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Interaction of cytochrome c and its precursor apocytochrome c with various phospholipids

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The effects of cytochrome c and apocytochrome c on the structural properties of various membrane phospholipids in model systems were compared by binding, calorimetric, permeability, ^{31}P n.m.r. and freeze-fracture experiments. Both cytochrome c and apocytochrome c experience strong interactions only with negatively charged phospholipids; apocytochrome c interacted more strongly than cytochrome c. These interactions are primarily electrostatic but also have a hydrophobic character. Cytochrome c as well as apocytochrome c induces changes in the structure of cardiolipin liposomes as is shown by ^{31}P n.m.r. and freeze-fracture electron microscopy. Cytochrome c does not affect the bilayer structure of phosphatidylserine. In contrast, interaction of apocytochrome c with this phospholipid results in changes of the ^{31}P n.m.r. bilayer spectrum of the liposomes and also particles are observed at the fracture faces. The results are discussed in relation to the import of the protein into the mitochondrion.
Key words: apocytochrome c/cytochrome c/mitochondrial protein import/model membrane/protein-lipid interaction

Introduction

Although the mitochondrion has a protein synthesizing capacity, the majority of the mitochondrial proteins are synthesized as precursors on free ribosomes in the cytosol (Schatz and Mason, 1974). During import into the mitochondrion, the precursors are converted into the mature proteins. At the molecular level very little is known about the translocation process which, depending on the type of protein, might involve insertion and transport across the outer or outer and inner membrane. It has been suggested for the inner mitochondrial membrane protein, cytochrome c_1, and for the intermembrane space protein, cytochrome b_2, that after recognition by and binding to the outer membrane, pores arise at the contact sites of inner and outer membrane through which the proteins are imported (Gasser *et al.*, 1982). Similarly, it has been demonstrated that the precursor of the extrinsic inner mitochondrial protein cytochrome c is transferred to the mitochondrion after being synthesized in haem-free apoform, probably *via* a receptor-mediated recognition of the outer membrane (Hennig and Neupert, 1981), followed by covalent attachment of the haem and translocation to the inner mitochondrial membrane.

The attachment of the haem group results in a quite different conformation of the protein (Stellwagen *et al.*, 1972; Fisher *et al.*, 1973; Cohen *et al.*, 1974), which might be the driving force for the translocation of the protein across the outer membrane (Hennig and Neupert, 1981).

Although proteins are clearly involved, it is conceptually difficult to imagine the translocation step to occur entirely *via* a protein type of channel without affecting the essential barrier function of the membrane, which is determined to a large extent by the lipid moiety. Therefore, we examined the possibility that lipid-protein interactions might be of importance in the mitochondrial membrane protein import process. For this reason, we compared, in model membrane systems, lipid-protein interactions as measured by binding, permeability, ^{31}P n.m.r., freeze-fracture electron microscopy and calorimetric experiments of apocytochrome c and cytochrome c.

Besides the extensive studies on the import of cytochrome c into mitochondria (Korb and Neupert, 1978; Hennig and Neupert, 1981; Matsuura *et al.*, 1981) the choice of this protein is based on a number of different reasons. The apocytochrome c can be chemically prepared in large quantities from the holoenzyme cytochrome c (Fisher *et al.*, 1973) which is essential for a physical-chemical characterization of the nature of the interaction. Both the proteins are well characterized (Stellwagen *et al.*, 1972; Fisher *et al.*, 1973; Cohen *et al.*, 1974; Takano and Dickerson, 1981a) and the *in vitro* prepared apocytochrome c competes with the natural apocytochrome c for uptake into mitochondria (Zimmermann *et al.*, 1981). Furthermore, cytochrome c experiences strong and specific interactions with membrane lipids (Nicholls, 1974) which, in the case of the inner mitochondrial membrane lipid cardiolipin, can result in the formation of non-bilayer lipid structures (de Kruijff and Cullis, 1980b). Non-bilayer lipid structures might be involved in the contact sites between outer and inner membrane (van Venetië and Verkleij, 1982) and can possibly provide a pathway for protein insertion and transport (de Kruijff *et al.*, 1981).

It will be shown that large differences exist in the magnitude and nature of the interaction of apo- and holocytochrome c with phospholipids, leading to the suggestion that lipid-protein interactions might be of importance in the import of cytochrome c into mitochondria.

Results

Binding experiments

To obtain a first insight into possible differences in lipid-protein interaction between cytochrome c and apocytochrome c we studied the binding of these proteins to different phospholipid vesicles. In agreement with previous observations (de Kruijff and Cullis, 1980b), addition of cytochrome c caused, in the case of the negatively charged lipids beef heart phosphatidylserine (PS), 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) and cardiolipin, an immediate precipitation of the multilamellar vesicles (MLV) which is accompanied by strong protein-lipid binding, and which reaches a plateau at high amounts of added protein (Figure 1, A-D). Apocytochrome c addition also resulted in direct precipitation of the liposomes prepared from the negatively charged lipids. No visual changes were noticed when apocytochrome c was added to the neutral phosphatidylcholine (PC) liposomes. The binding of apocytochrome c to the negatively charged liposomes, as judged from the saturation level, appears to be stronger than in the case of cytochrome c. Virtual-

