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Investigations on the insertion of the mitochondrial precursor protein apocytochrome *c* into model membranes

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Different aspects of the interaction of apocytochrome *c* and model membranes composed of negatively charged lipids, were studied in order to get insight into the nature of this interaction. The effect of the protein on the lipid packing properties are revealed by DSC, ESR and monolayer techniques. These experiments clearly demonstrate that upon electrostatic interaction with the negatively charged phospholipids, apocytochrome *c* is able to penetrate into the hydrophobic region of the model membrane. In the case of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, this results in a perturbation of 160 lipid molecules per apocytochrome *c* molecule. Most likely, apocytochrome *c* disrupts the formation of the gel phase and restricts the lipid chain motion above the gel to liquid-crystalline phase transition. Tryptophan fluorescence measurements confirm that at least a part of the protein penetrates into the bilayer, and suggest that after this penetration, the tryptophan (residue no. 59) is located in the glycerol backbone region of the phospholipids. Although the secondary structure of apocytochrome *c* is predicted to contain about 35% of α-helical structure, the CD pattern of an aqueous solution of the protein is featureless. However, negatively charged lipids are able to express this α-helical potency in the apocytochrome *c*, which might be important for the insertion of the protein into lipid membranes.

Introduction

The majority of the mitochondrial proteins is coded on nuclear genes, translated on cytoplasmic polysomes as a precursor form and somehow imported by the organelle [1,2]. One of the steps in the import of the cytoplasmically synthesized precursor proteins is the translocation across or insertion into one or both mitochondrial membranes, depending on the suborganellar localization of the protein. Very little is known about the actual mechanism of protein translocation across membranes, including the mitochondrial system. With respect to the properties of the components of a biological membrane, it is possible that different pathways might exist. First, outer mitochondrial membrane proteins have been shown to probably

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; bovine brain PS, bovine brain phosphatidylserine; egg PC, egg phosphatidylcholine; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine; 5-PG-SL, 1-acyl-2-[5-(4',4'-dimethyloxazolidine-*N*-oxyl)-stearoyl]-*sn*-glycero-3-phosphoglycerol; SDS, sodium dodecyl sulfate; Pipes, 1,2-piperazinediethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

play a role in the specific recognition of the precursor proteins [2] and possibly these receptor-type of proteins also could be involved in the translocation process. Secondly, lipids which determine to a large extent the barrier properties of a membrane might have a function in protein translocation. In our view [3], lipids are involved in protein translocation, possibly via local modulation of membrane structure by protein-lipid interactions. To get more insight into the nature of such interactions, studies on mitochondrial protein precursor molecules and lipids are essential. Apocytochrome *c* (molecular weight 12000) is the cytoplasmically synthesized precursor of the inner mitochondrial membrane protein cytochrome *c*. After synthesis of the precursor, which has a disordered structure [4], the protein is bound to specific receptor-type binding sites [5] on the outer mitochondrial membrane and translocated across this membrane. During or immediately after translocation, the heme group is covalently attached to the apoprotein [6], to form the highly structured holocytochrome *c* [7]. Because of the absence of an additional polypeptide sequence in the precursor protein, apocytochrome *c* can be easily chemically prepared from cytochrome *c* [4]. This chemically prepared apocytochrome *c* competes with apocytochrome *c* translated in a cell free system [8,9]. The abundant availability of this precursor protein and the strong apocytochrome *c*-phospholipid interaction [10], makes this protein a very suitable candidate to investigate the nature of the lipid-precursor protein interaction. In an early study [10], we showed that apocytochrome *c* specifically binds to negatively charged lipids, thereby influencing the barrier properties of the membrane and the macroscopic structural organization of the phospholipids. The penetration of apocytochrome *c* into the phosphatidylserine bilayer, as was also revealed by freeze-fracture electron microscopy experiments [10], even can result in a translocation of the protein across the phospholipid barrier present in large unilamellar vesicles [11].

In this study we report the results on two aspects of the apocytochrome *c*-lipid interaction. With DSC, ESR and monolayer techniques using different synthetic phospholipids, the lipid packing perturbing properties of the protein molecule are revealed. Tryptophan fluorescence and CD measurements, using different phospholipid- and detergent model systems, demonstrate that (a part of) the protein penetrates into the lipid bilayer which process is attended with a change in secondary structure, resulting in an increase of α-helical content.

Materials and Methods

Materials 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) were synthesized according to established methods [12,13]. Bovine brain phosphatidylserine (bovine brain PS) was essentially isolated and purified as described [14]. Egg phosphatidylcholine (egg PC) was obtained from hen eggs [14]. 1,2-Dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) was synthesized as described previously [15], and 1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS) was a kind gift from Dr. R.F.A. Zwaal from the State University of Limburg, Maastricht, The Netherlands.

