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Binding and Release of Cytochrome c in Brain Mitochondria Is Influenced by Membrane Potential and Hydrophobic Interactions with Cardiolipin

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Abstract. Factors influencing the release and anchorage of cytochrome c to the inner membrane of brain mitochondria have been investigated. Metabolic activity of mitochondria caused a decrease in the membrane potential $\Delta\psi_m$, accompanied by detachment of the protein from the inner membrane. In a model system of cytochrome c reconstituted in cardiolipin (CL) liposomes, phosphate was used to breach the hydrophilic lipid-protein interactions. About 44% cytochrome c was removable when heart CL (80% 18:2n-6) was employed, whereas the remaining protein accounted for the tightly bound conformation characterized by hydrophobic lipid-protein interactions. Cytochrome c release from brain CL liposomes was higher compared to heart CL, consistent with lower polyunsaturated fatty acid content. The release was even higher with CL extracted from metabolically stressed mitochondria, exhibiting more saturated fatty acid profile compared to control (30% *vs.*17%). Therefore, weakening of the hydrophobic interactions due to saturation of CL may account for the observed cytochrome c release from mitochondria following metabolic stress. Moreover, mitochondria enriched with polyunsaturated CL exhibited higher $\Delta\psi_m$, compared to less unsaturated species, suggesting that CL fatty acid composition influences $\Delta\psi_m$. Mitochondria incorporated exogenous cytochrome c without protease-sensitive factors or $\Delta\psi_m$. The internalized protein anchored to the inner membrane without producing swelling, as monitored by forward and side light scattering, but produced $\Delta\psi_m$ consumption, suggesting recovery of respiratory activity. The $\Delta\psi_m$ decrease is ascribed to a selected mitochondrial population containing the incorporated cytochrome c.

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Key words: Brain mitochondria — Mitochondrial membrane potential — Cardiolipin — Phosphatidylglycerol — Cytochrome c

Introduction

Inherited or acquired mitochondrial defects are the cause of neuronal degeneration as a consequence of energy defects and oxidative damage (Manfredi & Beal, 2000). There is much evidence showing both metabolic and oxidative damage in Alzheimer's disease. Studies on cybrid cell lines show reduced cytochrome c oxidase (Beal, 2000). Brain mitochondria become vulnerable to H_2O_2-induced oxidative stress when complex I of the respiratory chain is inhibited, resulting in a gradual loss of membrane potential ($\Delta\psi_m$). As the combined presence of complex I deficiency and H_2O_2-induced oxidative stress is characteristic of dopaminergic neurons in Parkinson's disease, the loss of $\Delta\psi_m$ could be a crucial factor in bioenergetic incompetence (Chinopoulos & Adam-Vizi, 2001). Recent human post-mortem studies suggest that dopaminergic neurons die from apoptosis in Parkinson's disease (Hirsch et al., 1999; Hartmann et al., 2001).

An important event in the apoptotic cascade is the release of cytochrome c from mitochondria into the cytoplasm, leading to sequential activation of caspase-9 and downstream executioner caspases (Liu et al., 1996; Budihardjo et al., 1999). It has been demonstrated that cytochrome c release in isolated liver mitochondria occurs through distinct mechanisms that are either Ca^{2+}-dependent or Ca^{2+}-independent (Gogvadze et al., 2001). In the first case, mitochondrial Ca^{2+} overload promotes mitochondrial permeability transition pore opening, swelling of the matrix, and rupture of the outer mitochondrial

membrane, thus allowing protein release. Whether Ca^{2+}-dependent cytochrome c release proceeds through the same pathway also in brain mitochondria is an open question (Andreyev & Fiskum, 1999; Brustovetsky et al., 2002). Ca^{2+}-independent cytochrome c release is likely to be governed by the Bcl-2 protein family. A recent report showed that cytochrome c release requires a two-step process that consists of detachment of the protein from its membrane-anchoring lipid, followed by Bax-promoted outer mitochondrial membrane permeabilization (Ott et al., 2002).

Cytochrome c binds to membranes containing acidic phospholipids, particularly cardiolipin (CL) either as such or complexed with cytochrome c oxidase (Das et al., 1962; Vanderkooi, Erecinska & Chance, 1973; Rytömaa & Kinnunen, 1995; Tuominen, Wallace & Kinnunen, 2002). Two different types of interactions have been characterized for binding of cytochrome c to the membrane through distinct sites in the protein, the A-site for electrostatic and the C-site for hydrophobic interactions. It has been suggested that the hydrophobic component of the C-site-mediated interaction is due to an extended lipid anchorage of one of the phospholipid acyl chains protruding from the membrane and accommodating within a hydrophobic channel in cytochrome c, whereas the other chain remains in the lipid bilayer (Tuominen et al., 2002).

The quantitative interactions of cytochrome c with CL and other anionic phospholipids, such as phosphatidylglycerol (PG), may be important in the equilibrium status between bound and unbound cytochrome c. Palmitate-induced cardiomyocyte apoptosis is accompanied by diminished CL synthesis due to PG fatty acid saturation that renders PG a poor substrate for CL synthase. In this system, decreased CL synthesis and cytochrome c release are directly correlated (Ostrander et al., 200la). Therefore, cytochrome c-CL interactions could be influenced not only by CL content but also by its fatty-acid composition, since the hydrophobic interaction is the main component in the association of cytochrome c to the inner mitochondrial membrane. In brain mitochondria, $\Delta\psi_m$ increased after fusion of exogenous CL to mitochondria, whereas $\Delta\psi_m$ decrease and cytochrome c release was observed during incubation of mitochondria at low respiratory rate (Piccotti et al., 2002).

