Biochimica et Biophysica Acta, 942 (1988) 115–124
Elsevier

BBA 74037

Influence of heme and importance of the N-terminal part of the protein and physical state of model membranes for the apocytochrome *c*–lipid interaction

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(Received 29 January 1988)

Key words: Apocytochrome *c*; Cytochrome *c*; Protein–lipid interaction; Phosphatidylserine; Protein translocation; Carboxyfluorescein release; Differential scanning calorimetry

The interaction between cytochrome *c* and its heme-free precursor apocytochrome *c* and chemically prepared fragments of these basic proteins with phosphatidylserine containing model membrane systems was studied by differential scanning calorimetry and carboxyfluorescein release experiments. Addition of apocytochrome *c* and fragments derived from the N-terminus cause a pronounced and linear decrease of the enthalpy (ΔH) of the gel to liquid-crystalline phase transition of dielaidoylphosphatidylserine. In contrast, fragments derived from the C-terminus cause a smaller reduction in ΔH; a similar trend was observed for the ability of the fragments to cause an increased carboxyfluorescein release from unilamellar vesicles. In addition, the covalent attachment of the heme at cysteine residues 14 and 17 greatly reduced the ability of both the intact protein and the N-terminal fragments to decrease ΔH. Using a protein translocation assay based on large unilamellar vesicles containing enclosed trypsin it was found that at gel state temperatures the ability of apocytochrome *c* to partially translocate the bilayer (reach the opposite membrane/water interface) was greatly reduced. The implications of these findings for the import mechanism of apocytochrome *c* in mitochondria are shortly indicated.

Introduction

Most mitochondrial proteins are synthesized in the cytosol as precursor proteins and then imported into mitochondria [1,2]. One such a protein is cytochrome *c*, a component of the inner membrane which is coded on a nuclear gene [3,4], and posttranslationally imported as apocytochrome *c* into mitochondria [5,6]. After passing through the outer membrane, the apoprotein is converted into holocytochrome *c* [7,8] by covalent attachment of the heme by the enzyme cytochrome *c* heme lyase [9–12]. For binding to the outer membrane, a putative (proteinaceous) receptor was proposed [13]. Our studies [14–16] indicate that the negatively charged lipid components of the membrane could function as such. Electron microscopic analysis of negatively stained crystalline channel arrays derived from the outer mitochondrial membrane of *Neurospora crassa* incubated with apocytochrome *c* lead to the suggestion that the protein binds to the lipid component and not the pore protein itself [17].

It was recently proposed that an apocytochrome *c* binding protein occurs in the intermem-

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brane space [12]. The conversion of apocytochrome *c* to holocytochrome *c* was found to be stimulated by NADH and a heat stable, protease insensitive, low molecular weight cytosolic factor [12].

The molecular mechanism of the actual translocation process across the outer mitochondrial membrane is still unknown. However, studies on apocytochrome *c*–lipid interactions in model systems summarized in [15,16,18–21], strongly suggest a lipid involvement in this process. Apocytochrome *c* has a high affinity for binding to negatively charged phospholipids [14–16]. The initial electrostatic interaction is followed by penetration of the protein into the hydrocarbon region [18–19], resulting in perturbation of acyl chain packing as inferred for instance from the large reduction in energy content of the gel to liquid-crystalline phase transition of different synthetic negatively charged phospholipids [18].

Because of its preferential interaction with the negatively charged lipid component in mixed bilayers, the protein can create a translocation site where the protein passes the hydrophobic core of the bilayer and reaches (partially) the inner surface of the vesicles [14,20,22]. The interaction between apocytochrome *c* and negatively charged phospholipids causes a conformational change from a largely disordered structure in aqueous solution to a partially α-helical structure in a hydrophobic environment [18,23].

In this study, we address two new aspects of the apocytochrome *c*–lipid interaction. Firstly, we investigated by differential scanning calorimetry and carboxyfluorescein release experiments the interaction of different chemically prepared fragments of horse heart apocytochrome *c* with negatively charged phospholipids to understand how the different parts of the protein interact with model membranes. Special attention is paid to the role of the covalently attached heme moiety by comparing heme-free and heme-containing peptides. Secondly, we studied the temperature dependence of the translocation process in large unilamellar vesicles, using a previously described translocation assay [15] and focussed on the possible influence of the physical state of the lipid on the translocation process. The results indicate that the N-terminus of the apoprotein is particularly effective in perturbing gel state lipid packing which effect is dramatically decreased by the presence of the heme moiety as in the holoprotein. Additionally, the N-terminus is efficient in inducing an increase in membrane permeability. Furthermore, apocytochrome *c* translocation is strongly temperature dependent and is particularly ineffective at gel state temperatures.

