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Lipid specificity for membrane mediated partial unfolding of cytochrome *c*

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Abstract In this study we investigated the lipid specificity for destabilization of the native structure of horse heart cytochrome *c* by model membranes. From (i) the enhanced release of deuterium from deuterium-labelled cytochrome *c* and (ii) the increased proteolytic digestion of the protein in the presence of anionic lipids, it was concluded that these lipids are able to destabilize the native structure of cytochrome *c*. Changes in the absorbance at 695 nm indicated that the destabilization was accompanied by a diminished ligation of Met-80 to the heme. Beef heart cardiolipin was found to be more effective than DOPS, DOPG or DOPA, while no protein destabilization was observed in the presence of the zwitterionic lipid DOPC or, surprisingly, in the presence of *E. coli* cardiolipin. Experiments with mitoplasts showed that the protein can also be destabilized in its native structure by a biological membrane.

Key words: Phospholipid; Cytochrome *c*; Protein folding; Exchange; Proteolytic digestion

1. Introduction

The detailed knowledge of cytochrome *c* with respect to its structure [1–3] has made it an attractive model for studies on protein folding and folding intermediates [4,5]. In order to characterize these folding intermediates, cytochrome *c* has been studied in an acid- [6–8] and urea-denatured form [9]. Also destabilization of the native structure of cytochrome *c* due to the presence of some phospholipid model membranes has been reported using calorimetry, by monitoring aspecific exchange of amide protons using IR spectroscopy, or indirectly by the effect on the ^{31}P relaxation behaviour using NMR [10–15].

Because folding events of cytochrome *c* are of importance for its functional activity and might play a role during the import process in mitochondria, the aim of our study was to obtain a better and comparative insight into the folding state of cytochrome *c* upon interaction with model membranes composed of phospholipids representative for those present in mitochondria and upon interaction with mitoplasts. For this purpose we used a technique of detecting deuterium–proton exchange of deuterium-labelled proteins, which was developed previously to investigate the folding state of micelle-associated cytochrome *c* [16]. The advantages of this technique are that (i) the rate of exchange directly reflects the folding state of the protein and (ii) it can be performed with a high specificity and on a relatively short time scale. In addition we used protease sensitivity and the visible light absorbance by the heme moiety to obtain complementary information on the folding state of the protein in the presence of various lipid systems.

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Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; (BH)CL, (beef heart) cardiolipin; PS, phosphatidylserine; PE, phosphatidylethanolamine; LUV, large unilamellar vesicle; IR, infrared; NMR, muclear magnetic resonance; TCA, trichloroacetic acid; 12-PN, dodecylphosphocholine; 12-Pglycol, dodecylphosphoglycol; L/P, lipid-to-protein; *E. coli, Escherichia coli.*

2. Materials and methods

2.1. Materials

The phospholipids DOPC, DOPG, DOPA, mitochondrial CL from beef heart, and DOPS were obtained from Avanti Polar Lipids (Alabaster, AL). Cardiolipin was purified from *E. coli* as described [17]. The detergents 12-PN and 12-Pglycol were synthesized as described previously [16]. Phospholipid concentrations were determined using the method of Rouser [18]. Horse heart cytochrome *c* (Type VI, Sigma) was purified on a Sephadex G-75 column using a 10 mM phosphate buffer (pH 7.0) as eluens. The protein was used in the oxidized form. Protein concentrations were determined according to Lowry [19]. L/P ratios are given as mol phosphate per mol protein.

2.2. Preparation of phospholipid vesicles

Phospholipid dispersions were prepared by hydrating a dry lipid film in 10 mM phosphate buffer (pH 7.0), if not mentioned otherwise, followed by ten cycles of freezing and thawing. Large unilamellar vesicles (LUVs) were obtained by the extrusion technique using polycarbonate filters with 400 nm pores at room temperature [20]. The volume of the vesicle stock solutions was adjusted to obtain final lipid concentrations of 20 mM.

Mixed micelles of 12-PN and 12-Pglycol (9/1 mol/mol) were prepared by dissolving the mixed powders in 10 mM phosphate buffer (pH 7.0).

2.3. Binding experiments

Cytochrome *c* (20 mM) was incubated for 15 min at room temperature with LUVs (3.0 mM phospholipid) in 10 mM Tris-buffer (pH 7.0, HCl) in a total volume of 150 μl containing 50 mM sodium chloride to facilitate pelleting of the vesicles upon centrifugation in a Beckman TL-100 ultracentrifuge (30 min at 20°C, 110,000 × *g*). Percentages of bound protein were calculated by determination of the amount of protein in the supernatant, corrected for the amount of non-pelletted lipids. The estimated error in the percentage is 3%.