In this study we demonstrate that highly unsaturated CL liposomes bind cytochrome c tighter than do less unsaturated species. This finding justifies the observed release of cytochrome c from brain mitochondria whose CL undergoes fatty-acid saturation after in vitro incubation at low respiratory rate. Cytochrome c detached from the inner membrane may be trapped in the intermembrane space or discharged out of mitochondria, depending on metabolic conditions. Exogenous cytochrome c added to mitochondria translocates across the outer mitochondrial membrane and localizes in the inner membrane, contributing to mitochondrial respiratory activity.

Materials and Methods

Chemicals

3,3′-diexyloxocarbocyanine iodide ($DiOC_6(3)$) was from Molecular Probes Europe BV; pronase, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and nigericin were from Fluka Chemie AG (Italy). HEPES, cytochrome c, ADP (K^+ salt), malic acid, and pyruvic acid were purchased from Roche Molecular Biochemicals (Italy). Pyruvic acid and malic acid solutions were adjusted to pH 7.0 with KOH. Heart CL, other phospholipids, protease inhibitor cocktail, and fatty acid methyl esters standards were obtained from Sigma Chemicals (Italy). Rabbit anti-(cytochrome c) polyclonal IgG and donkey anti-rabbit HRP-conjugated IgG were from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of Mitochondria

Mitochondria were prepared from rat brain cortex as previously described (Monni et al., 2000). The mitochondrial pellet was resuspended in 0.32 M sucrose, 2 mM HEPES (pH 7.4) (S/H buffer) and biochemical characterization was performed as described (Monni et al., 2000). In some experiments, pronase treatment of mitochondria was performed by incubating mitochondria (1 mg of protein in 1 ml of S/H buffer) with pronase (25 μg) at room temperature for 10 min. After pelletting by centrifugation for 10 min at $9{,}000 \times g$, mitochondria were resuspended in S/H buffer. Respiratory activity of mitochondria was modulated with ADP, pyruvate, and malate (0.8, 1.5, and 3.0 mM, respectively) in the presence of 5 mM potassium phosphate. Substrates and phosphate were omitted in de-energized state. When specified, 20 μM nigericin or 50 μM CCCP were added.

Treatment of Mitochondria with Digitonin

To permeabilize the outer membrane, mitochondria were treated with digitonin (0.4 mg/mg of mitochondrial protein, 10 min at 0°C) following a described procedure (Camici & Corazzi, 1995). The integrity of the inner mitochondrial membrane after digitonin treatment was evaluated by measuring the activity of cytochrome c oxidase (Corazzi et al., 1993). The content of CL in the post-digitonin supernatant was determined as an index of contamination of inner mitochondrial membrane fragments.

Isolation of CL and PG from Brain Mitochondria

Lipids were extracted as described (Folch, Lees & Sloane Stanley, 1957) with 50 μM butylated hydroxytuolene added as an antioxidant. After phase separation, the chloroform phase was collected and the hydro-methanolic phase and the interphase were extracted two additional times with chloroform. The combined extracts were evaporated to dryness and lipids were dissolved in chloroform. Isolated total lipids were then applied to silica gel-G plates and developed with chloroform/methanol/18% ammonium hydroxide (65:25:4) for the purification of CL, or chloroform/methanol/acetic acid (65:25:10) for the purification of PG. Lipids were identified by co-chromatography with authentic standards and eluted from the silica with chloroform/methanol/acetic acid/water (50:39:1:10). Phospholipid phosphate was assayed by the method of Bartlett (1959).

Preparation of Liposomes

CL and PG unilamellar vesicles were prepared in S/H buffer by reverse-phase evaporation (Szoka & Papahadjopoulus, 1978). Liposomes were sized by extrusion using polycarbonate Unipore membranes (0.1 μM pore size, Millipore).

Flow Cytometry Analysis

The energized state of mitochondria was monitored by flow cytometry, measuring the fluorescence of the membrane potential-sensitive probe $DiOC_6(3)$. The mean fluorescence of $DiOC_6(3)$-stained mitochondria correlates with $\Delta\psi_m$. The merits as well as limitations of the use of this probe in $\Delta\psi_m$ measurement have been reviewed by Nicholls and Budd (2000). Mitochondria (0.3 mg of protein) were incubated in different respiratory conditions and then loaded with 0.03 μM $DiOC_6(3)$ for 5 min at room temperature. Suspensions were analyzed immediately by flow cytometry analysis using a FACScan flow cytometer (Beckman Coulter Epics XL-MCL) equipped with a focused argon laser. For complete depletion of $\Delta\psi_m$ (positive control), the mitochondrial uncoupler CCCP (50 μM) was used. Data were analyzed and stored in a data management system (LYSYS software). $DiOC_6(3)$ green fluorescence (FL1) was plotted on a logarithmic scale *vs*. the frequency of events. The mean value of the integral of fluorescence (IGFL) was also evaluated (Monni et al., 2000). In other experiments, mitochondria were loaded with $DiOC_6(3)$ as described, in the presence of respiratory substrates. CL liposomes (10 nmol lipid) were then added and fusion was allowed for 5 min at room temperature in the presence of 5 mM potassium phosphate (Piccotti et al., 2002). Flow cytometry analysis was performed as described. Forward (FS) and side (SS) light scatter were recorded as indices of particle size and granularity, respectively.