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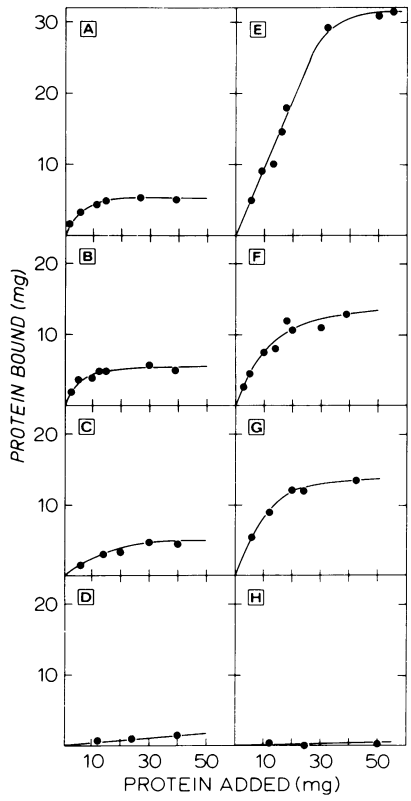

Fig. 1. Binding of cytochrome c **(A – D)** and apocytochrome c **(E – H)** to MLV of DMPG **(A,E)**, beef heart cardiolipin **(B,F)**, beef heart PS **(C,G)** and egg PC **(D,H)**. The binding experiments were performed as described in Materials and methods. The amount of protein added and the amount of protein bound is represented in mg protein per 10 μmol lipid phosphate.

ly no binding could be detected to the neutral PC liposomes. Using the binding model $P + L_N \rightleftharpoons PL_N$, where P and L are the concentration of free protein and lipid, respectively, and N is the number of lipid molecules that provide one binding site for the protein molecule P, the dissociation constant K_D is equal to $\frac{(P)(L_N)}{[PL_N]}$. The K_Ds were calculated from the binding curves reported in Figure 1 by using iterative non-linear regression methods as described previously (Hille *et al.*, 1981). In this model it is supposed that all lipid molecules are accessible for the protein. The results below show that in MLV of cardiolipin not all lipids are available for the protein and, in this case, this approach results in an overestimation of the K_Ds. The K_Ds in the case of cytochrome c binding to DMPG, cardiolipin, beef heart PS and egg PC were found to be 30.1, 13.3, 164.1 and 485 μM, respectively. In the case of apocytochrome c, the K_Ds were found to be 30 μM for DMPG, 35.5 μM for cardiolipin, 19.3 μM for beef heart PS and 979.1 μM for egg PC. The differences in the saturation level of binding of cytochrome c and apocytochrome c could, in part, be due to differences in availability of the lipids in the MLV. Therefore, we compared the binding of cytochrome c to different types of cardiolipin vesicles. Addition of cytochrome c to large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV) also caused precipitation of the vesicles and increased the saturation binding level nearly 2-fold (Figure 2, Table I). A similar level was reached when the MLV were prepared in a cytochrome c-containing solution in which case cytochrome c can potentially interact with all lipid molecules present (Figure 2B). Similar differences in

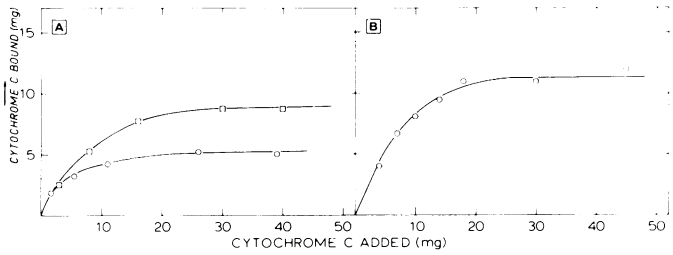

Fig. 2. Binding of cytochrome c to MLV of cardiolipin (○——○) performed with the external **(A)** or internal **(B)** method and to LUV (□–□), of cardiolipin. Experimental conditions are as described in Materials and methods. The amount of cytochrome c is represented in mg per 5 μmol cardiolipin.

Table I. Stoichiometry of cytochrome c and apocytochrome c binding to different liposomal systems

Lipid	Vesicles	Method	mol cytochrome c/mol P_i (x 10^{-2})	mol apocytochrome c/mol P_i (x 10^{-2})
PG	MLV	external	4.6	24.4
PG	MLV	internal	4.6	
CL	MLV	external	4.4	10.5
CL	MLV	internal	9.5	14.3
CL	LUV	external	8.3	
CL	SUV	external	9.1	
PS	MLV	external	4.0	11.1
PS	MLV	internal	4.0	11.1
PC	MLV	external	1.6	<0.2
PC	MLV	internal	3.3	<0.2

The protein-lipid ratio is calculated at the level where 50 mg protein is added to 10 μmol lipid phosphate.

binding to MLV of cardiolipin, but to a smaller extent, were noticed when apocytochrome c was added to the vesicles or incorporated into the vesicles during the preparation of the model membranes. This demonstrates that in the MLV a part of the cardiolipin molecules cannot interact with the protein but that, in the case of the LUV and SUV, all molecules, including those initially present in the inner monolayer, can interact with the protein. In the case of beef heart PS and DMPG MLV, the saturation binding level is independent of the way the proteins are added, showing that in those multilayered structures all potential binding sites are available to externally added protein (Table I). From a comparison of the stoichiometry at maximal binding it can be concluded that more apocytochrome c than cytochrome c can be bound per negatively charged lipid molecule and that apocytochrome c has the highest affinity for DMPG.