2.4. Preparation of mitoplasts

Mitoplasts were isolated from rat liver mitochondria according to the swell–shrink–sonicate procedure as described by Hovius and colleagues [21] and stored in 0.25 M sucrose in liquid nitrogen. The latency of cytochrome *c* oxidase, defined as the ratio of the rate of oxidation of ferrocytochrome *c* in the absence relative to the rate in the presence of 0.2% Lubrol PX [21], was found to be 0.97, indicating negligible shielding of the inner membrane. To compensate for the intrinsic molarity of the mitoplasts, all experiments were performed in a solution containing 130 mM potassium chloride and 10 mM Tris-HCl (pH 7.0), or 0.25 M sucrose solution (pH 7.0) in the case of the protease experiments.

2.5. Measurement of cytochrome c oxidase activity

The activity of cytochrome *c* oxidase was tested as described [21]. Briefly, 10 mM cytochrome *c* was reduced by incubation for 15 min with 100 mM ascorbic acid in 10 mM phosphate buffer (pH 7.0) and separated from the reducing agent by elution on a Sephadex G-50 column. The rate of oxidation of 50 μM reduced cytochrome *c* due to

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the presence of mitoplasts was obtained by fitting the monitored changes in absorbance at 550 nm in time by a single exponential decay curve.

2.6. Preparation of partially deuterated cytochrome c

Deuterated cytochrome *c* was prepared as described [16]. Prior to an exchange experiment, deuterons well exposed to the solvent were exchanged by eluting 20 mg of deuterated protein at room temperature over a Sephadex G-75 column with a 10 mM phosphate buffer (pH 7.0) in H_2O. The total volume of the collected protein was adjusted to 4 ml (protein concentrations of approximately 0.3 mM) and the protein solution was used immediately for an experiment.

2.7. Deuterium to proton exchange experiments

One aliquot of 0.5 ml of the partially deuterated cytochrome *c* solution was frozen immediately in liquid nitrogen. The amount of deuterium in this sample was taken as the amount present at $t = 0$ in the exchange experiment. Next, 0.5 ml aliquots of the protein solution were added to 0.25 ml of one of the vesicle solutions, 20 mM 12-PN/12-Pglycol (9/1) solution or to 10 mM phosphate buffer (pH 7.0). The exchange was quenched by freezing the samples in liquid nitrogen, after 5, 15, 30, 45 or 60 min of incubation time at room temperature. After removal of the exchanged deuterons by lyophilization, the samples were dispersed in 0.16 ml 10 M deuterium-depleted urea [16], and incubated for 3 h at 56°C to obtain a complete release of all non-exchanged deuterons from the protein. Next deuterium-depleted water was added to a total volume of 0.2 ml and the deuterium content of the samples was determined using ^2H NMR as described [16]. Corrections for natural abundance deuterium in protein-free samples were taken into account (approximately 5% of the initial amount of deuterium in cytochrome *c*). The accuracy in determining the amount of deuterium by this procedure was estimated to be 2%. The deuterium content of cytochrome *c* at $t = 0$ was found to be 31 ± 4 mol deuterium per mol protein. Determination of the protein and lipid concentrations in these samples yielded L/P ratios ranging from 115 to 137 for different experiments.

Since mitoplasts have a high background level of naturally abundant deuterium, we changed the protocol for this system as follows. Deuterated cytochrome *c* (56.5 nmol) was added to mitoplasts (25 mg protein, corresponding to 8.5 μmol phospholipid [21]) in a volume of 0.75 ml and incubated for 1 h. Next the mitoplasts were pelletted (5 min, 110,000 × *g* at 4°C), resuspended in 0.3 ml buffer and pelletted again (5 min at 110,000 × *g*). In this way a nearly 100% recovery of the added cytochrome *c* could be obtained in the collected supernatants as concluded from protein quantification. In a control sample without externally added cytochrome *c*, no protein could be detected in the supernatant. The supernatants were subsequently frozen in liquid nitrogen and treated in the same way as described above for the other lipid systems.