Bovine heart cardiolipin (disodium salt) was obtained from Avanti-Polar Lipids (Birmingham, U.S.A.). 1-Acyl-2-[5-(4′, 4′-dimethyloxazolidine-*N*-oxyl)stearoyl]-*sn*-glycero-3-phosphoglycerol (5-PG-SL) was synthesized as described by Marsh and Watts [16]. Trypsin (treated with L-1-tosylamido-2-phenylethylchloromethylketone) and sodium dodecyl sulfate (SDS) were obtained from Merck (Darmstadt, F.R.G.), and octylglucoside (octyl-β-D-glucopyranoside) from Calbiochem (San Diego, U.S.A.). Sodium cholate was purchased from Fluka AG (Buchs, Switzerland), and Lubrol PX from Sigma (St. Louis, U.S.A.).

Apocytochrome *c* was prepared from cytochrome *c* (type VI; Sigma), by removal of the heme moiety [4] and next subjected to a renaturation procedure [17]. The protein was stored at -20°C in 0.5 ml aliquots at a concentration of about 1.5 mg/ml in 100 mM NaCl, 10 mM 1,2-piperazinediethanesulfonic acid (Pipes), pH 7.0, 0.01% (v/v) β-mercaptoethanol. The protein was always used immediately after thawing.

DSC. Lipid dispersions were prepared by hydrating a dry film of 4 μmol lipid with 1.5 ml of 100 mM NaCl, 10 mM Pipes (pH 7.0) as previously described [18]. For the preparation of lipid-protein recombinants, the lipid was hydrated with

1.5 ml of the buffer mentioned above, which, in addition, contained increasing amounts of apocytochrome *c*. After 30 min incubation at 40°C, the lipid-protein recombinants were collected by spinning the samples for 20 min at 27000 × *g* at 4°C. The pellets were transferred to the commercial 200 μl stainless steel sample pans of the Setaram III high sensitivity calorimeter. Thermograms were obtained from heating runs over the temperature range 2–40°C using a scan rate of 2 Cdeg/min and a sensitivity setting of 100 μV. The thermograms show a reversible pattern under these conditions. For the detection of ΔH, the exact amount of lipid was determined afterwards by a phosphate determination.

ESR. ESR experiments were performed with lipid dispersions of DMPG, containing 1% (mol/mol) 5-PG-SL, with and without apocytochrome *c*. The dispersions were prepared from a mixed film of 1.5 μmol DMPG and 0.015 μmol of 5-PG-SL as described in the DSC-section. The buffer, in addition, contained 0.01% β-mercaptoethanol.

The pelleted complex was further concentrated in 100 μl glass capillaries by centrifugation (20 min, 3000 × *g*) before the ESR measurements. ESR spectra were recorded on a Varian E-12 9-GHz spectrometer equipped with nitrogen gas flow temperature regulation.

Monolayer experiments. Monomolecular lipid layers were formed at the air/water interface in a Teflon trough, 6 × 4.2 cm wide and 1 cm deep [19]. The through was filled with 100 mM NaCl, 10 mM Pipes (pH 7.0). The subphase was stirred with a magnetic bar. Apocytochrome *c* was injected underneath the monolayer from a solution of 1 mg/ml via a small bypass connection.

The area of the monolayer was kept constant and the surface pressure was measured with an LM 500 electrobalance. The experiments were performed at room temperature.

Tryptophan fluorescence. Intrinsic fluorescence of the single tryptophan residue (residue no. 59) of apocytochrome *c* was measured before and after addition of different amounts of phospholipid vesicles or a detergent solution to an 8.3 μM apocytochrome *c* solution. The detergent was dissolved in 100 mM NaCl, 10 mM Pipes (pH 7.0). The vesicles were prepared by ultrasonication of dispersions of lipids [20] in this buffer. One minute after mixing an emission spectrum was measured at 30°C in a Perkin-Elmer LS-5 spectrofluorimeter, equipped with a thermostatically controlled cuvette holder. Upon incubation for longer time periods, no further spectral changes were observed. The measurements were performed at an excitation wavelength of 290 nm and slit widths of 5 nm. Each measurement was corrected for the light scattering contribution of the signal due to the vesicles or micelles alone. The relative increase of the quantum yield was calculated according to the following equation:

$$\Delta\Phi(\%) = \frac{F_0 T_1}{F_1 T_0} \times 100\%$$

where Φ is the quantum yield, the subscripts 1 and 0 denote the presence and absence of lipid, respectively, F is the height of the fluorescence peak maximum after volume correction, and T is the transmission at the excitation wavelength. Transmission measurements were carried out in a Perkin-Elmer 356 double-beam spectrometer with the cuvettes placed against the photomultiplier to reduce light scattering. The intensities of the tryptophan fluorescence signal measured under the conditions described, were found to be proportional to the apocytochrome *c* concentration in the range 0–12.5 μM. Fluorescence quenching experiments were performed by titration of 8.3 μM apocytochrome *c* with aliquots of 4 M sodium iodide solution in buffer, containing 1 mM $Na_2S_2O_3$ to prevent I_3^- formation.

