no longer shows a phase transition and is considered to be in a fully condensed state. The surface pressure increase observed for DMPS at 18° C, having a limiting pressure of 33 mN/m, is only slightly higher than for DPPS at 30° C. Absolutely no interaction could be detected for DPPC. The importance of the molecular packing is also demonstrated in Fig. 2, where the most unsaturated phosphatidylserines show the highest pressure increase. The limiting pressures for DOPS, POPS, bovine brain PS, DDPS and DEPS are 50, 46, 43, 42 and 37 mN/m., respectively. A limited pressure increase can even be detected for the unsaturated phosphatidylcholine, DOPC. How-

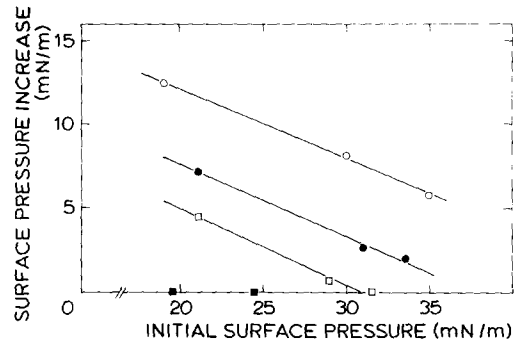

Fig. 1. Surface pressure increase after injection of apocytochrome *c* underneath monomolecular layers of phosphatidylserines of different chain length, DLPS (○), DMPS (●), DPPS (□) and the phosphatidylcholine DPPC (■) at different initial pressures. The final protein concentration in the subphase was 0.05 μM.

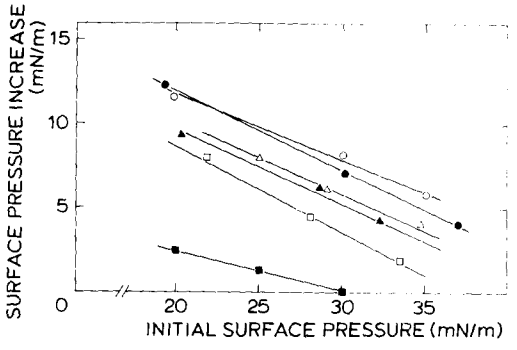

Fig. 2. Surface pressure increase after injection of apocytochrome *c* underneath monomolecular layers of phosphatidylserines of different unsaturation of the acyl chains, DOPS (○), POPS (●), bovine brain PS (△), DDPS (▲), DEPS (□) and the phosphatidylcholine DOPC (■) at different initial pressures. The final protein concentration in the subphase was 0.05 μM.

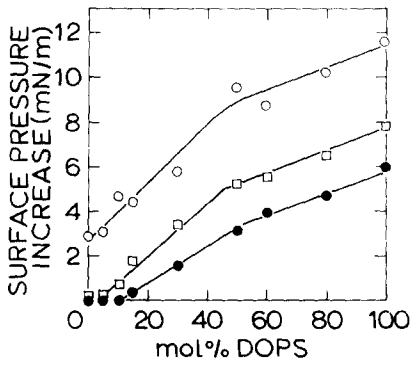

Fig. 3. Surface pressure increase after injection of apocytochrome *c* underneath mixed monomolecular layers of DOPC and increasing concentrations of DOPS at an initial pressure of 20 (○), 30 (□) and 35 mN/m (●). The final protein concentration in the subphase was 0.05 μM.

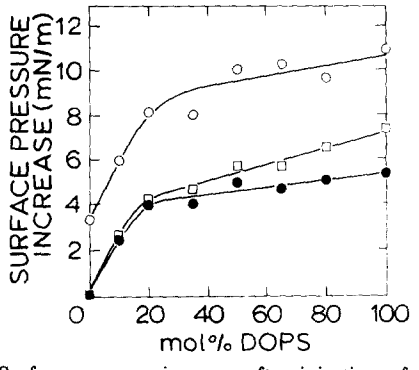

Fig. 4. Surface pressure increase after injection of apocytochrome *c* underneath mixed monomolecular layers of DOPC/cholesterol (molar ratio 1 : 1) and increasing concentrations of DOPS, at initial pressures of 20 (○), 30 (□) and 35 mN/m (●). The final protein concentration in the subphase was 0.05 μM.

ever the limiting pressure for this lipid is 30 mN/m.