2.8. Protease sensitivity experiments

Typically 20 μg of protein was incubated in the absence or presence of lipid micelles or vesicles (L/P ratio of 150) for 5 min at 37°C in a total of 40 μl 10 mM phosphate buffer (pH 7.0). At time point $t = 0$ 10 μl (0.02 U) of thermolysin (Type X; Sigma) solution was added and the mixture was subsequently incubated for different times at 37°C. The activity of the protease was quenched by addition of 50 μl 30% TCA. The material was pelletted by centrifugation (5 min, 15,000 × *g*), washed with acetone, and analyzed using 15% tricine SDS-PAGE under reducing conditions as described [22]. In the experiments where mitoplasts were involved, a lipid-to-cytochrome *c* ratio of 75 was used to avoid overloading of the gel (i.e. 0.36 g mitoplast protein) and 11% tricine gels were used to analyze the samples. Control experiments revealed that the proteolytic digestion of cytochrome *c* by thermolysin, both in the absence and presence of DOPS LUVs, was similar in 10 mM phosphate buffer (pH 7.0), as used in the experiments with model membranes, and in 0.25 M sucrose (pH 7.0), the medium used for the mitoplasts. The gels were stained (7% v/v acetic acid, 30% v/v methanol, 0.25% w/v Coomassie brilliant blue), and the amount of cytochrome *c* was quantified with an accuracy of 5% using a densitometer, where the intensity at $t = 0$ was set to 100%. The data presented are corrected for background intensities.

2.9. Spectrophotometric experiments

Absorbance spectra of protein samples in 10 mM phosphate buffer (pH 7.0) were recorded on a Hitachi U-3200 spectrophotómeter in the 650–750 nm region at room temperature in cells with a path length of 1 cm, and were corrected for the corresponding protein-free samples. Protein concentrations of 50 μM were used in the absence or presence of lipid micelles or LUVs in an L/P ratio of 150.

3. Results

3.1. Deuterium–proton exchange experiments

To analyze whether membrane phospholipids can destabilize the native structure of cytochrome *c* we monitored the release of deuterium from the protein after dilution in buffer containing LUVs of different phospholipids. Fig. 1 shows that in the presence of vesicles composed of DOPS, deuterium can be released from the protein depending on the L/P ratio. Whereas below L/P ratios of 80 this release increases with the lipid concentration, no effect of additional lipid on the release is observed at higher ratios. Therefore L/P ratios above 100 were used to investigate the lipid specificity of this release. Fig. 2 shows that the zwitterionic lipid DOPC has no effect on the deuterium–proton exchange of the protein as compared to the lipid-free situation. For both the lipid-free and DOPC systems during the first 10 min an exchange of approximately 20% of the initial amount of deuterium takes place, probably due to exchange of deuterons less deeply buried in the protein. After 1 h of exchange still 65% of the initial amount of deuterium is present in the tightly folded protein. The lack of binding of the basic cytochrome *c* to the DOPC LUVs (Table 1, first column) is most likely responsible for the unaltered ^2H–^1H exchange. In contrast, when the negatively charged lipids DOPS, DOPG or DOPA are present, only about 40% of the deuterons within the protein remains non-exchanged after 1 h. An even faster exchange was found for the negatively charged (BH)CL. In agreement with previous observations [16], the presence of 12-PN/12-Pglycol (9/1) micelles results in a complete release of all deuterons from the protein within 10 min. These data are summarized in the second column of Table 1.

Cardiolipin from beef heart is a unique lipid, both with respect to its overall chemical structure as a 'double phospholipid' and its acyl chain composition with a high content of polyunsaturated fatty acids (6.7% mono- and 92.3% polyunsaturation [23]). Therefore we also tested cardiolipin from

Table 1
Summary of the binding, amide exchange, protease sensitivity and photometric properties of cytochrome *c* in the absence or presence of various lipids

	% bound	Residual % ^2H after 60 min of exchange	% non-digested after 60 min	Presence of 695 nm band
No lipid	–	65	95	+
+12-PN/12-Pglycol (9/1)	100[a]	6	3	–
+DOPC	<5	71	98	+
+DOPS	73	40	47	–
+DOPG	96	41	49	–
+DOPA	98	42	44	–
+(BH)CL	97	18	17	–
+*E. coli* CL	65	64	84	+
+Mitoplasts	5	63	29	nd

nd, not determined.
[a]Based on ^1H NMR data published previously [19].