Materials

1,2-Dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dielaidoyl-*sn*-glycero-3-phosphoserine (DEPS) and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) were synthesized according to established methods [24,25]. Bovine brain phosphatidylserine was isolated and purified as described in [26]. Egg phosphatidylcholine was obtained from hen eggs [27]. 6-Carboxyfluorescein was obtained from Eastman-Kodak (Rochester, NY, U.S.A.) and purified by the method of Ralston et al. [28]. Trypsin and soybean trypsin inhibitor were purchased from Merck (Darmstadt, F.R.G.) and trypsin substrate carbobenzoxy-Val-Gly-Arg-*p*-nitroanilide acetate from Boehringer, Mannheim (F.R.G.). Horse heart cytochrome *c* (type VI) was from Sigma (St. Louis, MO, U.S.A.). Apocytochrome *c* was prepared from cytochrome *c* by removal of the heme moiety [29], then subjected to a renaturation procedure [30]. The protein was stored at −20 °C in 50 mM NaCl, 10 mM Pipes (pH 7.0), 0.01% β-mercaptoethanol buffer (Pipes buffer) at a concentration of 1–2 mg/ml. The protein was always used immediately after thawing.

Fragments (1–38)H (fragment 1–38 containing the heme), 1–38, and 39–104 were prepared according to the method of Juillerat et al. [31]. Fragments (1–65)H, 66–80, and 81–104 were obtained by cyanogen bromide cleavage of cytochrome *c* as described by Corradin et al. [32]. Fragment 1–65 was obtained by removal of the heme moiety from fragment (1–65)H [29]. The fragments 1–59 and 60–104 were prepared by cleavage of the single tryptophanyl peptide bond in cytochrome *c* by BNPS-skatole (3-bromo-3-methyl-2-[(2-nitrophenyl)thio]3H-indole) [33] followed by treatment with DTT to break disulfide

bridges. The reductant was subsequently removed by gel filtration on a Sephadex G-25 column. All the fragments mentioned above showed one band on polyacrylamide gel electrophoresis. Amino acid analysis of the purified fragments were carried out with a LKB 4151 Alpha plus amino acid analyzer after 24 h hydrolysis at 110 °C in vacuum tubes in 5.8 M HCl. The results were in full agreement with the expected amino acid composition.

Methods

Differential scanning calorimetry (DSC)

A dry film of 2 μmol DEPS was hydrated by the addition of 1.5 ml Pipes buffer containing no or increasing amounts of polypeptide and the lipid was dispersed by 5 min shaking by hand at room temperature. After 30 min incubation at 30 °C (above the gel-liquid crystalline phase transition temperature of DEPS), the material was collected by centrifugation at 27000 × *g* at 4 °C for 30 min. The pellets were transferred into 75 μl stainless steel sample pans. The samples were scanned with a heating rate of 2 °C per min in a Perkin-Elmer DSC-4 calorimeter equipped with a thermal analysis data station. All samples were scanned at least two times in the 15–35 °C temperature range, yielding identical thermograms. The exact amounts of protein and lipid present in the sample pan were determined afterwards.

Carboxyfluorescein release

Large unilamellar vesicles were prepared in 15 mM carboxyfluorescein, 25 mM NaCl, 10 mM Pipes (pH 7.0) buffer by extrusion techniques using filters with 100 nm pore size as described by Hope et al. [34]. The outside carboxyfluorescein was removed by gel filtration over a Sephadex G-50 column (1.5 × 15 cm) and elution with Pipes buffer. Increasing amounts of polypeptide (0–0.7 nmol) were incubated with carboxyfluorescein containing vesicles (10 nmol P_i) for the indicated times in a total volume of 70 μl at 25 °C. Then the sample was transferred to a cuvette containing 3 ml Pipes buffer. The carboxyfluorescein fluorescence was measured before and after addition of Triton X-100 (final concentration 0.1% (w/v)) at 25 °C in a Perkin-Elmer LS-5 fluorimeter with excitation at 430 nm and emission at 513 nm [35]. The intensity of the fluorescence signal was linear with the concentration of non-trapped carboxyfluorescein.