By the same methods the involvement of charge interactions were studied. Mixtures of DOPC and DOPS were formed to vary the charge distribution at the interface. Since DOPC and DOPS occupy practically the same molecular areas, in mixtures only the charge density is changed and not the molecular packing. Fig. 3 shows that the pressure increase becomes greater with increasing DOPS concentration, with a break at 50 mol% DOPS. At an initial pressure of 35 mN/m there is no measurable pressure increase at DOPS concentrations of less than 10 mol%.

In mixtures containing cholesterol the molecular packing will increase, and consequently the charge density, whereas the compressibility of the monolayer will decrease.

Fig. 4 shows that in the presence of cholesterol a saturation of the apocytochrome *c* penetration is shifted to DOPS concentrations of approximately 20 mol%. It should be noticed also that at high initial pressures (30 and 35 mN/m) pressure increases can be measured already at very low DOPS concentrations. The limiting pressure for apocytochrome *c* penetration in both DOPC and DOPC-cholesterol (molar ratio, 1 : 1) monolayers is 30 mN/m. To determine whether the higher pressure increase in the presence of cholesterol is due to changes in the charge density and molecular packing or binding of more protein the binding of radiolabelled apocytochrome *c* is measured.

It had to be verified first whether the labelled protein behaved identically to unlabelled apocytochrome *c*. The labelled protein was renatured by incubation in 3 M guanidine hydrochloride for 1 h and was directly injected into the subphase. It was verified with unlabelled apocytochrome *c* that guanidine hydrochloride concentrations in the subphase of 3 or even 50 mM have no effect. The pressure increase attained by the injection of labelled and unlabelled apocytochrome *c* underneath DOPS monolayers was identical. Different dilutions of labelled apocytochrome *c* with unlabelled apocytochrome *c* showed that a proportional incorporation of labelled apocytochrome *c* could be observed, and consequently the labelled protein behaves similarly to the parent compound. This conclusion was also confirmed by carrying out binding studies, as described in Ref. 12, of labelled and unlabelled apocytochrome *c* to bovine brain PS large unilamellar vesicles. However, the renaturation of the protein is essential. The binding of unlabelled apocytochrome *c* (renatured as indicated in Materials and Methods) and labelled apocytochrome *c* (renatured by preincubation in 3 M guanidine) was found to be identical. However, when the labelled apocytochrome *c* was not renatured a 4-times lower binding was found. Without renaturation also a much lower surface pressure increase was attained. This indicates that the monomeric or the more unfolded form of apocytochrome *c* is more potent in penetrating the monomolecular layer. Fig. 5 shows that the bind-

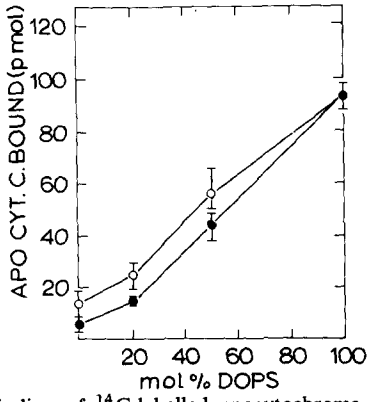

Fig. 5. Binding of ^{14}C-labelled apocytochrome *c* to monomolecular layers of DOPC (●) and DOPC/cholesterol (molar ratio, 1:1) (○) at increasing concentrations of DOPS. The initial surface pressure was 28 mN/m. The surface area was 29.61 cm^2. The final protein concentration in the subphase was 0.1 μM.

ing of apocytochrome *c* increases with increasing concentrations of DOPS. For determining the surface radioactivity the monolayer was collected after washing the subphase. No discontinuities are found at 50 or 20 mol% DOPS. This could indicate that at high DOPS concentrations not all apocytochrome *c* molecules can penetrate fully. The presence of cholesterol (molar ratio DOPC/cholesterol, 1:1) results in an increased change in surface pressure (see Fig. 4) but also an enhanced binding of apocytochrome *c*, even at 0 mol% DOPS (Fig. 5). At low cholesterol concentrations (molar ratio DOPC/cholesterol, 8:1) the binding of labelled apocytochrome *c* was slightly increased.