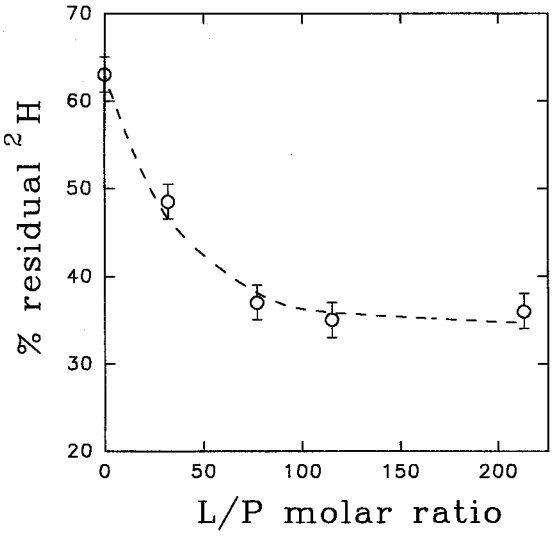

Fig. 1. The amount of deuterium present in cytochrome *c* expressed as the percentage of the initial amount at $t = 0$, after 60 min of exchange with the solvent at room temperature as a function of the amount of DOPS LUVs present in 10 mM phosphate buffer (pH 7.0).

E. coli, the fatty acids of which are more saturated (23.2% mono- and 0% polyunsaturation [17]) than those of (BH)CL, and also more saturated than those of the synthetic anionic phospholipids used (all 100% mono-unsaturated). Interestingly, no enhanced release of deuterium is observed in the presence of these vesicles compared to the lipid-free situation (Table 1), which is not due to a lack of binding (Table 1).

To investigate whether a biological membrane could destabilize the native structure of cytochrome *c* we incubated partially deuterated cytochrome *c* for 1 h in the presence of mitoplasts. No enhanced release is detected compared to a sample without mitoplasts treated in the same way (Table 1).

3.2. Protease sensitivity experiments

Native cytochrome *c* is a stable folded protein which is highly resistant to proteases like thermolysin, as demonstrated in Fig. 3A. Anionic mitochondrial phospholipids induce a conformational change in cytochrome *c* such that the protein becomes susceptible to the protease. Degradation is found in the presence of DOPS (Fig. 3B), DOPG and DOPA (not shown), (BH)CL (Fig. 3D), and in the presence of 12-PN/12-Pglycol (9/1 mol/mol) micelles (Fig. 3C), with main digestion products of 4.7 and 3.6 kDa, based on comparison with molecular weight markers. In contrast, vesicles composed of *E. coli* CL (Fig. 3E) or DOPC (not shown) do not affect the resistance of cytochrome *c* to proteolytic digestion. In the presence of mitoplasts, cytochrome *c* is proteolytically degraded (Fig. 3F) but no clear digestion products are observed. In the lane where no exogenous cytochrome *c* is present it can be seen that the mitoplasts do not contain proteins of comparable size to cytochrome *c*, which could possibly have interfered with the quantification of the data.

Fig. 4 shows the time-dependence of the extent of protein degradation, quantified by density scanning of the cytochrome *c* band on the gels, while Table 1 (third column) lists the percentage cytochrome *c* still present after 1 h of exposure to thermolysin in the presence of the various lipids. From these data it can be concluded that degradation is fastest for the micelles, followed by (BH)CL, mitoplasts and DOPS, DOPG and DOPA.

Similar experiments using the protease clostripain at room temperature revealed comparable results (data not shown).

3.3. Oxidation of reduced cytochrome c by mitoplasts

First the conditions were established under which the oxidation rate for cytochrome *c* is independent of the amount of mitoplasts added and therefore saturation of the enzyme can be excluded. Using 50 μM reduced cytochrome *c* an oxidation rate of 0.75 ± 0.05 s^{-1} per mg mitoplast protein is found, independent of the amount of mitoplasts added in the range 10–500 μg mitoplast protein (data not shown). Because in the deuterium–proton exchange and protease sensitivity experiments, respectively, 25 and 360 mg mitoplast protein were present, while a non-saturating condition of cytochrome *c* was met, oxidation rates of, respectively, 18.75 and 270 s^{-1} would be expected, resulting in a complete oxidation of cytochrome *c* on the sub-second time scale.