CD. The CD spectra were run on a home-built apparatus equipped with a Jobin-Yvon double monochrometer (type H-20) with spectral bandpass approx. 2.2 nm, a CaF_2 fotoelastic modulator (Morrue Electr. Syst.), and a lock-in amplifier (P.A.R. Synchro-het Model 186). The apparatus and data collection were controlled by a microcomputer (Apple IIe). Measurements were made at room temperature in quartz cells with a path length of 1 cm. Spectra of apocytochrome *c* and cytochrome *c* were recorded at a protein concentration of 50 μg/ml in 1 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.0)/100 mM NaCl. The spectra were corrected for baseline- and solvent effects. Wavelength calibration was done by referring to

the absorption spectrum of benzene vapour and the CD signal was calibrated with (+)-camphor-10-sulfonic acid monohydrate ($\Delta\varepsilon^{290} = +2.37$ (Ref. 21)).

Analytical methods. Apocytochrome *c* was quantitated by a protein assay, according to Lowry et al. [22] and lipid phosphorus was determined after perchloric acid destruction of the lipids [23].

Results

DSC

In an earlier study [10] we showed that incubation of DMPG with saturating amounts of apocytochrome *c* resulted in a total disappearance of the gel to liquid-crystalline transition of the phospholipid, suggesting a profound perturbation of the lipid packing by the protein. In order to get more quantitative insight into this effect, we titrated DMPG with increasing amounts of apocytochrome *c*. The phase transition of the pure lipid is centered around 22°C (Fig. 1A) and has an enthalpy content of 7.8 kcal/mol, in agreement with literature data [27]. The peak is rather broad due to the use of relatively large samples. Low amounts of apocytochrome *c* hardly change the transition temperature of DMPG (Fig. 1), but cause a dramatic reduction of the heat content of the gel to liquid-crystalline transition (Figs. 1 and 2). Previous binding studies [10] showed that nearly all apocytochrome *c* is bound to the lipid at the protein and lipid concentrations used in this experiment. Since PS is a major negatively charged phospholipid of the outer mitochondrial membrane [28], we next studied the effect of apocytochrome *c* on the phase transition of DMPS. Also for this negatively charged phospholipid, apocytochrome *c* causes a profound decrease in transition enthalpy (Fig. 2), without affecting the transition temperature (data not shown). In Fig. 2 it is shown that the ΔH of both DMPG and DMPS decreases linearly with the amount of protein added, and already at a protein-lipid ratio (nmol/μmol) of 6.1 and 17.4 for DMPG and DMPS, respectively, the transition has completely disappeared. This means that per apocytochrome *c* molecule added, 160 molecules of DMPG or 60 molecules of DMPS have been withdrawn from the gel to liquid-crystalline transition. These data

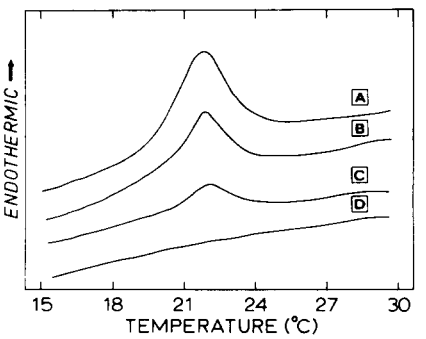

Fig. 1. DSC heating curves of a lipid dispersion of 4 μmol DMPG (A), of lipid-protein recombinants obtained by hydrating 4 μmol DMPG in the presence of 8.3 nmol (B), 16.7 nmol (C) and 45.8 nmol (D) apocytochrome *c*. Experimental details are presented in Materials and Methods.

suggest that the apocytochrome *c*-DMPG interaction is more effective in disturbing the lipid packing than the apocytochrome *c*-DMPS interaction.

ESR

The gel to liquid crystalline transition of a phospholipid is accompanied by a significant increase in lipid mobility. Fig. 3 shows that the hyperfine outer splitting (A_{max}) of the ESR spectrum of 5-PG-SL in DMPG liposomes is a sensitive parameter for this mobility increase. The pretransition of the pure DMPG, which was already observed before by ESR measurements [29], is reflected as a weak drop of A_{max} at 10–14°C and

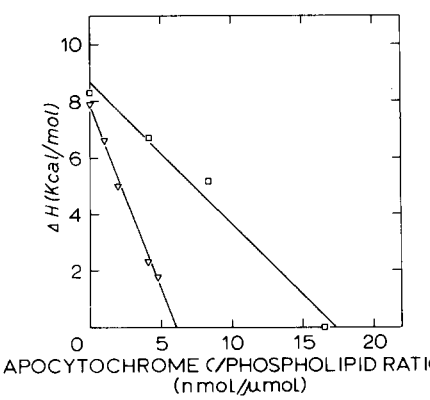

Fig. 2. Influence of increasing amounts of apocytochrome *c* on the enthalpy of the gel to liquid-crystalline phase transition of DMPG (▽———▽) and DMPS (□———□). Experimental conditions as described in Materials and Methods and in the legend to Fig. 1.