The importance of charge interactions for the penetration of apocytochrome *c* into DOPS monomolecular layers is also illustrated in Fig. 6. NaCl concentrations of more than 100 mM reduce the surface pressure increase significantly. For concentrations less than 100 mM NaCl the limiting pressure is 50 mN/m. At 200 and 300 mM NaCl the limiting pressure has decreased to 41 and 36 mN/m, respectively. The effects of pH on the interaction of apocytochrome *c* and DOPS are given in Fig. 7. In the pH range 4–8 especially the charge profile of the protein is affected. To cover the pH range from 4 to 8 a phosphate buffer was used for pH 5–8 and an acetate buffer for pH 4–5. At pH 7.0 the surface pressure increase was

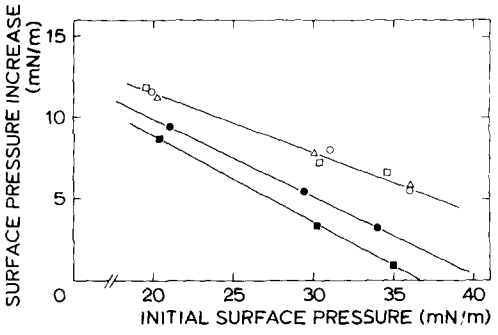

Fig. 6. Effect of NaCl concentration on the surface pressure increase of DOPS monolayers after the addition of apocytochrome *c* to the subphase. The subphase contained 10 mM Pipes (pH 7.0) and: no NaCl (□), 50 (○), 100 (△) 200 (●), or 300 mM NaCl (■). The final protein concentration in the subphase was 0.05 μM.

identical on phosphate buffer and on Pipes buffer. Lowering of the pH from pH 6.0 to 4.6 gives a significant increase in the surface pressure. This effect is slightly reduced at pH 4.0. There are no significant changes between pH 6 and 8.

In the absence of a lipid monolayer radiolabelled apocytochrome *c* collected at the interphase, up to a surface pressure of 16.2 mN/m, which is in agreement with previous results for unlabelled apocytochrome *c* [9]. The surface radioactivity culminated at the same time. Further addition of protein did not change the equilibrium

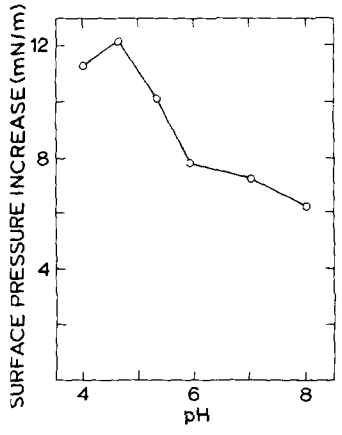

Fig. 7. pH dependence of apocytochrome *c* penetration in DOPS monolayers. At pH 5–8 a phosphate buffer and at pH 4 an acetate buffer (both 66 mM) were used. The initial surface pressure was 30 mN/m. The final protein concentration in the subphase was 0.05 μM.