3.4. Spectrophotometric experiments

Changes in the direct chemical environment of the heme can be monitored spectrophotometrically [9]. Fig. 5 shows the spectral region around 695 nm where for native cytochrome *c* an absorption band is present arising from the ligation of the heme to Met-80 of cytochrome *c* [9,24]. The decrease in the intensity of this band with increasing urea concentrations correlates to the detachment of the axial ligand of Met-80 from the heme group. In agreement with the exchange and protease sensitivity data, in the presence of the micelles (curve b), (BH)CL (curve c), or other anionic phospholipids (not shown), this band is absent, pointing to a tertiary unfolding in these systems. However, in the presence of DOPC (spectrum a) or CL from *E. coli*

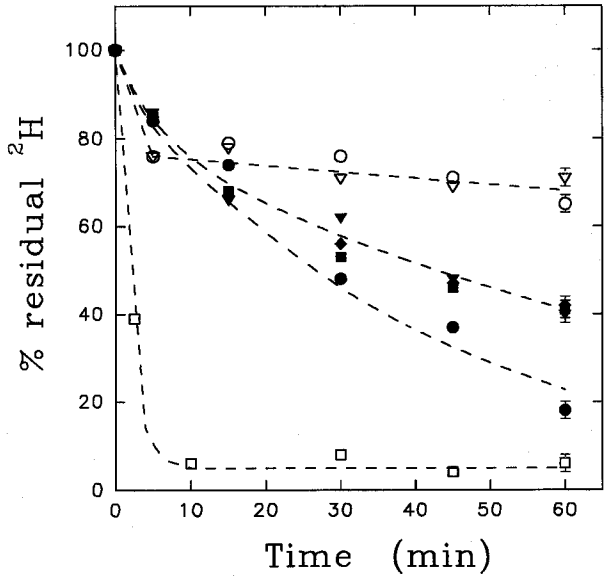

Fig. 2. Deuterium–proton exchange of ^{2}H-labeled cytochrome *c* with the solvent upon dilution of the protein at room temperature in 10 mM phosphate buffer (pH 7.0) in the absence of lipid (○), and in the presence of 12-PN/12-Pglycol (9/1) (□) micelles, and LUVs of DOPC (▽), DOPS (▼), DOPG (■), DOPA (♦), and (BH)CL (●). L/P ratios ranged from 115 to 137. The content of deuterium in cytochrome *c* is expressed as the percentage of the amount at $t = 0$.