Preparation of vesicles for translocation experiments

Large unilamellar vesicles were prepared by the reverse phase evaporation method according to Ref. 36 as adapted and described in Ref. 15. In case of trypsin-containing vesicles, the non-enclosed trypsin was removed by washing the vesicles three times by centrifugation (27000 × *g*, 30 min, 4 °C) and resuspension of the pellets. For translocation experiments using equimolar DEPS/DEPC vesicles at temperatures of 30 °C and higher, the vesicles were washed and kept at 30 °C. The final vesicle pellet was suspended in 0.1–0.5 ml Pipes buffer. To estimate the internal volume of vesicles, samples were drawn from the suspensions for the determination of the trypsin activity [20] and phospholipid content. In both equimolar DEPS/DEPC and DOPS/DOPC vesicles, the internal volume was found to be 10 μl/ml.

Translocation assay

Polypeptide translocation was measured using large unilamellar vesicles containing enclosed trypsin as described in detail in Ref. 15. Translocation is defined as the process by which apocytochrome *c* becomes (at least) partially exposed to trypsin action at the inner vesicular membrane interface. All the assays were performed at the temperature indicated in the figure legend. In order to test the reliability of the translocation assay, some control experiments were carried out as described in Ref. 14 with some modifications which are detailed below. Firstly, in case of the translocation experiments using equimolar DEPS/DEPC vesicles at 5 °C or lower temperatures, the control experiments were carried out at 5 °C. Secondly, to test whether in case of a possible trypsin leak from the vesicles, there is sufficient trypsin inhibitor present outside the vesicles, to immediately inhibit all trypsin activity present in vesicles, 10 μg trypsin inhibitor was added to the trypsin-containing vesicles (containing 10 nmol phospholipid in a volume of 15 μl). After 10 min incubation at 30 °C, chromogenic substrate was added followed by the addition of 1 μl of 10% Triton X-100 (w/v) to lyse the vesicles.

General methods

Binding experiments were performed by incubation of large unilamellar vesicles (containing 56 nmol P_i) with 0–0.3 mg apocytochrome *c* in a total volume of 140 μl according to Ref. 15 and the data were analyzed as described in Ref. 37. Lipid phosphate was assayed as described by Rouser et al. [38]. Protein was determined according to Lowry et al. [39], using cytochrome *c* and fragments (1–65)H, 1–65, 1–59, (1–38)H and 81–104 as standards.

Results

Differential scanning calorimetry

To get insight into which parts of apocytochrome *c* influence gel state packing of negatively charged lipids, we studied the effect of different chemically prepared fragments of apocytochrome *c* on the thermotropic phase behavior of DEPS by DSC. In addition the role of the heme moiety which in horse heart cytochrome *c* is covalently attached to the cysteine residues 14 and 17 was addressed by comparing the effects of heme free and heme containing polypeptides. Representative scans are presented in Fig. 1, quantitative data are summarized in Figs. 2 and 3. DEPS was chosen as test lipid because it undergoes an endothermic transition at the convenient temperature of 25 °C with a ΔH of 5.7 kcal/mol (Figs. 1, 2). The addition of increasing amounts of apocytochrome *c* to DEPS dispersion causes like in case of dimyristoylphosphatidylserine (DMPS) and dimyristoylphosphatidylglycerol (DMPG) [18] a dramatic linear decrease in the ΔH of the gel to liquid-crystalline transition (Fig. 2A), without significantly affecting the transition temperatures. The cooperative transition can be completely removed by the addition of 40 nmol apocytochrome *c* per μmol DEPS (Fig. 2A). Similar results were obtained with the N-terminal fragments 1–59 and 1–65. The addition of the fragment 1–38 has slightly less effect, but also this peptide causes a linear decrease in ΔH. However, in strong contrast with these results addition of increasing amounts of cytochrome *c* or fragment (1–38)H has no effect on both the transition temperature and ΔH of the transition of DEPS (Figs. 1, 2A). The heme containing fragment (1–65)H causes a decrease in ΔH, but is also much less effective than the heme-free fragment 1–65. Because the differences in effect of the various peptides could be due to differences in affinity for the lipid, the calorimetric data are also shown in Fig. 2B as a function of the amount of peptide bound to DEPS. A full account of the binding characteristics of the various peptides to different model membrane sys-

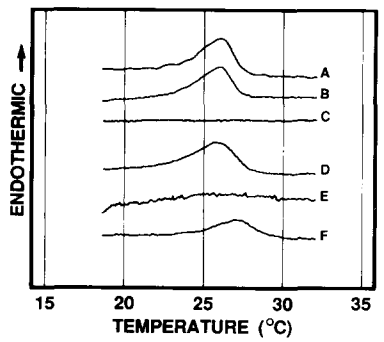

Fig. 1. The influence of different polypeptides on the calorimetric heating curves of DEPS dispersions. A, DEPS; B, DEPS + cytochrome *c*, C, DEPS + apocytochrome *c*; D, DEPS + (1–38)H; E, DEPS + (1–38); F, DEPS + (81–104). In all cases 80 nmol peptide was added per μmol of DEPS.