K^+ permeability studies

For studying the nature of protein-lipid interactions, investigations on the barrier properties of the protein-containing model membranes can be useful. Early ion permeability studies (Papahadjopoulos *et al.*, 1975) showed that cytochrome c induces a Na^+ efflux in sonicated PS vesicles. It would be interesting to compare the effect of the cytochrome c and apocytochrome c on the ion permeability of the different model membranes. To MLV of beef heart PS, cardiolipin and egg PC, in which K^+ ions have been enclosed, cytochrome c or apocytochrome c was added and the K^+ release was measured as a function of time. Figure 3

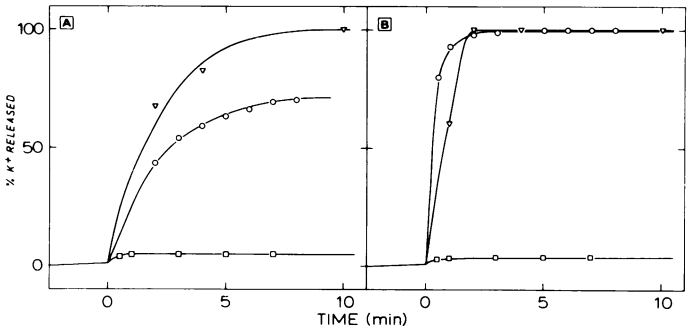

Fig. 3. K^+ release of MLV of beef heart PS (▽——▽), cardiolipin (○——○) and egg PC (□—□), after adding cytochrome c **(A)** or apocytochrome c **(B)** as a function of time at 30°C. 13 mg protein in 250 μl buffer was added at $t = 0$ to 10 μmol lipid in 7.5 ml buffer.

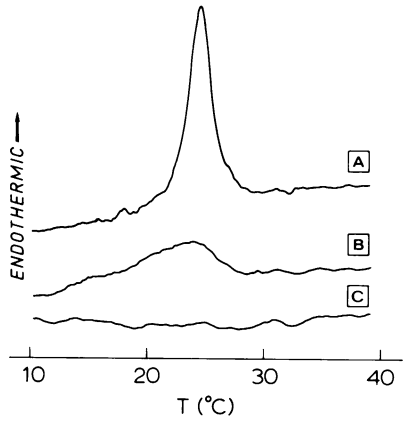

Fig. 4. DSC heating curves of MLV of DMPG **(A)**, MLV of DMPG after incubation with cytochrome c **(B)** and after incubation with apocytochrome c **(C)**. Experimental conditions are as described in Materials and methods.

shows that both proteins cause a fast K^+ efflux in the MLV of beef heart PS and cardiolipin, the strongest release being caused by apocytochrome c. The proteins are not able to induce a K^+ leakage in MLV of the neutral PC, which agrees with the low affinity of the proteins for this phospholipid. While addition of cytochrome c to cardiolipin MLV results in 60% release of the total K^+ content, suggesting that cytochrome c cannot reach all bilayers in the model membranes, addition of cytochrome c to cardiolipin LUV induces a 100% K^+ efflux within 2 min (data not shown).

Differential scanning calorimetry (DSC)

The ability of proteins to increase the permeability of the bilayers of phospholipids is related to their ability to penetrate the bilayer and/or perturb the acyl chain packing within the bilayers (Kimelberg and Papahadjopoulos, 1971). The extent of bilayer penetration can be determined *via* DSC by studying the effect of the protein on the temperature, width and energy content of the gel ⇒ liquid crystalline transition of synthetic phospholipids. In view of the affinity of the proteins for negatively charged lipids and the possibility of protein denaturation, we decided to study the effect of the protein on the thermotropic properties of DMPG, which in the absence of the proteins undergoes a reversible transition at 21°C with a ΔH of 7.7 kcal/mol (Figure 4A), in agreement with previous observations (Mombers *et al.*, 1979).

The addition of cytochrome c to MLV of DMPG (Figure 4B) causes, in agreement with earlier observations (Kimelberg

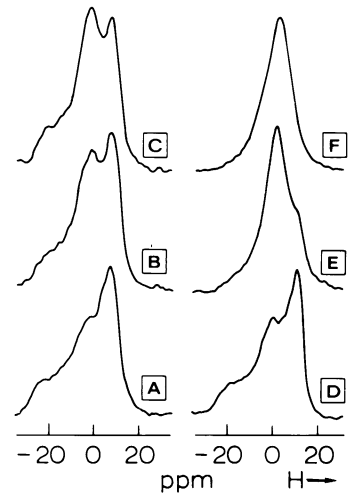

Fig. 5. ^{31}P n.m.r. spectra at 30°C of MLV of cardiolipin in the presence of cytochrome c **(A–C)** and apocytochrome c **(D–F)**. 40 μmol cardiolipin was dispersed in 1.0 ml buffer **(A,D)** and 13 mg **(B,E)**, respectively, 50 mg **(C)** cytochrome c or apocytochrome c **(F)** was added as a solution of 50 mg protein per ml buffer. The spectra were recorded 10 min after adding the protein. The 0 p.p.m. position in this and subsequent figures, represents the chemical shift position of the signal from sonicated egg PC vesicles.

and Papahadjopoulos, 1971, a decrease of the gel ⇒ liquid crystalline transition temperature, a broadening of this transition and a lowering of the ΔH to 2.9 kcal/mol. Figure 4C shows that addition of apocytochrome c to MLV of DMPG results in a total disappearance of the phase transition of the lipid. From these effects it can be concluded that both proteins have a strong electrostatic and hydrophobic interaction with negatively charged phospholipids and that apocytochrome c induces the strongest perturbation in the lipid packing.