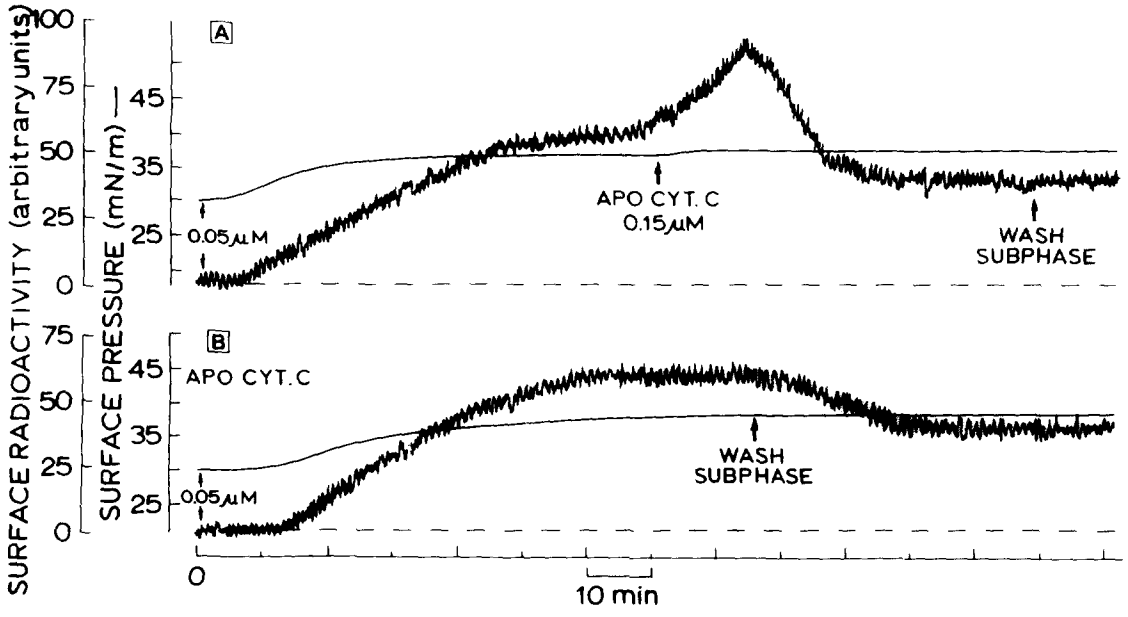

Fig. 8. The effect of apocytochrome *c* concentration on the surface pressure and surface radioactivity. A monolayer of DOPS was spread to an initial pressure of 30 mN/m. (A) ^{14}C-labelled apocytochrome c was injected to final concentrations of 0.05 and 0.15 μM. (B) ^{14}C-labelled apocytochrome c was injected to a final concentration of 0.05 μM. The start of washing the subphase with buffer at a rate of 10 ml/min is indicated.

value. After determining the surface radioactivity, it could be calculated that in the absence of lipid the molecular area of the protein is about 1300 Å^2.

The time dependency of the protein penetration in DOPS monolayers is given in Fig. 8. After injection of apocytochrome *c*, giving a subphase concentration of 0.05 μM, there is a rapid increase in surface pressure and a slower increase in surface radioactivity. The further addition of protein, giving a subphase concentration of 0.15 μM, caused no significant change in the surface pressure, but there was a further increase in the surface radioactivity (Fig. 8A). After about 10 min the surface radioactivity started to drop with about 55%, to result in an equilibrium value. The surface radioactivity did not change further, even after washing the subphase with buffer. The surface pressure was not affected. This experiment shows that high protein concentrations lead to the formation of adsorbed protein monolayers which spontaneously desorb. Also at lower protein concentrations of 0.05 μM an adsorbed monolayer will be formed, although to a lesser extent (Fig. 8B). This layer can only be removed after washing the subphase with buffer. The equilibrium value of the surface radioactivity is the same as in Fig. 8A, representing the penetrated protein. The formation of an adsorbed layer is also connected with the DOPS concentration in the monolayer. For DOPC-DOPS monolayers containing 50 mol% DOPS the effect is already strongly reduced, and at 20 mol% DOPS an adsorbed protein layer cannot be demonstrated. To test whether covalent oligomer formation, which has been described previously [12], occurs in the adsorbed layer the monolayer was collected, and it could be demonstrated by SDS-polyacrylamide gel electrophoresis and silver staining that oligomer formation at the interface depended on time and protein concentration. After 80 min oligomers could also be detected in the subphase. No oligomers were present in the interfacial layer after washing the subphase.

Proteins can be spread at the air/water interface by the method of Trurnit [24]. Compression of 3.45 nmol apocytochrome *c* resulted in a pressure-area curve with a collapse point at 14.4 mN/m (Fig. 9A). This corresponds to a molecular area of 1225 Å^2/molecule, which is in agreement with the molecular area determined for the adsorbed film at the air/water interface (see above).

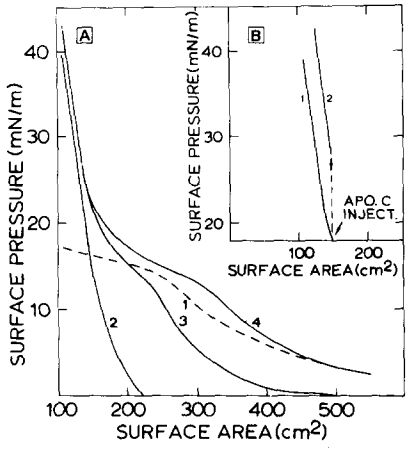

Fig. 9. (A) Force-area curves for apocytochrome *c* and mixed monolayers with DOPS. Apocytochrome *c* was spread by the method of Trurnit [24]. Curve 1, 3.45 nmol apocytochrome *c*; curve 2, 30 nmol DOPS; curve 3, 30 nmol DOPS and 1.72 nmol apocytochrome *c*; curve 4, 30 nmol DOPS and 3.45 nmol apocytochrome *c*. (B) Force area curve for DOPS and a mixed monolayer of DOPS and apocytochrome *c*. Apocytochrome *c* was injected to the subphase to a concentration of 0.05 μM underneath a DOPS monolayer at 18.4 mN/m. Curve 1, 30 nmol DOPS; curve 2, 30 nmol DOPS after penetration of apocytochrome *c*.