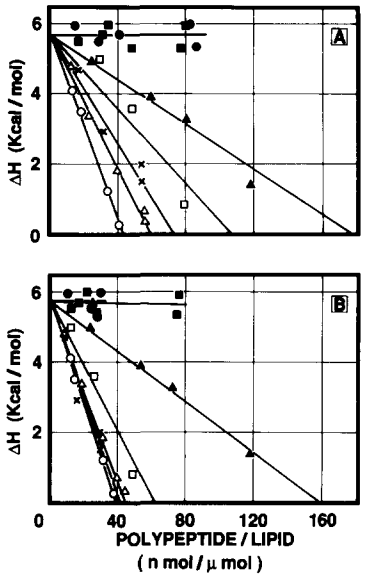

Fig. 2. Influence of increasing amounts of added (A) and bound (B) apocytochrome *c* (○), cytochrome *c* (●), (1–65) (△), (1–65)H (▲), (1–38) (□), (1–38)H ■ and (1–59) (×) on the energy content of the gel to liquid-crystalline phase transition of DEPS.

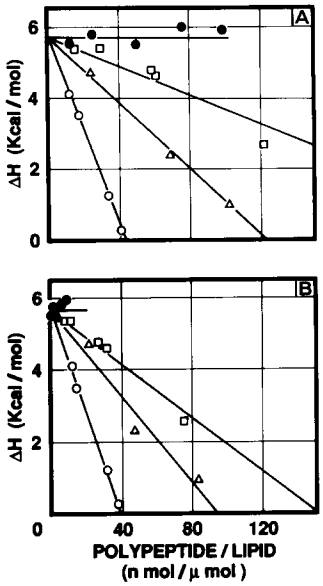

Fig. 3. Influence of increasing amounts of added (A) and bound (B) fragments (39–104) (△), (81–104) (□) and (60–104) (●) on the energy content of the gel to liquid-crystalline phase transition of DEPS. The curve of apocytochrome *c* (○) is included for comparison.

tems will be given elsewhere. From Figs. 2A and B, it can be concluded that the large differences in effect of the various peptides cannot be explained by differences in binding but predominantly reflect differences in mode of interaction with the lipid.

The effect of fragments of the C-terminal part of apocytochrome *c* on the phase transition of DEPS is summarized in Fig. 3. The behavior of the intact protein is included for comparative purposes. Addition of increasing amounts of fragment 39–104 or 81–104 causes a linear reduction in ΔH, but their effect is much less than that of apocytochrome *c* (Figs. 1, 3A). Fragment 81–104 causes a slight increase in transition temperature of the lipid (Fig. 1). All other fragments did not significantly affect the temperature of the transition. Fragment 60–104 has no measurable effect on the ΔH of DEPS. Fragment 66–80 did not bind to DEPS and therefore did not show an effect on the phase transition (data not shown). When the data are normalized for the amount of peptide bound (Fig. 3B), it becomes clear that the fragments 39–104 and 81–104 in lipid bound form are also less efficient in reducing ΔH than the intact protein and the N-terminal fragments. The absence of effect of added fragment 60–104 is largely due to the low extent of binding of this fragment to DEPS, but in addition it appears that this fragment causes much less perturbation of gel state packing. From an extrapolation of the plots of ΔH versus the amount of protein bound per lipid (Fig. 2B and 3B), the stoichiometry of the peptide–DEPS interaction in terms of the number of DEPS molecules withdrawn from the cooperative gel to liquid-crystalline transition by each bound peptide molecule can be calculated. The results of such calculations are presented in Table I. For apocytochrome *c* this number is 25, which is less than previously reported for DMPS and DMPG [18], possibly due to the looser gel state packing of this *trans*-double band containing PS species. The presence of the heme caused a very large reduction in N.