Release of Cytochrome c From Isolated Mitochondria

First, mitochondria (0.3 mg of protein) incubated in different respiratory conditions were centrifuged for 10 min at 9,000 × *g*. The supernatant (S) was recovered, whereas the mitochondrial pellet was resuspended in S/H buffer and treated with digitonin as described earlier to permeabilize the outer mitochondrial membrane. After centrifugation for 10 min at 9,000 × *g* the pellet (M) and post-digitonin supernatant (I) were recovered. Western blot analysis of cytochrome c was performed in M, S, and I fractions. Next, control or pronase-treated mitochondria (0.3 mg of protein) were incubated for 10 min at 20°C with or without 20 mM potassium phosphate (pH 7.4). The mitochondrial pellet and the extramitochondrial medium were recovered by centrifugation for 10 min at 9,000 × *g* and subjected to Western blot analysis using anti-(cytochrome c) IgG.

Reconstitution and Topology of Cytochrome c in Liposomes

Reconstitution of cytochrome c in CL liposomes was performed by mixing liposomes (10 nmol lipid in 200 μl of S/H buffer) with cytochrome c (1 nmol). The solutions were incubated for 10 min at room temperature and unbound cytochrome c filtered through Microcon YM-100 (Amicon) by centrifuging at 10,000 × *g* for 10 min. Filtered and retained material was recovered and analyzed by immunoblotting for the detection of cytochrome c. In some experiments liposomes were prepared in the presence of cytochrome c (10 nmol CL and 1 nmol cytochrome c). To study the localization of cytochrome c in the lipid bilayer, reconstituted liposomes were treated with pronase (0.75 μg, 5 min at room temperature). After the addition of a protease inhibitor cocktail, liposomes were filtered and cytochrome c was analyzed as described above.

Release of Cytochrome c from Liposomes

Cytochrome c-CL liposomes obtained as described were incubated 10 min at room temperature in the presence of increasing potassium phosphate concentrations.

Samples were then filtered through Microcon YM-100 by centrifuging at 10,000 × *g* for 10 min and cytochrome c in the filtrate was detected by Western blotting. Individual band densities of immunoblots were integrated by using Quantiscan software (Biosoft, Cambridge, U.K.).

Fatty Acid Analysis of CLs and PGs

Purified CLs and PGs were transesterified using HCl/methanol (1:20, v/v). Fatty acid methyl esters were separated by gas-liquid chromatography on a glass column (0.2 × 240 cm) packed with GP 10% SP-2330 on 100/120 Chromosorb W AWW (Supelco). Separation of fatty acid methyl esters of 18 carbon atoms or less was achieved by isothermal chromatography at 200°C for 10 min. The temperature was then increased to 230°C at a rate of 10°C/min to separate fatty acid methyl esters longer then 18 carbon atoms. All the analyses were performed on HRGC 5300 (Carlo Erba Instruments) gas chromatograph. Eluted peaks were integrated with a Borwin chromatography software program. Identification of each methyl ester was based on chromatography with authentic standards.

Enrichment of Mitochondria with Exogenous Cytochrome c

Control or pronase-treated mitochondria (0.3 mg of protein) were incubated with cytochrome c (1 nmol) for 10 min at room temperature in different respiratory conditions. Residual external cytochrome c was removed by incubation for 5 min with pronase (2.5 μg) followed by treatment with protease inhibitors. Mitochondria were then recovered by centrifuging at 9,000 × *g* for 10 min and cytochrome c was evaluated by Western blot. In other experiments, respiratory substrates, 5 mM potassium phosphate, and cytochrome c (1 nmol), were added to mitochondria, preincubated or not at 37°C for different times in de-energized state. Cytochrome c was omitted in controls. Mitochondria were then loaded with $DiOC_6(3)$ and $\Delta\psi_m$, FS, and SS were determined.

Abbreviations

S/H, sucrose/HEPES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CL, cardiolipin; PG, phosphatidylglycerol; $\Delta\psi_m$, mitochondrial membrane potential; IGFL, integral of fluorescence; FS, forward light scatter; SS, side light scatter; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TOM, trans-locase of the outer membrane.

Results

Characterization of Purified Mitochondria

Biochemical parameters of mitochondrial preparations were previously reported (Monni et al., 2000). Respiratory properties of mitochondria were studied

in the presence of pyruvate and malate. Mitochondria were metabolically active with a respiratory control ratio (ratio of state 3 to state 4) in the range 5–6. This value is in agreement with data reported in the literature (Lai, Sheu & Carlson, 1985). After digitonin treatment cytochrome c oxidase was quantitatively recovered. CL, which is only present in the inner mitochondrial membrane, was not detectable in the post-digitonin supernatant. All these findings suggest that in our experimental conditions digitonin acts on the outer mitochondrial membrane, without damaging the inner membrane.