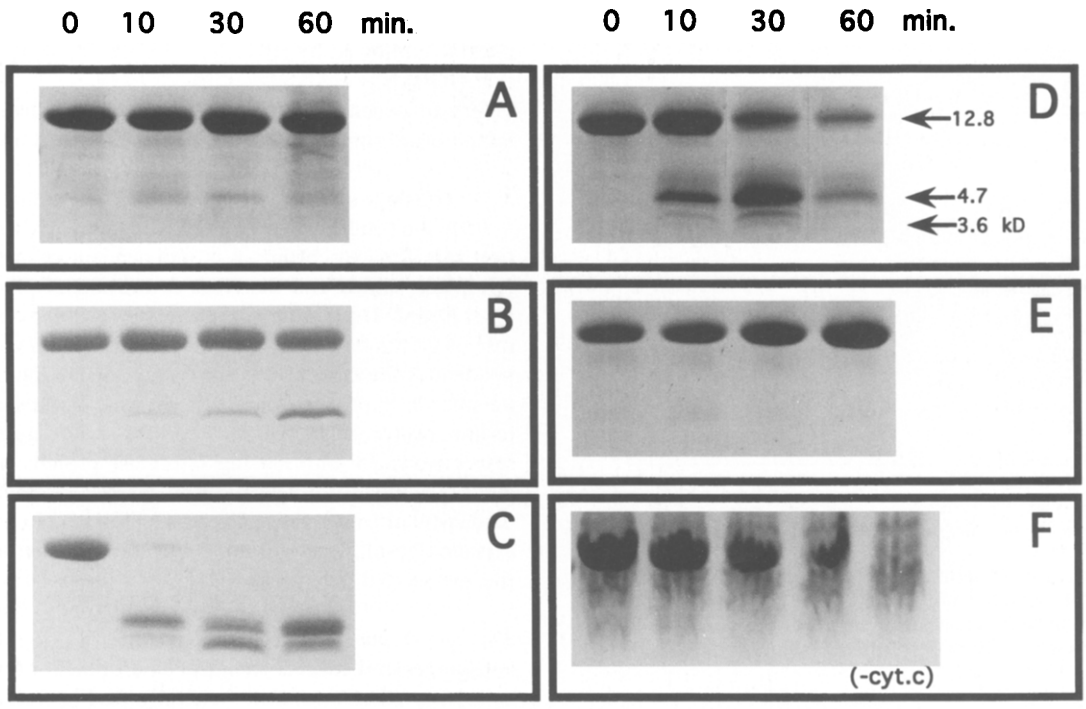

Fig. 3. Effect of thermolysin on cytochrome *c* in the absence of lipids (A) and in the presence of DOPS (B), 12-PN/12-Pglycol (9/1) micelles (C), (BH)CL (D), *E. coli* CL (E) (all L/P ratios of 150 in 10 mM phosphate-buffer pH 7.0), and rat liver mitoplasts (F) (L/P ratio of 75 in 0.25 M sucrose), where for this latter system in the right lane no external cytochrome *c* was added. All experiments were carried out at 37°C.

(spectrum d) clearly an absorption can be detected in this region, comparable to the lipid-free situation. These data are qualitatively summarized in Table 1 (fourth column).

4. Discussion

In this work we investigated the destabilization of the native structure of cytochrome *c* in the presence of membranes composed of various phospholipids or in the presence of inner mitochondrial membranes. By measuring the release of deuterium from cytochrome *c* (Fig. 2), specifically labelled at the interior of the protein, and the sensitivity of cytochrome *c* for proteolytic digestion by proteases (Figs. 3 and 4), it was shown that vesicles composed of anionic mitochondrial phospholipids are able to destabilize the native structure of cytochrome *c*. Tertiary destabilization of cytochrome *c* by PG and (BH)CL was already reported previously [10–15]. However, Table 1 shows that beef heart CL is more effective in inducing structural destabilization compared to other anionic lipids. Because in the presence of *E. coli* CL no destabilization of the protein could be observed, it is likely that the degree of unsaturation of the acyl chains plays a dominant role in the unfolding process, as suggested previously [13]. Whether this is related to the fluidity of the membrane, the potential for membrane curvature, or a lipid-specific effect, is unclear.

The first step in unfolding of cytochrome *c*, which in its native form is tightly folded around the heme group, is the detachment of Met-80 from the heme [25]. That this step indeed occurs in the presence of most anionic phospholipids is concluded from the absorbance measurements, which showed that in these lipid systems the ligation of Met-80 with the heme is diminished (Table 1). These observations agree with previous suggestions made in the presence of (BH)CL [11,14]. The fact that the protein is digested in two main products with sizes corresponding to 28 and 37% of that of the native protein indicates that, as described for the first unfolding step [9,26], a major conformational change takes place in the presence of these lipids. In a second and faster step of unfolding, the ligation of His-18 is detached from the heme [9]. It is possible that

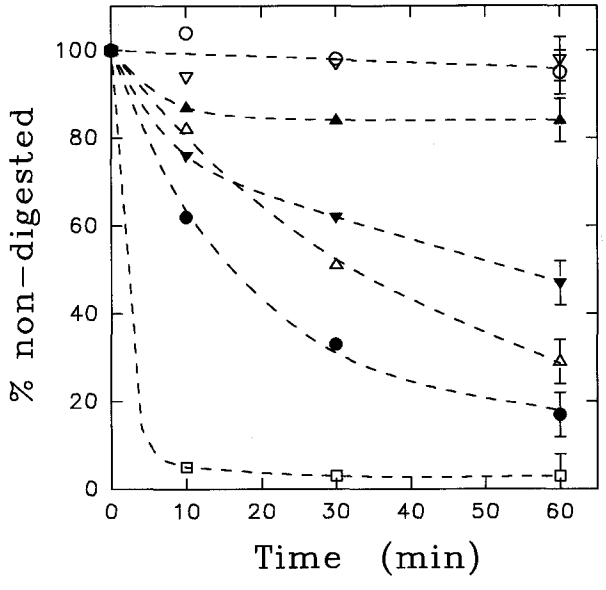

Fig. 4. Analysis of the gels of Fig. 3 by densitometry, monitoring proteolytic digestion of cytochrome *c* by thermolysin in the absence of lipids (○) and in the presence of LUVs of DOPC (▽), DOPS (▼), (BH)CL (●), *E. coli* CL (▲), 12-PN/12-Pglycol micelles (□) and rat liver mitoplasts (△).