Carboxyfluorescein release

To investigate whether (apo)cytochrome *c* and the derived fragments affect the barrier properties of PS containing model membranes, we studied

TABLE I

EFFECT OF APOCYTOCHROME *c*, CYTOCHROME *c* AND DERIVED FRAGMENTS ON THE GEL TO LIQUID-CRYSTALLINE PHASE TRANSITION OF DEPS

N is the number of molecules of DEPS removed from the cooperative gel to liquid crystalline phase transition by each molecule of bound peptide.

Protein	*N*	Total positive [b] charge	Net positive [b] charge
Apocytochrome *c*	25	21	9
Cytochrome *c*	0	21	9
(1–38)	16.1	8	5
(1–38)H	0	8	5
(1–59)	23.3	11	7
(1–65)	22.2	12	6
(1–65)H	6.3	12	6
(39–104)	10.6	13	4
(60–104)	0	10	2
(66–80)	– [a]	3	1
(81–104)	6.6	6	2

[a] This fragment does not bind to DEPS vesicles.
[b] Charge calculated from the amino acid sequence of the polypeptide at pH 7.0.

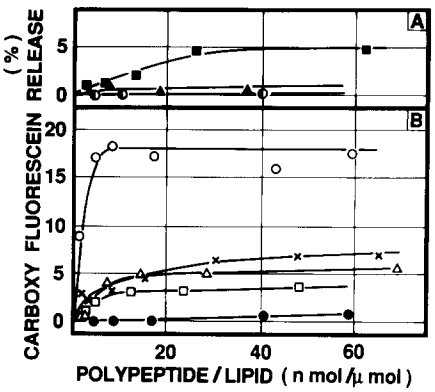

Fig. 4. The release of carboxyfluorescein from vesicles prepared of an equimolar mixture of bovine brain PS and egg PC 90 min after the addition of the different polypeptides. (A) 39–104 (■); 81–104 (▲); 60–104 (◐). (B) Apocytochrome *c* (○); cytochrome *c* (●); 1–38 (□); 1–65 (△); 1–59 (×).

the ability of these polypeptides to permeabilize large unilamellar vesicles composed of equimolar bovine brain PS and egg PC with respect to enclosed carboxyfluorescein. This particular lipid system was selected to facilitate a comparison with previous apocytochrome *c* translocation experiments [15]. Release of carboxyfluorescein enclosed in high concentration in vesicles causes dilution of the chromophore and dequenching of its fluorescence. Addition of the various polypeptides caused a polypeptide specific increase in carboxyfluorescein permeability. At low polypeptide concentrations (protein to lipid ratio's below 70 nmol/μmol), the release increased linear with time (data not shown). At higher concentrations in case of apocytochrome *c*, and the heme containing fragments (1–38)H and (1–65)H the kinetics are complicated and will not further be described or discussed. Fig. 4 shows the release of carboxyfluorescein from the vesicles after 90 min incubation with polypeptides in the low concentration range. Apocytochrome *c* causes a maximal release of approx. 18% of carboxyfluorescein whereas cytochrome *c* virtually has no effect. The other polypeptides shown were much less effective and caused up to maximally 7% of carboxyfluorescein release in 90 min. Interestingly also with respect to carboxyfluorescein release the fragments derived from the N-terminus are more effective than the fragments derived from the C-terminus.

Translocation experiments

To understand how the physical state of lipids influences apocytochrome *c* translocation, we investigated the temperature dependency of apocytochrome *c* translocation in equimolar trypsin containing DEPS/DEPC and DOPS/DOPC large unilamellar vesicles. Lipid mixtures were chosen because with pure PS vesicles apocytochrome *c* addition causes a substantial trypsin release [15]. The gel to liquid-crystalline phase transition of DEPC and DEPS occurs at 10 °C and 25 °C, respectively. The equimolar DEPS/DEPC mixture undergoes a broad gel to liquid-crystalline transition composed of two overlapping peaks in the temperature range of 10–20 °C (data not shown). Coexisting gel and liquid-crystalline lipids in a bilayer can cause permeation pathways for normally impermeable large polar solutes [40].