the gel to liquid-crystalline transition is coupled with a steep decrease of A_{max} in the temperature range of 20–22°C, which transition temperature corresponds with that obtained by DSC (Fig. 1), and Ref. 27. In agreement with the DSC data the phase transition in the protein/lipid recombinants containing high amounts of apocytochrome *c* (initial lipid-protein molar ratio 5 : 1 and 10 : 1, respectively) has disappeared, whilst the recombinant with a lipid/protein ratio of 150 : 1 still shows a broad transition as a decrease of A_{max} in the temperature range of 17–31°C (Fig. 3). Because of the rather low thermal resolution of the DSC experiments, this broad transition was not observable anymore in the DSC scans. Fig. 3, in addition, shows that below the phase transition of DMPG, the value and the temperature dependency of A_{max} of 5-PG-SL in DMPG-apocytochrome *c* recombinants is similar to that of the spin-label in pure DMPG. Above the phase transition apocytochrome *c* causes a decrease in spin-label mobility, which is most pronounced at the high protein/lipid ratio, indicating that the protein restricts the lipid motion in the liquid-crystalline phase. Both DSC and ESR experiments indicate that apocytochrome *c* has a large perturbing effect on the packing properties of the bilayer.

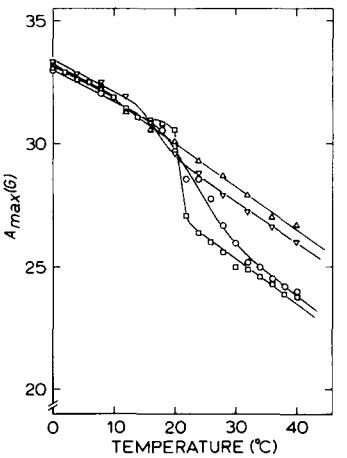

Fig. 3. Temperature dependency of the maximum outer hyperfine splitting A_{max} of 5-PG-SL in a pure DMPG dispersion (□———□) and in apocytochrome *c*-DMPG recombinants. Initial apocytochrome *c*/DMPG ratio (nmol/μmol): 6 (○———○), 100 (▽———▽) and 200 (△———△), respectively. Experimental details and sample preparations are described in Materials and Methods.

Monolayer studies

To test whether the lipid perturbing ability of apocytochrome *c* is due to penetration of the protein into the hydrophobic region of the model membrane, the effect of apocytochrome *c* addition on the surface pressure of different monolayers was studied. Apocytochrome itself is surface-active, as in the absence of a lipid monolayer, it gives rise to a surface pressure increase of 16.4 $mN \cdot m^{-1}$ (data not shown). Fig. 4 shows that the protein causes a large increase in surface pressure for monolayers composed of the negatively charged phospholipids cardiolipin and DOPS. The surface pressure increase is much less in case of the neutral DOPC, indicating the specificity of the apocytochrome *c* interaction with negatively charged lipids, in agreement with previous observations [10]. The surface pressure increase induced by the protein, decreases with increasing initial surface pressure (Fig. 4A). The extent of pressure increase induced by apocytochrome *c* is higher at higher protein concentration (Fig. 4B). The effects of different apocytochrome *c* concentrations on the magnitude of the surface pressure increase, is shown for two different initial surface pressures in Fig. 5. It is clearly shown that high pressure increases are only observed for the negatively charged phospholipids both at the initial surface pressure of 20 $mN \cdot m^{-1}$ (Fig. 5A), and of 35 $mN \cdot m^{-1}$ (Fig. 5B). At the lower initial surface pressure the amount of apocytochrome *c* required to saturate the lipid film is similar for cardiolipin and DOPS, while at the higher initial surface pressure DOPS requires a higher protein concentration for saturation.

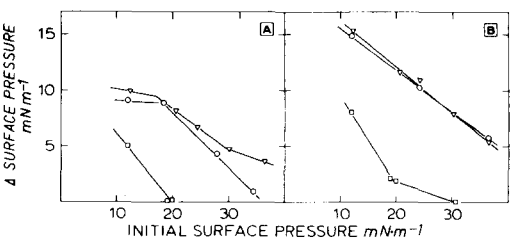

Fig. 4. Surface pressure increase after the injection of apocytochrome *c* underneath a monolayer of DOPC (□———□), DOPS (○———○) or cardiolipin (▽———▽) at different initial pressures. The final protein concentrations in the subphase were 0.6 μg/ml (A) and 6.6 μg/ml (B), respectively.

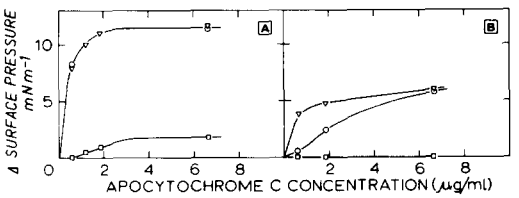

Fig. 5. Surface pressure increase after the injection of different amounts of apocytochrome *c* underneath a monolayer of DOPC (□——□), DOPS (○——○) or cardiolipin (▽——▽) at an initial film pressure of 20 $mN \cdot m^{-1}$ (A) and 35 $mN \cdot m^{-1}$ (B).