Influence of Mitochondrial Respiratory Activity on $\Delta\psi_m$ and on the Release of Cytochrome c from Mitochondria

We evaluated the possible correlation between the respiratory activity of mitochondria and the release of cytochrome c in the extramitochondrial medium. The $\Delta\psi_m$ of mitochondria incubated in different respiratory conditions was evaluated after loading with the membrane potential-sensitive probe $DiOC_6(3)$. IGFL values (arbitrary units) were calculated from single-parameter frequency histograms after flow cytometry analysis. In parallel experiments, mitochondrial suspensions were analyzed by Western blot for cytochrome c bound to the inner membrane, released outside mitochondria, and in the post-digitonin supernatant. In the de-energized state, an IGFL value of 67 was measured at zero time and cytochrome c was retained in mitochondria (Fig. 1). IGFL decreased to 51 after 60 min incubation at 37°C. When mitochondrial potential was sustained by the addition of respiratory substrates and 5 mM phosphate, an initial IGFL value of 114 was measured that decreased to 60 after 60 min incubation at 37°C. Release of cytochrome c beyond the outer mitochondrial membrane in the supernatant was observed in both experimental conditions. In every case, soluble cytochrome c was found in the post-digitonin supernatant (Fig. 1). Pretreatment of mitochondria with ionophore nigericin produced an increase of $\Delta\psi_m$ (IGFL = 150) without detachment of cytochrome c. Complete depletion of $\Delta\psi_m$ in the presence of the mitochondrial uncoupler CCCP resulted in release of cytochrome c, most of which was found in the post-digitonin supernatant (Fig. 1).

Phosphate is an important factor in respiratory activity. In a previous paper we described the displacement of cytochrome c from mitochondria triggered by phosphate concentrations higher than 10 mM (Piccotti et al., 2002). To investigate the mechanism of phosphate-dependent cytochrome c release, control and pronase-treated mitochondria were incubated with 20 mM potassium phosphate for 10 min at room temperature in the presence of respiratory substrates. The phosphate-dependent release of cytochrome c monitored by Western blot analysis of mitochondrial pellet and extramitochondrial medium is shown in Fig. 2. The entrance of 20 mM phosphate inside the mitochondrion caused cytochrome c release, interfering with hydrophilic interactions existing between cytochrome c and CL. The pre-treatment of mitochondria with pronase prevents the phosphate-dependent release of cytochrome c, possibly by degrading the phosphate transporter, thus hindering the entrance of the anion. This result suggests that treatment of mitochondria with 20 mM phosphate occurs without disrupting the outer mitochondrial membrane.

Interaction of Cytochrome c with Liposomes

The mode of membrane association of cytochrome c was studied in a model system of cytochrome c reconstituted in CL liposomes. Heart CL liposomes (10 nmol lipid) were mixed with cytochrome c (1 nmol) in S/H buffer and incubated at room temperature for 10 min. In these experimental conditions the binding of protein to CL was complete, since no residual cytochrome c was detected in the filtrate after centrifugation of liposomes through Microcon YM-100. To evaluate the localization of cytochrome c in liposomes, reconstituted vesicles were treated with pronase, and cytochrome c in the recovered liposomes was evaluated by Western blotting. Fig. 3 shows that no cytochrome c was present in pronase-treated liposomes, indicating that protein localizes on the external surface of liposomes. In the experiments performed by preparing CL liposomes in the presence of cytochrome c, Western blot analysis of pronase-treated vesicles showed that about 30% of cytochrome c was still present in liposomes (Fig. 3). This indicates that part of cytochrome c was entrapped inside liposomes and could not become a substrate for pronase, demonstrating that pronase acts only on the surface of liposomes.

Displacement of cytochrome c by phosphate was evaluated in CL liposomes. Cytochrome c-reconstituted liposomes were incubated for 10 min at room temperature in the presence of increasing phosphate concentrations. Samples were centrifuged for 10 min at $10{,}000 \times g$ through Microcon YM-100 and released cytochrome c was evaluated in the filtrate by Western blot analysis. Cytochrome c release was observed for phosphate concentrations ≥10 mM (Fig. 4*A*), as previously observed also in mitochondria (Piccotti et al., 2002). The amount of released cytochrome c did not follow a linear relationship with phosphate concentration. For phosphate concentrations higher than 100 mM the release of cytochrome c reached a plateau (Fig. 4*B*) corresponding to about 44% of total cytochrome c in liposomes.

The ability of phosphate to displace cytochrome c from liposomes made with different CLs was tested.