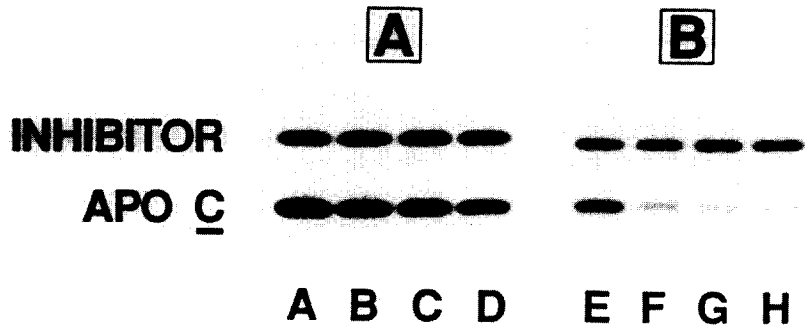

Fig. 5. Digestion of apocytochrome *c* by trypsin enclosed in equimolar DEPS/DEPC vesicles. To DEPS/DEPC vesicles (containing 100 nmol P_i), 80 μg apocytochrome *c* was added and the vesicles were incubated at 0 °C (A) or 37 °C (B), respectively (total volume 150 μl). After 0, 30, 60 and 120 min (lanes A–D or lanes E–H), 10-μl samples was drawn and analysed by gel electrophoresis.

Consistent with this idea we observed marked trypsin leakage from the vesicles in the temperature range of the phase transition in the DEPS/DEPC vesicles (data not shown). We therefore carried out the translocation experiments either below or above the transition temperature. As control system equimolar DOPS/DOPC vesicles were used which are in the liquid-crystalline state above 0 °C. Fig. 5 shows the actual electrophoresis pattern of a typical experiment, using trypsin enclosed in equimolar DEPS/DEPC vesicles, incubated with apocytochrome *c* at 0 °C and 37 °C. From the intensity of the apocytochrome *c* band relative to that of the trypsin inhibitor it can be concluded that the digestion of apocytochrome *c* at 0 °C is very slow and much more rapid at 37 °C. To test whether the limited apocytochrome *c* digestion at 0 °C is caused by an insufficient trypsin activity, we lysed the vesicles at 0 °C and 37 °C by 1% (w/v) Triton X-100 (final concentration) in the absence of trypsin inhibitor, immediately followed by the addition of apocytochrome *c*. Within 3 min, all apocytochrome *c* was digested. This demonstrates that, there is sufficient trypsin activity enclosed in the vesicles to digest all the externally added apocytochrome *c*. Additionally, some other control experiments were carried out. Firstly, apocytochrome *c* was incubated with the extravesicular solution by pelleting the vesicles by centrifugation. After two hours of incubation, the amount of apocytochrome *c* was found to be unchanged, demonstrating the effective blocking of the activity of the residual traces of outside trypsin by the inhibitor. Secondly, upon lysing the vesicles with 1% Triton X-100 (final concentration), the released trypsin was immediately inactivated by trypsin inhibitor. This proves that a transient release of trypsin by the apocytochrome *c* addition cannot cause the observed apocytochrome *c* digestion. Thirdly, trypsin-free vesicles were prepared and then incubated with trypsin whereafter the vesicles were washed and incubated with trypsin inhibitor and apocytochrome *c* at 30 °C. Under these conditions no apocytochrome *c* digestion was observed excluding the possibility of apocytochrome *c* digestion at the outside surface of the vesicles by bound trypsin. From these control experiments, it can be concluded that the digestion of apocytochrome *c* represents the translocation of at least a part of apocytochrome *c* across the lipid bilayer till it can be digested at the inner surface of the vesicles by trypsin. Fig. 6 shows that the amount of digested apocytochrome *c* in both equimolar DEPS/DEPC and DOPS/DOPC vesicles is strongly temperature-dependent and that translocation is virtually blocked at 0 °C. From a comparison of apocytochrome *c* digestion in both vesicles, it can be concluded that at low temperatures the translocation of apocytochrome *c* is more reduced in case of the gel state DEPS/DEPC vesicles. Above the transition temperature the difference in apocytochrome *c* digestion is much less. To know whether the inefficient apocytochrome *c*

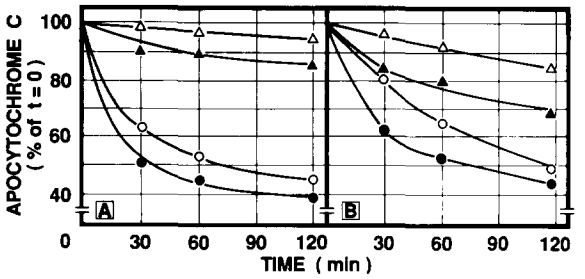

Fig. 6. Time dependence of apocytochrome *c* translocation across model membranes composed of (A) equimolar DEPS/DEPC or (B) equimolar DOPS/DOPC. The trypsin-containing vesicles were incubated at 0 °C (△), 5 °C (▲), 30 °C (○) or 37 °C (▲). The incubations were performed as described in Materials and Methods.