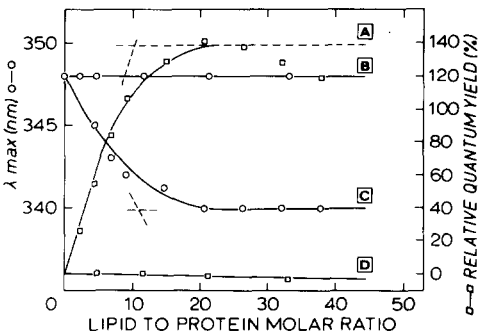

Fig. 7. Effect of the addition of sonicated vesicles of PS (A, C) and PC (B, D) on the tryptophan fluorescence quantum yield (□——□) and on the wavelength at the fluorescence peak maximum (○——○).

Fluorescence experiments

One of the factors which determine the intensity and emission wavelength of tryptophan fluorescence is the dielectric constant of the environment in which the indole group is located [30]. Therefore, fluorescence experiments can be used to monitor the transfer of that part of the apocytochrome *c* molecule, in which the single tryptophan residue at position 59 (see for sequence Fig. 10) is located, from an aqueous to more hydrophobic surroundings. Upon addition of sodium cholate, which was used as a model for a negatively charged membrane phospholipid, a blue shift and an increase of the intensity of the fluorescence is observed (Fig. 6), both indicating a more hydrophobic environment of the tryptophan [30]. The same effects were observed after interaction of apocytochrome *c* with sonicated vesicles of bovine brain PS (Fig. 7), and bovine heart cardiolipin

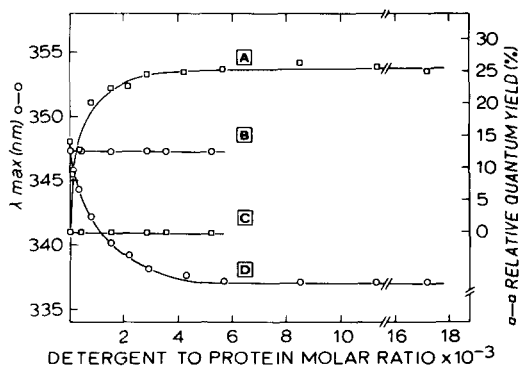

Fig. 6. Effect of sodium cholate (A, D) or octylglucoside (B, C) addition on the tryptophan fluorescence quantum yield (□——□) and on the wavelength of maximal fluorescence (○——○).

(data not shown). Both the increase in intensity and the decrease in λ_{max} reach a plateau at a lipid-to-protein molar ratio of 10:1 both for PS and for cardiolipin, which is in rather good agreement with the stoichiometry established by previous binding studies [10]. K_d values were calculated from the increase in fluorescence intensity as described before [31] and found to be 6.3 μM for PS, and 6.6 μM for cardiolipin, respectively. The decrease in fluorescence intensity at higher lipid-protein ratios (Fig. 7) is probably due to some precipitation of protein-lipid aggregates in the cuvette. Titration of apocytochrome *c* with either the neutral detergent octylglucoside (Fig. 6) or with sonicated vesicles of PC (Fig. 7) do not give rise to fluorescence changes, indicating the absence of interaction of apocytochrome *c* with uncharged lipids, which is in agreement with earlier observations [10]. The creation of a more hydrophobic environment for the tryptophan residue after the apocytochrome *c*-negatively charged phospholipid interaction can be due to a penetration of at least a part of the protein into the lipid matrix or to a structural change of the protein, possibly accompanied by protein aggregation, by which the tryptophan becomes more buried inside the protein or inside the protein aggregate or to a combination of both. In order to get more insight into the factors determining the tryptophan fluorescence of apocytochrome *c* some additional experiments were performed. First, the effect of the collisional quencher I^- on the tryptophan fluorescence was studied. The decrease of tryptophan

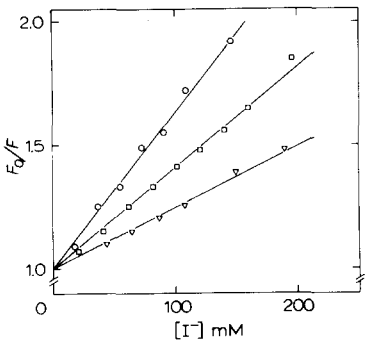

Fig. 8. Stern-Volmer plots of the quenching by iodide of tryptophan fluorescence in apocytochrome *c* in buffer (○——○) and after addition of bovine brain PS (□——□) or sodium cholate (▽——▽). Initial concentrations of apocytochrome *c*, bovine brain PS and sodium cholate, 8.3 μM, 150 μM and 39.5 mM, respectively.