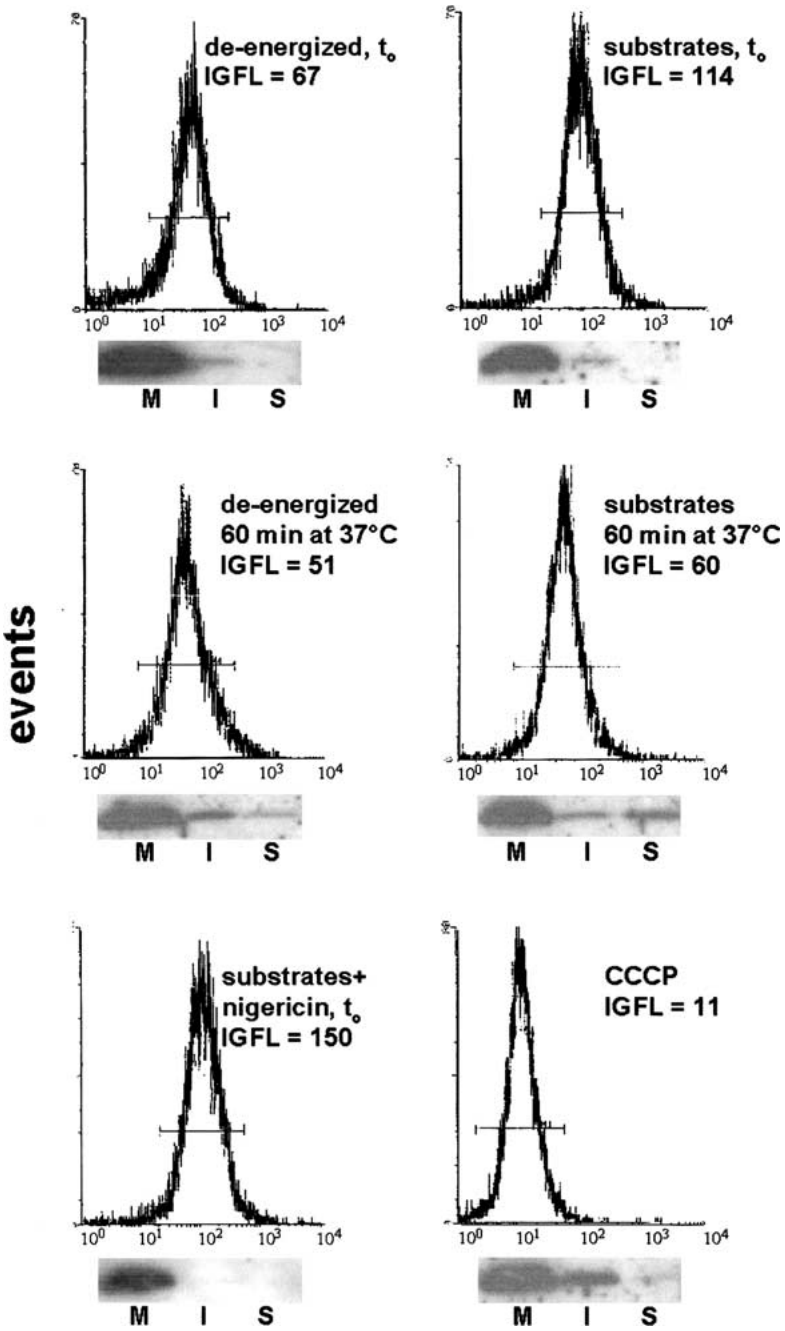


Fig. 1. Influence of mitochondrial respiratory activity on $\Delta\psi_m$ and on the release of cytochrome c. Mitochondria (0.3 mg of protein) incubated in different respiratory conditions were loaded with $DiOC_6(3)$ (0.03 μM) before flow cytometry analysis. In parallel experiments, mitochondria incubated as described above were centrifuged at 9,000 × *g* for Western blot analysis of cytochrome c in the supernatant (*S*). The mitochondrial pellet was then resuspended and treated with digitonin (0.4 mg/mg of mitochondrial protein, 10 min at 0°C). After centrifugation (9,000 × *g* for 10 min), cytochrome c in pellet (*M*) and in supernatant was analyzed. Cytochrome c in the supernatant was taken as the protein present in the intermembrane space (*I*). A representative experiment of three is shown.

Binding of cytochrome c to CL from heart mitochondria is stronger than that to CL from brain mitochondria (Fig. 5). Moreover, CL prepared from brain mitochondria previously incubated for 60 min at 37°C favors the release of cytochrome c, compared to CL prepared from non-incubated mitochondria. To better understand these results, we analyzed the fatty-acid composition of the different CLs. Heart mitochondria CL contains about 14% saturated fatty acids (SFA), 6% monounsaturated fatty acids (MUFA), and 80% 18:2n-6. In contrast, CL from brain mitochondria contains about 17% SFA, 34% MUFA, and 48% polyunsaturated fatty acids (PUFA), about 20% of which is 18:2n-6, 22% 20:4n-6, and 6% 22:6n-3. CL from brain mitochondria underwent modifications following incubation of mitochondria for 60 min at 37°C. SFA increased to 24% and PUFA decreased to 41%. PUFA decrease was mainly due to 18:2-n6.

PG is the precursor for CL synthesis through CL synthase (Ostrander et al., 2001a). We purified PG from brain and heart mitochondria and determined the fatty-acid composition. In brain PG, 65% was SFA, 17% MUFA, and 18% PUFA (only 2% 18:2-n6), whereas in heart PG, 53% was SFA, 31% MUFA, and 16% PUFA (14% 18:2-n6). The extent of

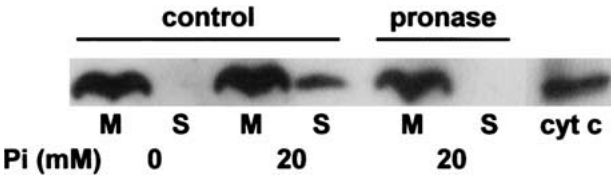


Fig. 2. Effect of pronase treatment of mitochondria on phosphate-dependent release of cytochrome c (*cyt c*). Aliquots of control and pronase-treated mitochondria (0.3 mg of protein) were incubated for 10 min at 20°C with or without 20 mM potassium phosphate and centrifuged for 10 min at 9,000 × *g*. Western blot analysis of cytochrome c was performed in the mitochondrial pellet (*M*) and in the extramitochondrial medium (*S*). A representative experiment of three is shown.