^{31}P n.m.r.

The introduction of ^{31}P n.m.r. has facilitated the discrimination between several phospholipid structures in natural and model membranes. Due to incomplete averaging of the chemical shift anisotropy, the line-shape of the proton-decoupled ^{31}P n.m.r. spectrum is sensitive to the mode of phospholipid organization. Phospholipids organized in an extended bilayer show a ^{31}P n.m.r. spectrum with a low field shoulder and a dominant high field peak, separated by ~40 p.p.m. This line-shape is the result of the rotational motion of the phospholipid-phosphate around the long axis of the molecules. In the hexagonal H_{II} phase the lipids can undergo, in addition, fast diffusion around the cylinders, of which this phase is composed, resulting in a further averaging of the chemical shift anisotropy. In this case, a ^{31}P n.m.r. spectrum of a reduced width and reversed symmetry is observed. Phospholipids organized in structures in which the molecules can undergo rapid isotropic motion (e.g., in SUV, micelles, inverted micelles) give rise to a narrow symmetrical line-shape. It should be noted that changes in local structure or motion in the phosphate region can cause similar spectral changes. Membrane proteins can influence the structural organization, as has been demonstrated before in the cytochrome c-cardiolipin system (de Kruijff and Cullis, 1980b). In agreement with these studies, cytochrome c addition to cardiolipin MLV resulted in the formation of an isotropic component in the ^{31}P n.m.r. spectrum (Figure 5A–C). Sometimes, in addition, spectral components indicative of the

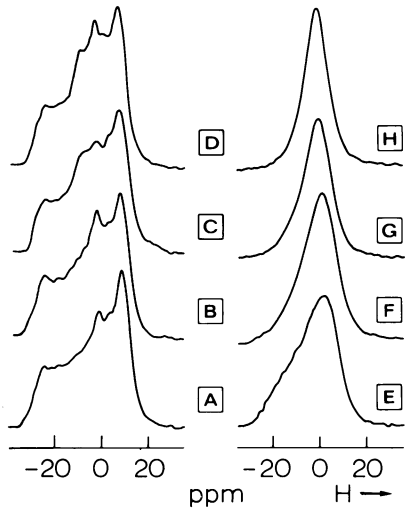

Fig. 6. ^{31}P n.m.r. spectra of cardiolipin-cytochrome c **(A–D)** and cardiolipin-apocytochrome c **(E–H)** reconstitutions, prepared by the internal method. 40 μmol cardiolipin was dispersed in 1.2 ml buffer, containing 50 mg protein and spectra were recorded at 0°C **(A,E)**, 20°C **(B,F)**, 30°C **(C,G)** and 40°C **(D,H)**.

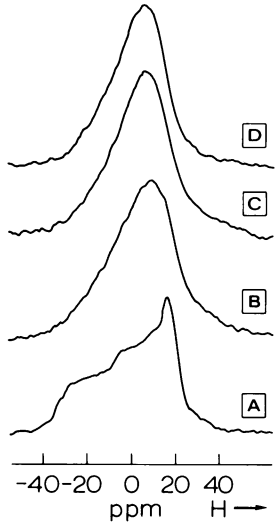

Fig. 7. ^{31}P n.m.r. spectra at 30°C of beef brain PS in the presence of apocytochrome c. 80 μmol beef brain PS was dispersed in 1.0 ml buffer **(A)** and 15 mg **(B)**, 30 mg **(C)** and 50 mg **(D)** apocytochrome c was added as a solution of 50 mg protein/ml buffer.

H_{II} phase were noticed. Addition of similar amounts of apocytochrome c induced a broad isotropic signal in the ^{31}P n.m.r. spectra of the cardiolipin MLV (Figure 5D–F). For the ^{31}P n.m.r. spectra, shown in Figure 6, the protein-lipid recombinants were prepared by adding the protein solution to the dry cardiolipin film. The ^{31}P n.m.r. spectrum of the cytochrome c-cardiolipin system showed at 0°C and 10°C (Figure 6A,B) besides the dominant peak at 11 p.p.m., which is characteristic for phospholipids in extended bilayers, a small component at 0 p.p.m., indicating isotropic motion of lipid molecules. The ^{31}P n.m.r. spectra at 30°C and 40°C (Figure 6C+D) were shown also to have a small peak at 6.5 p.p.m., which is typical for phospholipids in the H_{II} phase. This peak disappeared by cooling the sample to 20°C, which indicates reversibility.