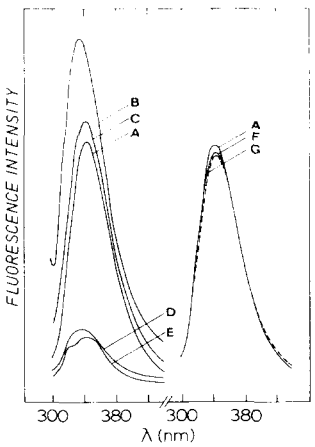

Fig. 9. Tryptophan fluorescence spectra of 8.3 μM apocytochrome *c* in buffer (A); after interaction with 150 μM of sonicated vesicles of bovine brain PS (B); in the PS-apocytochrome *c* complex after 30 min incubation with trypsin (trypsin/apocytochrome *c* ratio 1:10, w/w) (C); after 30 min incubation with trypsin in the absence of lipids (G); and after mixing of digested apocytochrome *c* with 150 μM PS (F). Complete digestion of apocytochrome *c* by trypsin was demonstrated by SDS gel electrophoresis as described before [11]. Tryptophan fluorescence spectra of trypsin with a concentration corresponding to that used in the above described digestion mixtures, in buffer (E) and after interaction with 150 μM bovine brain PS (D). The spectra are not corrected for volume increase (2%).

fluorescence intensity of apocytochrome *c* in buffer is proportional to the I^- concentration (Fig. 8). The quenching efficiency of the water-soluble I^- is decreased by the presence of bovine brain PS and even stronger decreased in the presence of sodium cholate, indicating a shielding of the tryptophan from its original environment. Second, the observed changes in fluorescence properties after interaction with PS, can be reversed by trypsin treatment (Figs. 9A, B, C). This digestion results in a total loss of the binding of the tryptophan containing part of the protein to the lipid, as was revealed by tryptophan fluorescence measurements in the supernatant of the apocytochrome *c*-PS mixture before and after trypsin treatment (data not shown). These experiments show that lipids are not able to protect the tryptophan containing part of the protein against trypsin digestion. Similar observations were published by Dumont and Richards [32], who showed that after interaction of apocytochrome *c* with lipid vesicles only the C-terminal part of the protein was protected against trypsin. Incubation of trypsin-digested apocytochrome *c* with PS vesicles does not result in fluorescence changes (Figs. 9A, F, G). Previous experiments have shown that the apocytochrome *c*-negatively charged phospholipid interaction can cause protein aggregation [10], resulting in chemical oligomerization of the protein [11]. Fluorescence experiments, performed in the presence of 0.01% mercaptoethanol which is able to inhibit the formation of these oligomers [11], yielded similar results as in the absence of mercaptoethanol (Rietveld, A., unpublished data). This indicates that this type of protein-protein interaction is not responsible for the change of hydrophobicity in the environment of the tryptophan after interaction of apocytochrome *c* and PS.

Conformational analysis

The conformation of apocytochrome *c* after interaction with phospholipids might play an important role in the penetration of the protein into the bilayer. Apocytochrome *c* has a disordered structure in aqueous solutions [4] and we wondered whether the protein, on base of its primary structure, contains the potency to adopt a structured conformation. The secondary structure of apocytochrome *c* is predicted with a computerized version of the method of Chou and Fasman [24,33]. Indeed, this method predicts different conforma-

tional possibilities like α-helical segments, β-strands and β-turns which are indicated in Fig. 10. There are some segments which have the potency to form both α-helices and β-strands. This suggests a structural flexibility of the protein. For three parts of apocytochrome *c*, corresponding with 35% of the total protein, α-helical segments (residues 4–17, 58–69, 88–101) are predicted. All commonly used prediction methods only take into account the contribution of the individual aminoacids to the structure [34–36]. This implies that predictions applied to both cytochrome *c* and apocytochrome *c* should result in identical predicted structures. The results we obtained with the Chou-Fasman method, which is based on the use of a statistical partition function of calculating the possibility that a given portion of the chain would coil spontaneously into an α-helix or β-strand, agrees rather well with respect to the predicted α-helix regions, obtained with a number of other predictions, based on different theories, applied to (apo)cytochrome *c* [34–36]. The mean hydrophobicity *H* (kcal/mol), calculated with the hydrophobicity scale according to Janin [37], and the hydrophobic moment $^{\mu}H$ (kcal) which was estimated as described by Eisenberg et al. [38], amounts to −0.36 and 0.33, respectively, for the predicted α-helical segment at the N-terminal part of the protein, −0.31 and 0.092, respectively, for the residues 58–69, and −0.51 and 0.24, respectively, for the predicted α-helical segment at the C-terminal part of the protein.

These values result in a position of the three segments in the globular region of a hydrophobic plot, as described by Eisenberg et al. [38,39]. Two of the predicted α-helix segments, namely the N-terminal part and the C-terminal part of the protein, are assumed to play a role in the interaction with the mitochondrion [8,40]. The hydrophobicity plot (Fig. 10) shows a rather hydrophilic protein with a mean hydrophobicity of 1.11 kcal/mol (according to the scale of Tanford [26]), which is comparable with the mean hydrophobicity of hemoglobin and serum albumin [41]. In addition, the plot shows some more extended hydrophobic segments at the C- and N-terminal part of the protein, and for the segment 70–78, which is the most conservative part of cytochrome *c* [7].