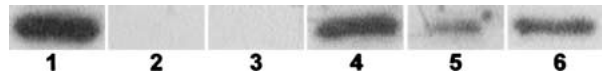


Fig. 3. Reconstitution and topology of cytochrome c in CL liposomes. *Blots 1–3:* Pre-formed CL liposomes (10 nmol lipid) were incubated with cytochrome c (1 nmol) for 10 min at room temperature and then filtered through Microcon YM-100. In blot *3*, reconstituted liposomes were treated with pronase (*see* Methods) before filtration. Cytochrome c was detected by immunoblotting in the retained portion and in the filtrate. Blot *1*, retained portion in control; blot *2*, filtrate in control; blot *3*, retained portion in pronase-treated; *blots 4 and 5*, CL (10 nmol lipid) was mixed with cytochrome c (1 nmol) and liposomes were prepared as described. Cytochrome c retained in control (blot *4*) and in pronase-treated (blot *5*) was detected by immunoblotting. *Blot 6:* standard cytochrome c.

phosphate-dependent release of cytochrome c from PG liposomes was higher with brain than with heart PG (*not shown*). These results suggest that the phosphate-dependent displacement of cytochrome c from CL and PG liposomes may be influenced by phospholipid fatty-acid composition that can modulate the extent of hydrophobic interactions between cytochrome c and phospholipids.

Enrichment of Mitochondria with CL: Effect on $\Delta\psi_m$

Fusion of CL liposomes to mitochondria was carried out as previously described (Piccotti et al., 2002). The fusion kinetics in the presence of phosphate was identical with both heart and brain CL, indicating that mitochondria were enriched to the same extent with exogenous CLs. The $\Delta\psi_m$ of CL-fused mitochondria was evaluated by cytofluorimetry using $DiOC_6(3)$. Fusion with heart CL noticeably increased $\Delta\psi_m$ compared to the basal value measured in control mitochondria (Fig. 6). $\Delta\psi_m$ increase was lower upon enrichment with brain CL, suggesting that the fatty-acid composition of CL may influence $\Delta\psi_m$. Treatment of mitochondria with CCCP produced a decrease of $\Delta\psi_m$, whereas FS did not increase in fused mitochondria compared to control, indicating the absence of mitochondrial swelling (*not shown*).

Incubation of Mitochondria with Exogenous Cytochrome c

Uptake of exogenous cytochrome c by mitochondria was investigated to evaluate the permeability of the outer mitochondrial membrane to this protein and the effect on mitochondrial respiratory activity. Control or pronase-treated mitochondria (0.3 mg of protein) were incubated with cytochrome c (1 nmol) for 10 min at room temperature. Non-incorporated cytochrome c was proteolyzed by treatment with pronase and mitochondria were recovered by centrifugation. The addition of cytochrome c to a sus-

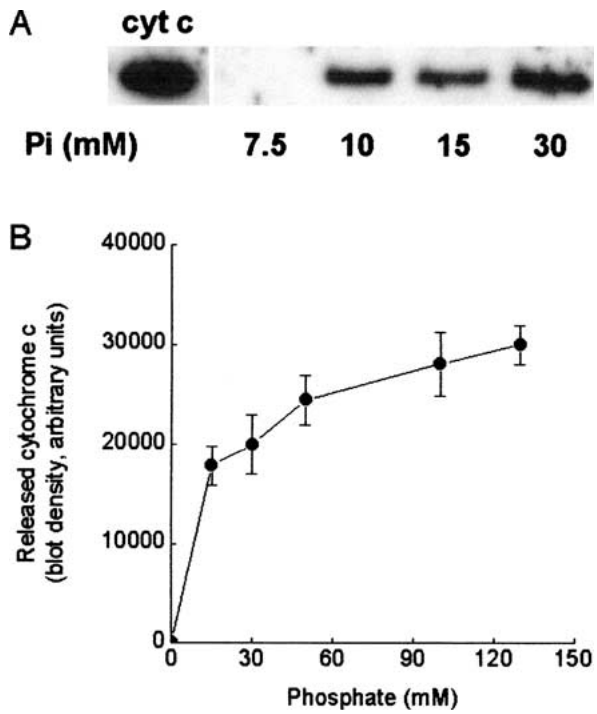


Fig. 4. Phosphate-dependent release of cytochrome c from CL liposomes. Cytochrome c-reconstituted liposomes (10 nmol CL, 1 nmol cytochrome c) were incubated for 10 min at room temperature in the presence of increasing phosphate concentrations and then filtered through Microcon YM-100. Western blot of cytochrome c was performed on the filtrate. (*A*) Phosphate concentration threshold. A representative experiment of three is shown. (*B*) Released cytochrome c is expressed as arbitrary units calculated by immunoblot densities. Values are the mean ± SD of four independent experiments.