The resulting recombinants obtained after hydration of the cariolipin with apocytochrome c showed broad symmetrical spectra (Figure 6E–H), as was observed after the addition of the proteins to preformed liposomes. At lower temperatures the spectra were slightly asymmetrical. In early studies (de Kruijff and Cullis, 1980b) it was found that addition of cytochrome c to beef brain PS liposomes did not change the ^{31}P n.m.r. spectrum of the phospholipids. In contrast, the ^{31}P n.m.r. spectra of beef brain PS MLV, which in the absence of protein has the characteristic asymmetrical line-shape (Figure 7A) has changed to a broad isotropic peak after adding 15 mg apocytochrome c to 80 μmol lipid (Figure 7B), which is hardly affected by further protein addition (Figure 7C,D).

Freeze-fracture electron microscopy

In agreement with early observations (de Kruijff and Cullis, 1980b), addition of cytochrome c to cardiolipin liposomes caused a number of changes in the freeze-fracture morphology of the liposomes. Besides stacking of the bilayers and the formation of tubular structures (Figure 8B) and occasionally hexagonal H_{II} type of structures, particulate structures are visible on the bilayer surfaces (Figure 8C) which is remarkable because cytochrome c is believed to be an extrinsic membrane protein (Papahadjopoulos *et al.*, 1975). That these morphological features are specific for the cardiolipin-cytochrome c interaction is indicated by the observation that cytochrome c (except for bilayer stacking) does not affect the morphology of PS liposomes despite the strong PS-cytochrome c interaction (Figure 8A). Apocytochrome c addition to cardiolipin-liposomes caused the formation of well defined relatively large particles (Figure 9A). No tubular or hexagonal H_{II} structures could be detected. In strong contrast to cytochrome c, addition of apocytochrome c also greatly affected the morphology of the PS liposomes. Addition of low amounts of apocytochrome c caused the appearance of particles on the fracture face of the bilayer (Figure 9B). The number of particles increased with increasing apocytochrome c concentrations. At high amounts of added apocytochrome c, smooth fracture faces could no longer be detected; instead the entire system consisted of a particulate structure (Figure 9C). Similar morphological changes were observed when the apocytochrome c was added to MLV of PS at a concentration of 1 mg/ml.

Discussion

Our results show that cytochrome c and apocytochrome c both interact strongly with negatively charged phospholipids in model membranes. From the absence of such interactions with neutral PC liposomes and the net positive charge of the protein at neutral pH, it can be concluded that these interactions are primarily electrostatic. However, since the barrier function of the vesicles is greatly reduced upon addition of the proteins, and the acyl chain packing is perturbed, as revealed by DSC, it can be suggested that the proteins subsequently penetrate into the bilayer, thus giving rise to hydrophobic protein-lipid interactions. Such suggestions have been made previously for cytochrome c (Papahadjopoulos *et al.*, 1975). Besides these local effects, the proteins also cause large macroscopic changes in phospholipid structure. In the case of cytochrome c, upon interaction with MLV of PS, precipitation of the lipids occurs in the form of closely packed multilayered structures. Massive structural reorganization of PS molecules must occur, since binding sites on the liposomes of the inner lamellae become more available to the added proteins. However, the phospholipids remain organized in ex-