When both lipid and protein are spread and compressed, the shift in pressure-area curve of the phospholipid will give the area occupied by the protein present at the interface. At low surface pressures, a change in protein molecular area can already be observed in the spread mixed monolayer. Fig. 9A shows that there is only a small shift in the pressure in the pressure-area curve at high surface pressures, which is the result of the squeezing out of the protein during compression. This shift did not increase by spreading more apocytochrome *c*. A much bigger shift in the pressure-area curve can be observed when the protein is incorporated in the lipid film by penetration from the subphase (Fig. 9B). DOPS was compressed to a surface pressure of 18.4 mN/m, and apocytochrome *c* injection in the subphase increased the surface pressure to 28 mN/m after 30 min. Further compression showed that the protein remained in the interface. It can be concluded that after spreading apocytochrome *c* at the interface by Trutnit method [24] the conformation is such that only very little protein will interact with DOPS compared to penetration of the lipid monolayer by protein from the subphase.

In order to determine the molecular area of apocytochrome *c* at the interface in the presence of lipid it is necessary to quantify the amount of protein at the interface using radiolabeled protein. To collect only the protein present in the monolayer without the adsorbed protein layer or the protein present in the subphase, the subphase was washed with buffer. At constant surface area the penetration of the protein causes a pressure increase. The corresponding decrease in DOPS molecular area can be calculated form its pressure-area curve. The difference in surface area at both pressures is the area occupied by the protein $S_A \cdot (A_0 - A_1)/A_0$ (A_0, A_1, molecular area DOPS at initial and final surface pressure, respectively; S_A, total surface area). For apocytochrome *c* injected at an initial surface pressure of 25 mN/m and giving a final surface pressure of 35 mN/m the average value of four measurements was 712 $\pm$ 15 Å^2/molecule. At constant surface area the pressure increase will limit the protein penetration, and in order to determine the stoichiometry, measurements at constant pressure have also been performed. The relative area increase as a function of the surface pressure showed a linear decrease. Fig. 10 shows the incorporation isotherms of apocytochrome *c* into DOPS and DOPC monolayers at 25 mN/m, with a strong area increase for DOPS. Under these conditions the molecular area of apocytochrome *c* is 890 $\pm$ 91 Å^2/molecule. According to the binding model described by Hille et al. [25], assuming independent and identical binding sites, the stoichiometry and K_d of apocy-

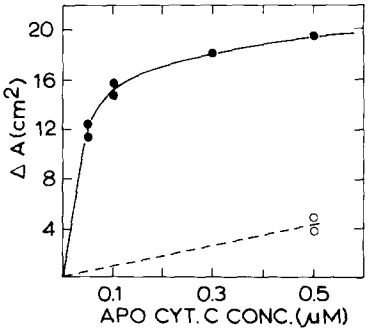

Fig. 10. The incorporation isotherms of apocytochrome *c* into DOPS (●) and DOPC (○) monolayers at a constant surface pressure of 25 mN/m.

tochrome *c* binding to DOPS is calculated. This yields a stoichiometry of 15 ± 2 PS per apocytochrome *c* molecule and a K_d of $0.017\,\mu M$.

Discussion

In agreement with previous results, a clear preference of apocytochrome *c* for negatively charged lipids as phosphatidylserines compared to zwitterionic lipids as phosphatidylcholines, can be seen. The results presented in Figs. 1 and 2 show also that the interaction is strongly dependent on the molecular packing of the monolayer. The increase in surface pressure correlates well with the molecular areas of the molecular species (Demel, R.A., Paltauf, F. and Hauser, H., Biochemistry, in press) It can be assumed that a decreased membrane lipid packing will also increase the insertion and translocation of apocytochrome *c*. This is contrary to the penetration of cytochrome b_5 into PC monolayers, which increased with increasing chain length in a homologous series of phosphatidylcholines [26]. The penetration of DMPS is reduced when the temperature is reduced, and a fully condensed monolayer is formed. Condensed monolayers show a lower compressibility, so that less protein at the interface would give the same pressure increase. Since the pressure increase has diminished, there is even a proportionally stronger reduction in apocytochrome *c* in the monolayer. Although the limiting pressure for condensed phosphatidylserines as DPPS is strongly reduced, there is still an interaction with apocytochrome *c* at pressures below 31 mN/m. On the other hand, condensed monolayers of phosphatidylcholines as DPPC show virtually no interaction. The results indicate that the charge interaction is not only required for binding the protein to the lipid but also for penetration in the hydrophobic phase.