pension of mitochondria resulted in incorporation of the protein inside mitochondria (Fig. 7). Neither the increase of $\Delta\psi_m$ in the presence of respiratory substrates nor the collapse of $\Delta\psi_m$ after the addition of CCCP appeared to influence entrance of the protein. Moreover, import of cytochrome c did not require

Fig. 5. Phosphate-dependent cytochrome c release from CL liposomes: influence of CLs. Cytochrome c-reconstituted liposomes (10 nmol lipid, 1 nmol cytochrome c) made with CL purified from heart mitochondria (blot *2*), or from brain mitochondria (blot *3*), or from brain mitochondria incubated 60 min at 37°C in de-energized state (blot *4*) were incubated for 10 min at room temperature in the presence of 50 mM potassium phosphate and then filtered through Microcon YM-100. Western blot analysis was performed in the filtrate. Blot *1* is standard cytochrome c. A representative experiment of three is shown.

pronase-sensitive components of the outer mitochondrial membrane. Digitonin permeabilization of cytochrome c-enriched mitochondria did not result in release outside of the imported protein (*not shown*).

Membrane Potential of Cytochrome c-enriched Mitochondria and Light-Scattering Properties

The $\Delta\psi_m$ was determined by cytofluorimetry of $DiOC_6(3)$ mitochondria after the addition of exogenous cytochrome c. IGFL values (arbitrary units) of 233 and 155 were measured at zero time for control and cytochrome c-enriched mitochondria, respectively. IGFL values decreased to 163 and 67 after incubation for 60 min at 37°C in the presence of respiratory substrates and 5 mM phosphate. These data indicate that acquired cytochrome c determines a consumption of $\Delta\psi_m$ that is more pronounced during the respiratory activity of mitochondria.

FS and SS of mitochondria were determined. FS reflects the size of mitochondria, whereas SS (light collected at 90°) depends on intensity and diffraction capacity of the mitochondrion, providing information on its structure (complexity/granularity). Mitochondrial size, determined by FS of individual particles, did not change following incubation in the presence of respiratory substrates or after the capture of cytochrome c (Fig. 8), indicating the lack of swelling. In contrast, compared to control, SS increased in mitochondria that incorporated cytochrome c, indicating a higher complexity in their inner structure. The distribution of mitochondrial complexity in the function of $DiOC_6(3)$-fluorescence accumulation was also evaluated. In the presence of respiratory substrates, about 40% mitochondria belong to a high-potential population. In the same conditions, the incorporation of cytochrome c inside mitochondria determines a reduction of the high-potential population to 15%, accompanied by a shift towards a low-potential population characterized by higher complexity (Fig. 8).

Discussion

Detachment of cytochrome c from the inner membrane of brain mitochondria was observed during metabolic stress. A model system of cytochrome c reconstituted in CL liposomes was investigated to evaluate the importance of hydrophobic interactions in the anchorage of cytochrome c to the inner mitochondrial membrane. We found that changes of CL fatty-acid composition occurring during the metabolic activity of mitochondria were responsible for the weakening of the hydrophobic interactions in the binding of the protein, resulting in cytochrome c release.

Decrement of $\Delta\psi_m$ was measured in mitochondria subjected to metabolic stress, as a result of catabolic and oxidative processes. These events were accompanied by cytochrome c release. Some of the cytochrome c, very likely representing a soluble pool restricted to the intermembrane space of mitochondria, was found in the post-digitonin supernatant. The increase of this pool during incubation of mitochondria was accompanied by extrusion of the protein outside mitochondria (Fig. 1). This finding is in accordance with the proposed two-step model for cytochrome c release, consisting in detachment of the protein from its membrane-anchoring lipid into a soluble pool, followed by permeabilization of the outer mitochondrial membrane (Ott et al., 2002). The size of the soluble cytochrome c pool may be regulated through $\Delta\psi_m$. In fact, when $\Delta\psi_m$ increased in the presence of nigericin, soluble cytochrome c moved to the inner membrane. In contrast, collapse of $\Delta\psi_m$ caused a shifting of the equilibrium towards the soluble form that was maintained in the intermembrane space (Fig. 1).

Exposure of mitochondria to 20 mM phosphate resulted in the release of cytochrome c outside mitochondria (Fig. 2). The system of cytochrome c reconstituted in CL liposomes is a useful model to investigate the effect of phosphate on the hydrophilic component of cytochrome c-CL interactions. As for intact mitochondria, phosphate-dependent cytochrome c release from reconstituted liposomes required at least 10 mM phosphate (Fig. 4*A*), indicating this value as the threshold phosphate concentration able to discharge the loosely bound cytochrome c pool. Tightly bound protein was released by increasing phosphate concentration, reaching about 44% of total cytochrome c at 100 mM (Fig. 4*B*). The remaining cytochrome c should account for the tightly bound conformation characterized by hydrophobic interactions. The relevance of the hydrophobic interaction component in cytochrome c–CL association was further demonstrated by comparing the phosphate-dependent cytochrome c detachment from liposomes made with CL extracted from heart or brain mitochondria, highly different for linoleic