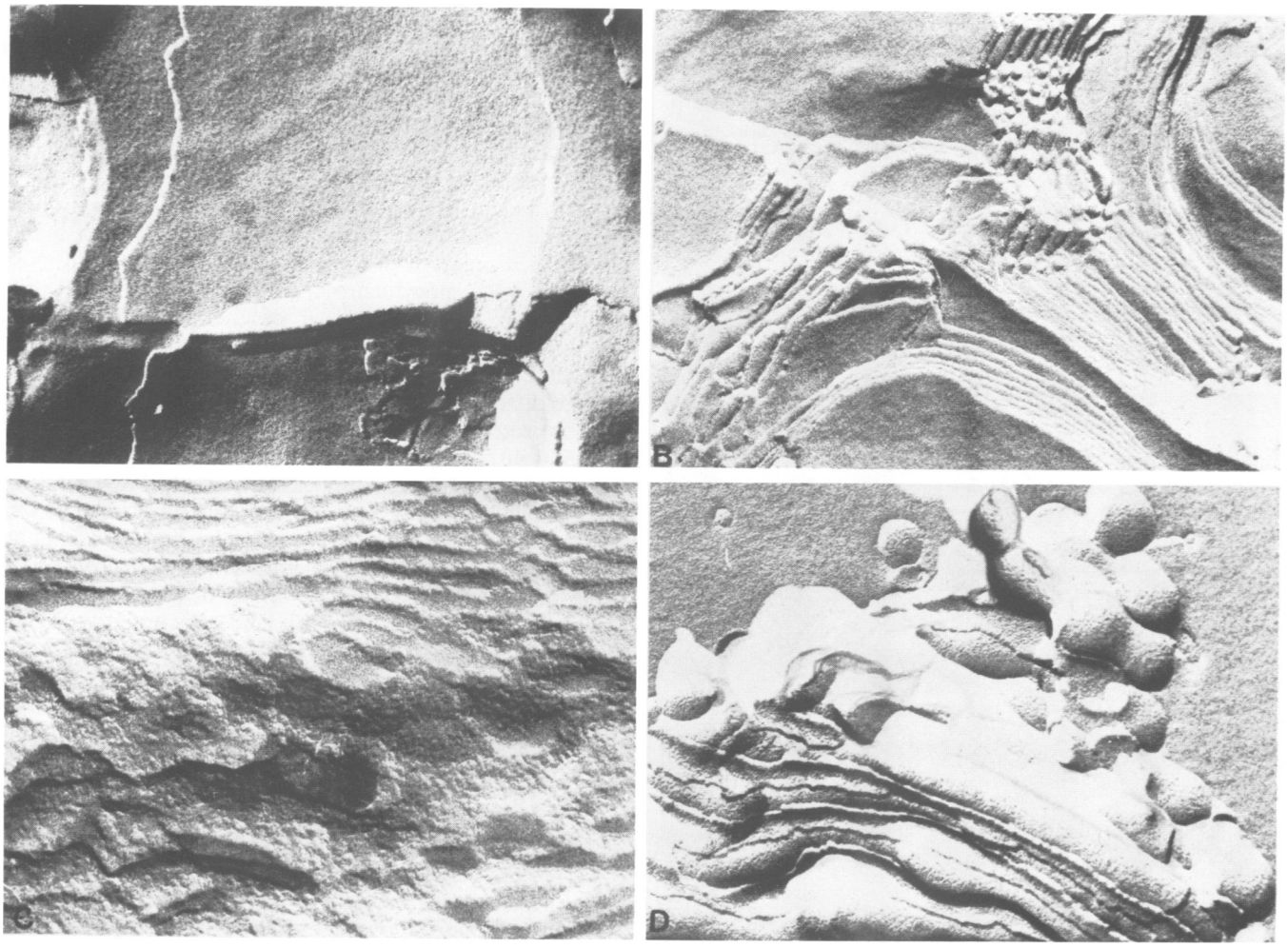

Fig. 8. Freeze-fracture electron microscopy of various cytochrome c-containing systems. 5 mg cytochrome c was added as a solution of 50 mg/ml to MLV of 5 μmol beef brain PS **(A)** or to MLV of 2.5 μmol of cardiolipin **(B – D)**. Magnification x 100 000.

tended lamellar structures and the local order in the phosphate region is not significantly affected (de Kruijff and Cullis, 1980b). Freeze-fracturing reveals smooth fracture faces demonstrating the absence of non-lamellar lipid structures as well as the absence of protein particles, typical for intrinsic membrane proteins. Cytochrome c addition to cardiolipin liposomes results in comparable changes but, in addition, ^{31}P n.m.r. indicates large changes in phospholipid order, consistent with the formation of non-lamellar lipid structures (de Kruijff and Cullis, 1980b). Also, on the fracture face of the cardiolipin liposomes, particulate structures are present, suggesting penetration of the proteins in the bilayer or the formation of protein-lipid complexes (see also de Kruijff and Cullis, 1980b).

The influence of apocytochrome c on the macroscopic structure of the lipids is quite different. Both in the case of cardiolipin and PS, the protein induces the formation of a broad isotropic peak in the ^{31}P n.m.r. spectrum of the phospholipids. Since the local structure of the phosphate region of the phospholipids in model and biological membranes is highly conserved (Seelig and Seelig, 1980), as a first approximation it is assumed that the 'bilayer' ⇒ 'isotropic' transition in the ^{31}P n.m.r. spectrum is due to increased isotropic motion of the phospholipid molecules. This could, for instance, be due to wobbling of the phospholipids around their long axis, diffusion of the phospholipids around curved small structures or due to the formation of non-lamellar lipid structures. Freeze-fracturing reveals that both in cardiolipin and PS systems apocytochrome c induces the formation of large particles on the fracture faces. These particles could reflect either apocytochrome c penetrated into the bilayer, or protein-lipid complexes possible in an intrabilayer non-lamellar lipid configuration. In view of the size of the particles (≈ 100 Å) and the relatively small size of the apoprotein, the possibility that protein aggregates give rise to the particles has to be considered. Freeze-fracturing of a 50 mg/ml apocytochrome c solution (data not shown), reveals similarly sized protein particles, in agreement with the suggestion that apocytochrome c shows a concentration-dependent aggregation (Stellwagen *et al.*, 1972). However, similar particles are found on the fracture faces of the PS bilayers when the apocytochrome c is added as a diluted solution in which the protein is either mono- or dimeric. Therefore, if these particles represent protein aggregates, the aggregates are caused by the interaction with the negatively charged lipid. It can be questioned whether the observed structural changes are specific for these proteins, or are the result of the interaction of any basic protein or polypeptide with negatively charged lipids. This latter possibility is unlikely as poly-L-lysine, which also strongly binds to negatively charged lipids, does not induce an isotropic ^{31}P n.m.r. signal (de Kruijff and Cullis, 1980a), nor particles on the fracture face (B.de