Mixtures of DOPC and DOPS show that the charge distribution at the interface is important for the interaction with apocytochrome *c*. There is a linear increase in surface pressure with DOPS concentration, showing a break at 50 mol%. It is possible that at high concentrations not all DOPS can interact with apocytochrome *c*. It has been shown that above 50 mol% PS there is no further increase in rate and extent of translocation [12]. At high initial pressures (30–35 mN/m) at least 5–10 mol% DOPS is required before an effect can be observed. Of particular interest in this respect is the effect of cholesterol. With mixtures of DOPC and cholesterol (1 : 1) the effect of apocytochrome *c* is nearly maximal at 20 mol% DOPS, and at low DOPS concentrations there is a significant enhancement of the surface pressure increase. In support of this, we found also that more protein is incorporated in DOPS monolayers when cholesterol is present. Due to its condensing effect [27], cholesterol can increase the surface charge density and stimulate the interaction at low PS concentrations. The increased binding in the absence of DOPS could indicate that cholesterol also favours the hydrophobic interaction. It is tempting to speculate that cholesterol present in the outer mitochondrial membrane could have a stimulatory function in the binding and penetration of cytochrome *c*. It has been shown recently that the presence of cholesterol can catalyze the insertion of integral membrane proteins into vesicles [28]. The effect of ion concentration on the PS-apocytochrome *c* interaction can be in response to the screening of the surface charge, as well as in response to the effect on the protein structure or aggregation. The results are in agreement with earlier results [29], which showed a decreasing apocytochrome *c* binding with increasing salt concentration. The pH dependence of the apocytochrome *c* effect on the surface pressure gives supportive evidence for electrostatic interactions. The rapid pressure increase from pH 6 to 4.6 is most likely explained by an increase in positive charge of the protein. This observation correlates with the increased number of lipids associated with each protein in vesicles on titration below pH 5 [29].

It has been noted before that the apocytochrome *c*-lipid interaction facilitates the formation of oligomers [12], which is sharply increased in the presence of over 50 mol% charged phospholipid. The monolayer experiments show also that oligomers are formed when apocytochrome *c* accumulates at the interface, depending on the protein concentration and phosphatidylserine concentration in the monolayer. The removal of the adsorbed layer does not affect the surface pressure, and the monolayer collected thereafter does not show the presence of oligomers. It can be

concluded that the monomeric form of apocytochrome *c* is penetrating the monolayer.

In experiments with PS vesicles oligomer translocation has also been found, although at a much lower rate than for the monomeric form [12]. However, in the vesicle experiments a protein concentration three orders of magnitude higher was used than in these monolayer experiments, which enhance oligomer formation. In the absence of a lipid layer apocytochrome *c* developed a molecular area of 1200–1300 $Å^2$/molecule. Compression of spread mixed monolayers of DOPS and apocytochrome *c* showed that this does not lead to a significant interaction. The approach of the monolayer by the protein from the subphase is required for a successful interaction. The apocytochrome *c* molecular area under these conditions is 700–900 $Å^2$/molecule. The lower value (712 ± 15 $Å^2$/molecule) measured at constant surface area is probably due to the fact that the pressure increase limits the full penetration of the protein. The higher value (890 ± 91 $Å^2$/molecule) is measured at constant surface pressure. In the presence of DOPS the molecular area of apocytochrome *c* is about 1.5-times smaller than in the absence of the lipid. The surface area of apocytochrome *c* in the monolayer with lipids would agree with a structure 30–34 Å in diameter. For holocytochrome *c*, which has an ellipsoid structure, similar dimensions have been found [4]. It has been suggested [30,31] that for insertion of proteins into membrane an α-helix formation is essential. Such an increase in α-helix structure [9] has been found after the interaction of apocytochrome *c* with negatively charged detergents. The stoichiometry of 15 indicates that under our conditions in a pure DOPS monolayer not all PS molecules are involved in a charge neutralization of apocytochrome *c*. The low K_d value of 0.017 μM demonstrates the very efficient binding of apocytochrome *c* to DOPS, a value which is in agreement with the K_d of 0.04 μM found for the binding of the protein to PS in small unilamellar vesicles [5]. A comparable value, K_d 0.05 μM, was reported for the binding of apocytochrome *c* to the putative receptor on the mitochondrial outer membrane [32]. These results emphasize the high degree of lipid specificities in apocytochrome *c* binding and penetration into lipid monolayers which could contribute to the spatial specificities of the import process.

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