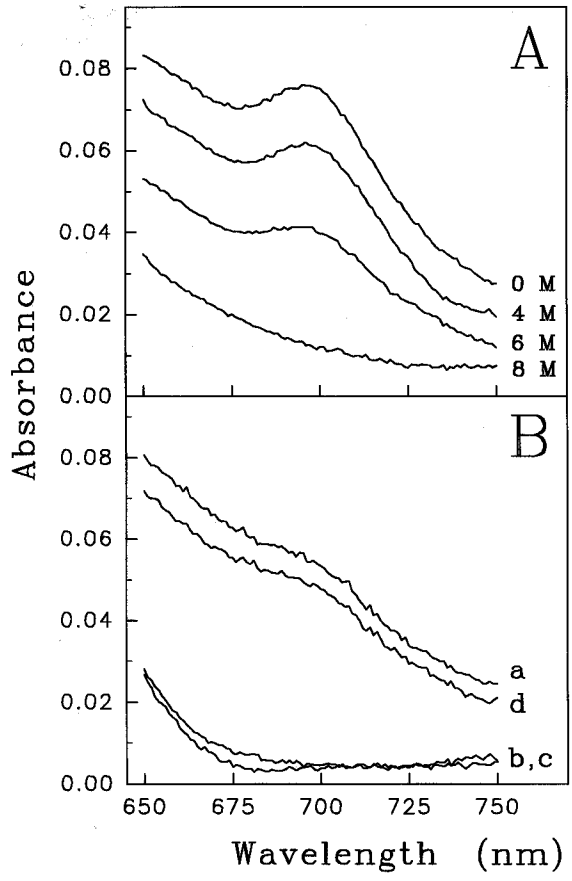

Fig. 5. (A) Absorbance spectra from 650 to 750 nm of cytochrome *c* at various concentrations of urea at pH 7.0, and (B) in the presence of LUVs of DOPC (a), 12-PN/12-Pglycol micelles (9/1 mol/mol) (b), or vesicles of (BH)CL (c), or *E. coli* CL (d), in 10 mM phosphate-buffer (pH 7.0).

in the micellar system His-18 also detaches from the heme, accompanied by a complete tertiary unfolding of the protein. Such a folding state would explain the reported structural and dynamical resemblance with its heme-free precursor in micellar systems [16]. It also could explain the almost 10 times faster exchange (Fig. 2) and proteolytic digestion (Fig. 3) of cytochrome *c* in the presence of micelles compared to (BH)CL. (BH)CL might be an intermediate between the detergents and the other negatively charged lipids in that to some extent this second unfolding step might occur.

It has been reported that the effect of negatively charged membranes on the native structure of cytochrome *c* strongly diminishes upon increasing the PC content [12,27], as we also observed by deuterium–proton exchange experiments where (BH)CL was diluted with 25 mol% DOPC (data not shown). Since approximately 27% of the lipids in the inner leaflet of the outer mitochondrial membrane are anionic and face the inter membrane space [28], we propose that this is insufficient to prevent Met-80 to ligate with the heme, and that therefore cytochrome *c* will be able to fold tertiary after translocation of the precursor apocytochrome *c* across the outer membrane and covalent attachment to the cysteines of the heme by the enzyme heme lyase. Because this enzyme has a high affinity binding site for apocytochrome *c* [29], it is possible that phospholipids as well as the heme lyase play a role in the folding pathway of cytochrome *c*.

In the native structure, ferrocytochrome *c* is well protected from oxidation because the lower dielectric constant near the iron atom in the heme of native cytochrome *c* makes it energetically more costly to remove an electron as compared to a water-exposed heme group. For electron transport an opening of the heme crevice becomes necessary, both for steric and energetical reasons. It was found that cytochrome *c* hardly binds to mitoplasts (Table 1), in agreement with the rather free diffusion of the protein over the mitochondrial inner membrane [30]. However, cytochrome *c* does become protease sensitive in the presence of mitoplasts (Figs. 3 and 4), and under these conditions the protein would be able to transfer its electron on the sub-second time scale, as determined by the measurement of cytochrome *c* oxidase activity. We speculate that upon collision of cytochrome *c* with its redox partners the interaction is long enough to allow detachment of Met-80 from the heme (a 100 ms step [9]), possibly facilitated by the specific binding of cardiolipin to cytochrome *c* oxidase [31], providing a destabilized protein which can be recognized by a protease. On the other hand the lifetime of the destabilized intermediate is not long enough to allow a free exchange of deuterium from the interior of the protein with the solvent.

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