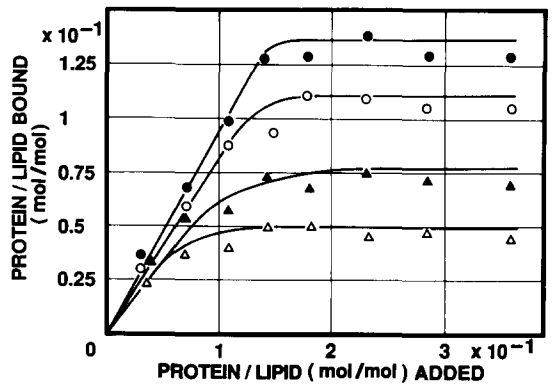

Fig. 7. Binding of apocytochrome *c* to large unilamellar vesicles at 5 °C (○, △) or 30 °C (●, ▲). Increasing amounts of apocytochrome *c* (0–0.2 mg) were added to vesicles (56 nmol P_i) composed of DEPS (○, ●) or equimolar DEPS/DEPC (△, ▲). Binding was determined as described in Materials and Methods.

TABLE II

APOCYTOCHROME *c* BINDING TO DEPS OR EQUIMOLAR DEPS/DEPC VESICLES

The binding data were calculated from the results in Fig. 7 using the method described in the experimental section.

Lipid	K_d (μM)	N
DEPS		
30 °C	1.2±0.2	7.3±1
5 °C	2.6±0.3	8.8±2
DEPS/DEPC		
30 °C	2.8±0.3	13 ±3
5 °C	2.8±0.4	20 ±4

translocation at low temperatures is caused by decreased binding of the protein to the gel state vesicles, we compared the binding of apocytochrome *c* to DEPS and equimolar DEPS/DEPC vesicles at 5 °C and 30 °C (Fig. 7). The binding shows saturation as described previously for other negatively charged lipids [15]. From these binding data a dissociation constant K_d and the number of lipid molecules (N) that provides one protein binding site can be calculated (Table II). For the pure DEPS vesicles, the stoichiometry of the apocytochrome *c*–PS interaction is very similar above and below the transition temperature. The binding affinity, however, is 2-fold reduced at gel-state temperatures. In the mixed DEPS/DEPC system the affinity of apocytochrome *c* for the vesicles is actually temperature independent. The approximately 2-fold increase in N for these vesicles as compared to the pure PS vesicles is due to the lower PS content of the vesicles. On PS basis, the stoichiometry is very similar as was reported before for other PS containing vesicles [15]. Since the lipid/protein molar ratio in translocation experiments is 16 and at this relatively low molar ratio no differences in the amount of protein bound to DEPS/DEPC vesicles is observed (Fig. 7), it can be concluded that the apocytochrome *c* translocation across lipid vesicles is reduced at gel-state temperatures.

Discussion

The reduction in ΔH of the main transition of phospholipids in model membranes upon incorporation of polypeptides can in case of a largely unaffected transition temperature most simply be interpreted as being the result of perturbation of gel state packing by membrane penetrated polypeptide [41].

The large difference in reduction of ΔH of $18{:}1_t/18{:}1_t$-PS between apo- and holocytochrome *c* can be understood along this line because apocytochrome *c* penetrates more extensively in negatively charged bilayers than cytochrome *c* [19]. Since the polypeptide chain of both proteins is identical this difference must be due to the presence of the heme moiety which structures the holoprotein [29]. Similar behavior was observed for the N-terminal fragments 1–38 and 1–65. Whereas the heme-free peptides were highly effective in perturbing gel state packing, the heme-containing fragments were much less efficient. These results therefore strongly suggest that the presence of the heme group prevents penetration of the N-terminal part of the peptide into the PS bilayer. Since the heme group itself is rather hydrophobic, which should favor penetration and perturbation of gel state packing opposite to what is observed, we suggest that this group folds the N-terminus in such a way that it is less capable of penetrating the model membrane.