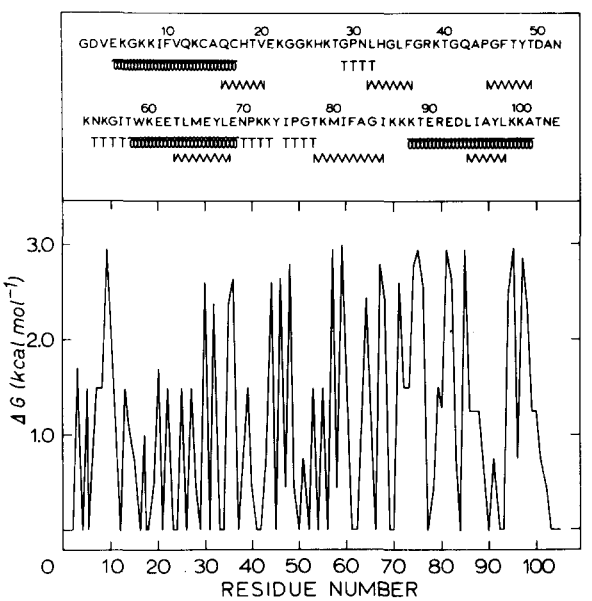

Fig. 10. Secondary structural elements predicted by the method of Chou and Fasman [24] and hydrophobicity profile as determined by the method of Rose [25,26], for apocytochrome *c*. Coils indicate α-helix, zig-zags β-strand, T represents β-turns and *G* indicates the free energy of transfer (kcal/mol) from water into ethanol.

CD

In agreement with a previous study [4] the CD pattern of an aqueous solution of apocytochrome *c* (Fig. 11A, curve 1) is featureless and resembles that of disordered fragments of other proteins [42].

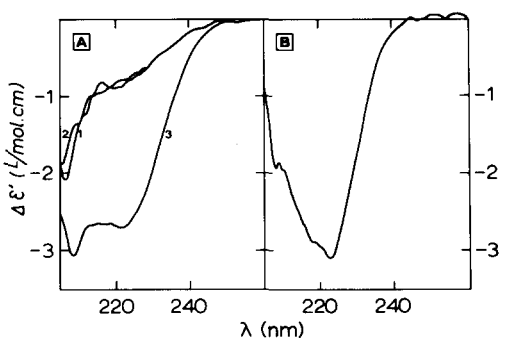

Fig. 11. CD spectra (A) of apocytochrome *c* in buffer (curve 1), in buffer containing 0.5% (w/v) Lubrol PX (curve 2), in buffer containing 0.5% SDS (curve 3); and (B) of cytochrome *c* in buffer. $\Delta\varepsilon'$ is the difference in absorbance of left and right circularly polarized light and is expressed per residue, using 115 as the average molecular weight per residue.

On the contrary, the CD spectrum of cytochrome *c* (Fig. 11B) shows the presence of α-helical structures, as is indicated by the extrema of $\Delta\varepsilon'$ at 209 and 222 nm. From the $\Delta\varepsilon'$ value of the spectrum at 222 nm the helical content was estimated [43] at 27%. These data show that the potency of a part of the apocytochrome *c* to adopt α-helical structures is expressed after coupling of the heme moiety. Because it has been suggested [44] that for insertion of proteins into membranes, α-helix formation is essential we studied whether the apocytochrome *c*-lipid interaction also would result in α-helical structures in the protein.

Since the interaction of apocytochrome *c* and vesicles of negatively charged phospholipids results into aggregation of the vesicles [10] which will give rise to light-scattering problems in the CD measurements, detergents were used as model for membrane phospholipids. Due to their spectral properties SDS and Lubrol have been chosen as negatively charged and neutral membrane lipid models, respectively. The effect of these detergents on the tryptophan fluorescence properties of apocytochrome *c* was similar, as described for sodium cholate and octylglucoside (data not shown). Fig. 11A shows that the presence of the neutral detergent Lubrol does not change the CD pattern of apocytochrome *c*; on the other hand, the presence of SDS (in a concentration of 0.5%) results in a CD spectrum which resembles that of cytochrome *c* with a helical component which amounts to 23% of the protein. SDS-induced conformational changes in water-soluble proteins have been well-documented [45,46]. At much lower SDS concentrations (0.001%) no changes in CD spectra were observed, whereas fluorescence experiments already showed an induction of a more hydrophobic environment of the tryptophan residue (Rietveld, A. and Jordi, W., unpublished data). Apparently, this increase in hydrophobicity of the environment of the tryptophan residue is not due to a structural reorganization of the protein, but created by the hydrophobic part of the detergent.