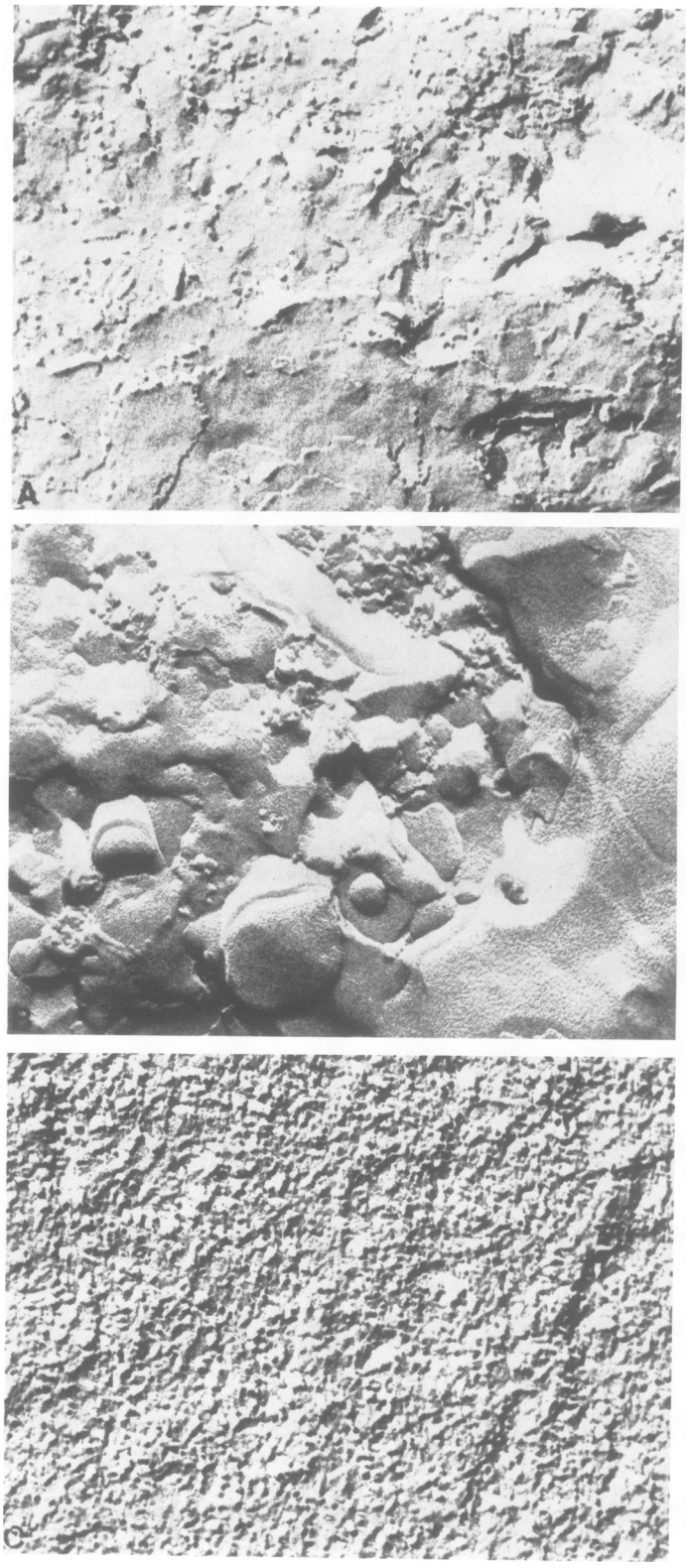

Fig. 9. Freeze-fracture electron microscopy of various apocytochrome c-containing systems. MLV of 5 μmol cardiolipin **(A)** and 2.5 μmol beef brain PS **(B,C)** after adding 5 mg **(A,C)** and 1.25 mg **(B)** apocytochrome c. The protein was added as a solution of 50 mg protein/ ml buffer. Magnification x 100 000.

Kruijff, unpublished observations). From the primary structure, it can be calculated that at neutral pH cytochrome c and apocytochrome c will have eight net positive charges on the protein. From the stoichiometry of the binding of the proteins to the negatively charged lipids, it can be concluded that, in the case of cytochrome c and apocytochrome c, neutralization occurs. However, in the case of apocytochrome c binding to DMPG liposomes, more protein is bound than can be accounted for by charge stoichiometry, which possibly indicates the presence of protein aggregates.

Most data suggest that the interaction between apocytochrome c and negatively charged lipids is stronger than that of cytochrome c with these lipids. Since the overall positive charge of the apocytochrome c must be less than that of cytochrome c, due to removal of the haem containing the positively charged iron, the stronger interaction must be the result of the differences in protein conformation. Indeed, large differences in tertiary structure have been reported for these proteins (Fisher *et al.*, 1973). Apocytochrome c is mainly in a disordered structure, whereas cytochrome c is highly structured (Takano and Dickerson, 1981a, 1981b). Since our data suggest that the apo-protein penetrated into the bilayer and import of apocytochrome c in mitochondria is thought to be related to changes in structure of apocytochrome c (Hennig and Neupert, 1981), it will be of great interest to see whether, upon interaction with lipids, the tertiary structure of apocytochrome c changes. The strong PS-apocytochrome c interaction is also of great interest for the import of the protein. Cardiolipin is confined to the inner mitochondrial membrane, where cytochrome c is located. However, PS is the major negatively charged lipid in the outer membrane (Stoffel and Schiefer, 1968), which is the first barrier the apocytochrome c has to pass. Recent freeze-fracture studies revealed that addition of apocytochrome c to rat liver mitochondria caused the formation of contact sites between the outer and inner membrane (van Venetië and Verkleij, 1982). It is, therefore, tempting to speculate that these sites formed by apocytochrome c-negatively charged phospholipid interactions are the pathways for the import of the protein.

Materials and methods

Lipids

Beef brain PS was isolated and purified as described before (Sanders, 1967), and egg PC was isolated from hen eggs. DMPG was synthesized as described previously (van Dijck *et al.*, 1975). A highly unsaturated PS was isolated from beef heart as will be described elsewhere. The sodium salt of cardiolipin from beef heart was obtained from Avanti (Birmingham). All lipids were chromatographically pure.