The different fragments of apocytochrome *c* display large differences in effectivity to decrease ΔH of the main transition of $18{:}1_t/18{:}1_t$-PS model membranes. In all cases the plots of ΔH versus polypeptide bound are linear, suggesting that at this level of detection no cooperative events (such as peptide aggregation) take place in the polypeptide–lipid interaction. The general trend is that the N-terminal fragments are more effective in perturbing gel state packing than the C-terminal fragments. For instance fragment 1–59 has almost the same effect as the intact protein whereas 60–104 has virtually no effect on the ΔH. Interestingly, combining the effect of corresponding N- and C-terminal fragments to yield the complete sequence results in stoichiometries of 26.7, 23.3 and 28.8, PS/peptide withdrawn from the transition for the various combinations which is close to the value of 25 for the intact protein. This suggests that the N- and C-terminal part of apocytochrome *c* affect gel state packing in a more of less independent way. That this is not necessarily true for sequences within the C-terminal part of the pro-

tein is indicated by the significant decrease in ΔH of fragment 81–104 whereas for 60–104 no effect is observed. In this case the sequence 60–80 apparently interacts with its sequence 81–104 within fragment 60–104 whereby the ability of 81–104 to perturb gel-state packing is lost.

Horse heart apocytochrome *c* does not contain hydrophobic sequences longer than six amino acids. The average hydrophobicity of the polypeptide is similar to that of water soluble globular proteins and with the exception of some small hydrophobic stretches (Residue numbers 74–78, 80–85 and 94–98) in the C-terminus, the hydrophobic and hydrophilic amino acids are evenly spread over the entire sequence [18]. Therefore, it is unlikely that the differences in effect of the C- and N-terminal parts of the protein arise from differences in hydrophobicity. Neither is there a correlation with the total number of positive charges (Table I). However, it appears that the net positive charge seems to be the most important factor determining the efficiency to perturb gel state packing of the anionic phospholipid model membranes. The C-terminal part has much less net positive charges than the N-terminal part. This then supports previous observations on the importance of electrostatic interactions in the ability of apocytochrome *c* to penetrate into model membranes [19,42]. Results of carboxyfluorescein release experiments using mixed PC/PS vesicles support the above interpretation of the calorimetric data. Apocytochrome *c* penetrates the bilayer of these vesicles [15] and caused a slight but significant increase in carboxyfluorescein permeability. This is much less for cytochrome *c*. The N-terminal fragments of apocytochrome *c* increase bilayer permeability towards carboxyfluorescein more than the C-terminal fragments. It should be realized, however, that there is no molecular basis to quantitatively link the two sets of data. For instance melittin, the major polypeptide from bee venom is at low concentrations highly lytic towards membranes [43] but only at much higher concentrations reduces the ΔH of the main transition of phospholipid model systems [44].

The biological implications of these findings for apocytochrome *c* import into mitochondria are that the N-terminal part of the protein by interaction with the lipids initiates translocation across the lipid barrier of the outer membrane. As soon as the heme group is covalently attached to the N-terminus in the intermembrane space the holoprotein is formed, which can no longer return to the cytosol because it has lost the ability to pass through the lipid part of the outer membrane and because of its high affinity binding to the cardiolipin and cytochrome *c* oxidase component of the inner mitochondrial membrane.

It is of interest to note that the basic cleavable N-terminal presequences of different mitochondrial proteins also have the ability to strongly interact with negatively charged lipids [45–48] which might suggest a similarity in function of the N-terminal part of the various mitochondrial precursor proteins, despite the fact that the import of these latter proteins shows differences with that of apocytochrome *c* [2].

Little is known about the importance of the various regions of apocytochrome *c* for import into mitochondria mainly because gene fusion experiments with this protein have not yet been performed. Matsuura et al. [49] reported that an excess of fragment 66–104 (and not the fragment 66–80 or 81–104) could compete for transport of newly synthesized apocytochrome *c* into mitochondria. Stuart et al. [50] reported that a cytochrome *c* mutant of *Neurospora crassa* synthesizes an apocytochrome *c* with a 19 amino acids longer C-terminus with the final 27 amino acids of an unrelated sequence. This mutant apocytochrome *c* was incompetent for binding to mitochondria and failed to be imported. These studies thus suggest that in addition the C-terminus is important for the import of this precursor protein.

The observed strong temperature dependency of the apocytochrome *c* translocation in pure lipid vesicles and the inhibition of this process at gel state temperatures reported in this study is in full agreement with previous studies [15,42] which revealed a strong influence of lipid packing and the phase transition on apocytochrome *c* penetration in a PS monolayer at the outer-water interface. Whereas no data are published on the temperature dependency of apocytochrome *c* import into mitochondria the import of precursors with N-terminal cleavable presequences appears to be also strongly temperature dependent. At low tempera-

tures translocation intermediates can even be arrested in translocation contact sites [51]. Also this similarity in behavior might point to a more general lipid involvement in mitochondrial protein import.

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