Discussion

The results clearly demonstrate that apocytochrome *c* has a profound interaction with negatively charged lipids, which is accompanied by a change in lipid packing and protein conformation. First, we want to discuss the influence of the apocytochrome *c*-lipid interaction on the lipid packing properties, as revealed by DSC, ESR and monolayer studies. The withdrawal of 60 PS and 160 PG molecules per molecule of apocytochrome *c* from the gel to liquid-crystalline transition as was shown by DSC, indicates that apocytochrome *c* causes a dramatic perturbation of the lipid packing. This effect is of the same magnitude and strength as compared to that induced by different integral proteins and peptides like glycophorin, cytochrome *c* oxidase and gramicidin, which are able to prevent the phase transition of about 90 [47], 70 [48] and 10 [49] lipid molecules. The influence of apocytochrome *c* on the phase transition of DMPG is very large compared to other water-soluble proteins. For both cytochrome *c* [10,50] and A_1 basic protein from myelin [51], the ΔH of the DMPG transition decreases only by maximally 30%. For this latter protein, this is paralleled by a lowering of the transition temperature. It is of interest to compare the stoichiometry obtained by binding and fluorescence data with that obtained by DSC experiments. The observed difference suggests that the lipid perturbation capacity of apocytochrome *c* includes much more lipids than those directly stoichiometrically (10 lipids per 1 apocytochrome *c* molecule) bound. The disappearance of the gel to liquid-crystalline transition of the phospholipids after interaction with apocytochrome *c* was confirmed by the behaviour of the 5-PG spin-label. The DSC and ESR results suggest that apocytochrome *c* prevents the formation of a closely packed gel phase and, in addition, restricts the chain motion in the liquid-crystalline phase. That this most likely is due to a deep penetration of the protein into the bilayer, is suggested by recent ESR experiments, which showed that apocytochrome *c* is able to strongly restrict mobility in the PG-SL molecule, even down to the C14th position of the fatty acid chain (Görissen, H., Marsh, D., Rietveld, A. and De Kruijff, B., unpublished data). Also the apocytochrome *c*-induced surface pressure increase of negatively charged lipid monolayers can be interpreted as a penetration of the (basic) protein into the monolayer. In this respect, apocytochrome *c* resembles the A_1 basic protein which also can

penetrate into a monolayer of negatively charged lipids [52], resulting in a large increase (20 $mN \cdot m^{-1}$) of surface pressure at an initial pressure of 10 $mN \cdot m^{-1}$ [53]. On the other hand, poly(L-lysine), which only binds electrostatically to the lipid surface [54], has nearly no effect on the surface pressure [53]. The combined DSC, ESR and monolayer studies, clearly suggest that at least a part of the apocytochrome *c* penetrates into the hydrophobic part of the membrane. Tryptophan fluorescence experiments show that this part of the protein contains the tryptophan residue, which is located at position 59 (Fig. 10). Quenching experiments with I^- confirm that the tryptophan is shielded to some extent from the aqueous phase. However, the shielding is much less than that observed for the amphipathic basic polypeptide cardiotoxin of which the tryptophan-containing part penetrates more deeply into cardiolipin model membranes (Batenburg, M., unpublished data). The amount of lipid protection against I^- of the tryptophan in apocytochrome *c* is comparable with the lipid shielding documented for the tryptophan in melittin [55] after interaction with phospholipids. For that protein a location of the tryptophan in the region of the glycerol backbone was suggested. Since trypsin is also able to reach the tryptophan-containing part of apocytochrome *c* bound to PS, the possibility that the tryptophan of apocytochrome *c* is also located in the region of the glycerol backbone is very likely. The interfacial location of tryptophan would be consistent with the structural prediction (Fig. 10) since the position of the tryptophan residue (residue no. 59) is just at the end of one of the predicted α-helical segments (residues no. 58–69), which have been assumed to play an important role in membrane penetration [44]. The position of the three predicted α-helical segments in apocytochrome *c* in water is about the same as that of the α-helical segments in horse heart cytochrome *c* as was established from X-ray studies [7,56]. These X-ray studies showed an α-helical content in cytochrome *c* of 34%. This is in rather good agreement with our CD results of cytochrome *c*. The CD experiments of apocytochrome *c* show that the interaction of the protein with a negatively charged lipid induces conformational changes in the apocytochrome *c*, resulting in an α-helical content of 23%. This by lipid induced structural change towards the conformation of cytochrome *c* possibly facilitates the heme coupling by the enzyme cytochrome *c* synthethase. Probably, this conformational change is also important for the insertion of the protein into the bilayer, as was reported recently for the signal sequence of the *Escherichia coli* λ receptor protein [57], and for the A fragment of diphtheria toxin [58]. Integration of the different aspects of the apocytochrome *c*-lipid interaction leads to the following schematic sequence of events: First, apocytochrome *c* binds in an electrostatic way to the phospholipids. This binding is accompanied by the penetration of a part of the protein into the hydrophobic part of the model membrane. This process is attended with a change towards a more helical structure of the protein. This protein insertion can result in the translocation of (a part of) the protein across the model membrane barrier [11]. It is very interesting that this translocation is not restricted to model membranes, composed of only negatively charged phospholipids, but also model membranes composed of mixtures of neutral and negatively charged phospholipids exhibit this capacity [32], leading to the speculation that the lipidic components of the mitochondrial membranes might be able to induce a pathway for apocytochrome *c* translocation.

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Our CD results on apocytochrome *c*-detergent systems are in good agreement with the CD measurements of Walter et al. [59] on apocytochrome *c*-PS recombinants.

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