Proteins

Cytochrome c (type VI) was purchased from Sigma (St. Louis, MO) and was in the oxidized form. Apocytochrome c was prepared from cytochrome c as described previously (Fisher *et al.*, 1973), but on a 10x larger scale. There was no detectable amount of cytochrome c present in the final product. On 12.5% polyacrylamide slab gel electrophoresis in 0.38 M glycine, 0.05 M Tris pH 8.3, containing 0.1% SDS, cytochrome c and apocytochrome c each migrated as a single band corresponding to a mol. wt. of 12 500. The fluorescence spectra of cytochrome c and apocytochrome c in the absence and presence of guanidine-HCl were identical to those published before (Fisher *et al.*, 1973). Gel filtration on Sephadex G-75 (superfine) column (50 x 1 cm) of a 3 mg/ml buffer apocytochrome c solution indicated an apparent mol. wt. of the protein of 27 500. Ferritin (mol. wt. 450 000), bovine albumin (45 000) and chymotrypsin (25 000), all from Boehringer, Mannheim, were used as marker proteins. Using the same column, poly-L-lysine (mol. wt. 20 000) obtained from Sigma, eluted with an apparent mol. wt. of 47 000.

Preparation of model membranes

MLV were prepared by hydrating the dry lipid film as described previously (de Kruijff and Cullis, 1980b). The preparation of SUV by ultrasonication of MLV was also described previously (de Kruijff *et al.*, 1975). LUV were prepared *via* an ether evaporation method (Deamer and Bangham, 1976). All types of model membranes were prepared in 100 mM NaCl, 10 mM Tris-HCl, pH 7.0.

Binding studies

The protein-lipid binding experiments were performed by incubation of the vesicles with increasing amounts of protein at 30°C during 30 min. Typically, 1.25 μmol lipid (based on lipid phosphate) was incubated with cytochrome c or apocytochrome c in 100 mM NaCl, 10 mM Tris-HCl, pH 7.0 and a total volume of 750 μl. In the case of the MLV, the dry lipid film was either hydrated with buffer, whereafter the protein solution was added (external method) or the lipid film was hydrated with the protein solution (internal method). In both cases the mixtures were incubated for 30 min.

The non-bound protein was separated from the MLV or LUV by spinning the samples for 20 min at 27 000 *g* at 4°C. The amounts of protein and P_i were determined in the supernatant. The P_i in the supernatant was <2% of the total P_i in the sample. In the case of SUV, the protein was separated from the vesicles by ultrafiltration of the mixture on an Amicon XM-100 membrane (Brown and Wüthrich, 1977), whereafter protein and P_i contents were determined in the effluent. More than 95% of the vesicles and no protein was retained on the filter.

K^+ permeability studies

For K^+ trap measurements, the model membranes were prepared in 100 mM KCl, 10 mM Tris/HCl, pH 7.0. The outside K^+ was removed by dialysis of the sample against 5 x 250 ml of the 100 mM choline chloride buffer analogue or (in the case of cardiolipin LUV), by gel filtration over a Sephadex G-50 (coarse) column (4.5 x 1 cm) and elution with the choline chloride, Tris/HCl buffer. The K^+ release of the model membranes was measured at 30°C with a K^+-specific glass electrode as described previously (Blok *et al.*, 1975). Corrections were made for the small electrode response caused by the addition of the protein itself.

^{31}P n.m.r.

^{31}P n.m.r. spectra were recorded at 34.6 MHz under conditions of proton decoupling as described before (Cullis and de Kruijff, 1976). Generally, 10 000 45° r.f. pulses with 0.17 s inter-pulse time were employed on 1.2 ml samples in 10 mm tubes containing 80 μmol lipid (based on P_i) in 100 mM NaCl, 0.2 mM EDTA, 10 mM Tris/HCl, pH 7.0 buffer, containing 25% ^2H_2O. To increase the signal-to-noise ratio, all free induction decays were exponentially filtered resulting in a 50 Hz line broadening.

Freeze-fracture electron microscopy

The samples were quenched with either the two-sided (Müller *et al.*, 1980) or one-sided (Pscheid *et al.*, 1981) jet-freezing technique. No cryoprotectants have been used. Freeze-fracture electron microscopy was performed according to established procedures.

DSC

Calorimetric experiments were carried out on DMPG dispersions in the absence and presence of cytochrome c and apocytochrome c. Typically, MLV of DMPG (8 μmol) were incubated with or without 20 mg protein in a total volume of 1.5 ml, whereafter the lipids were pelleted and transferred to the commercial 200 μl stainless steel sample pans of the Setaram III high sensitivity calorimeter. Thermograms were obtained from heating runs using a scan rate of 2°C/min and a sensitivity setting of 100 μV. For the detection of the ΔH, the exact amount of lipid in the pan was afterwards determined by a phosphate determination.

Analytical methods

Cytochrome c was quantitated by absorbance measurement at 410 nm (Nichols, 1974) and apocytochrome c was determined *via* a protein assay (Lowry *et al.*, 1951). Lipid phosphorus was determined after perchloric acid destruction of the lipids (Fiske and Subbarow, 1925).

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