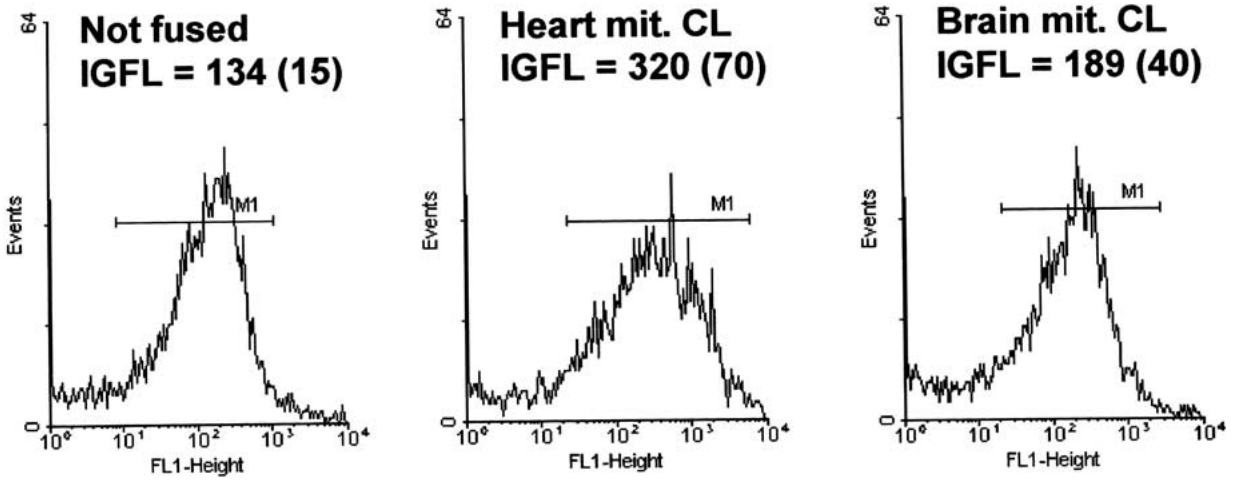
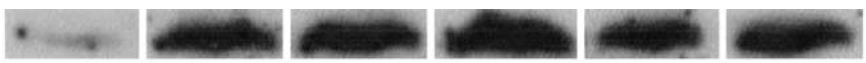


Fig. 6. Flow cytometry analysis of $\Delta\psi_m$ in $DiOC_6(3)$-stained mitochondria: effect of fused CLs. Mitochondria (0.3 mg of protein) were loaded with $DiOC_6(3)$ (0.03 μM) and incubated for 5 min at room temperature in the presence of respiratory substrates plus 5 mM potassium phosphate. Liposomes (10 nmol lipid) made with CL purified from heart or from brain mitochondria were added and fusion was allowed for 5 min at room temperature before flow cytometry analysis. Numbers in parentheses indicate the IGFL value in the presence of CCCP. A representative experiment of three is shown.



Cytochrome c	-	+	+	+	+	+
Phosphate	-	-	+	+	-	+
Respiratory substrates	-	-	-	+	-	+
CCCP	-	-	-	-	+	-
Pronase treatment	-	-	-	-	-	+

Fig. 7. Enrichment of mitochondria with exogenous cytochrome c. Control or pronase-treated mitochondria (0.3 mg of protein) were incubated with cytochrome c (1 nmol) for 10 min at room temperature, in different respiratory conditions. Residual external cytochrome c was removed by incubation for 5 min with pronase (2.5 μg), followed by treatment with protease inhibitors. Mitochondria were recovered by centrifugation (9,000 × *g*, 10 min) and cytochrome c was evaluated by immunoblot. A representative experiment of three is shown.

acid content. In cytochrome c–lipid interactions, the adoption of the extended conformation (Rytömaa & Kinnunen, 1995) requires the protrusion of the *sn*-2 acyl chain, in which cis double bonds allow for greater conformational flexibility (Kinnunen et al., 1994; Tuominen et al., 2002). Therefore, the stronger cytochrome c–heart CL hydrophobic interaction is imputable to 18:2n-6, which should represent the fitting acyl chain in this type of interaction. Consequently, the weaker hydrophobic interaction of cytochrome c with CL purified from brain mitochondria can be explained by the presence of more saturated acyl chains and less linoleic acid (Fig. 5).

The role of CL in mitochondrial function is a subject of increasing interest. The lack of mitochondrial anionic phospholipids causes an inhibition of translation of protein components of the electron transport chain (Ostrander et al., 2001b). CL is also required for supercomplex formation in the inner mitochondrial membrane (Zhang, Mileykovskaya & Dowhan, 2002). Lack of CL in yeast results in decreased $\Delta\psi_m$ and reduced mitochondrial function (Jiang et al., 2000). CL prevents the rate-dependent uncoupling and provides osmotic stability of mitochondria (Koshkin & Greenberg, 2002). In our studies, $DiOC_6(3)$-accumulated fluorescence and the size of mitochondria were measured after supplementing with exogenous CL. For cationic dyes, fluorescence accumulation is dependent not only on $\Delta\psi_m$ increase but also on the increase of mitochondrial volume (Vander Heiden et al., 1997). We found that forward angle light scattering, the direct measure of particle size (Allman et al., 1990), was not affected by the fused lipid, indicating that the observed fluorescence increase was not due to mitochondrial swelling but only to an increase of $\Delta\psi_m$ (Fig. 6). In confirmation of this, the uncoupler CCCP, which dissipates the H^+ ion generated by the electron transport chain,

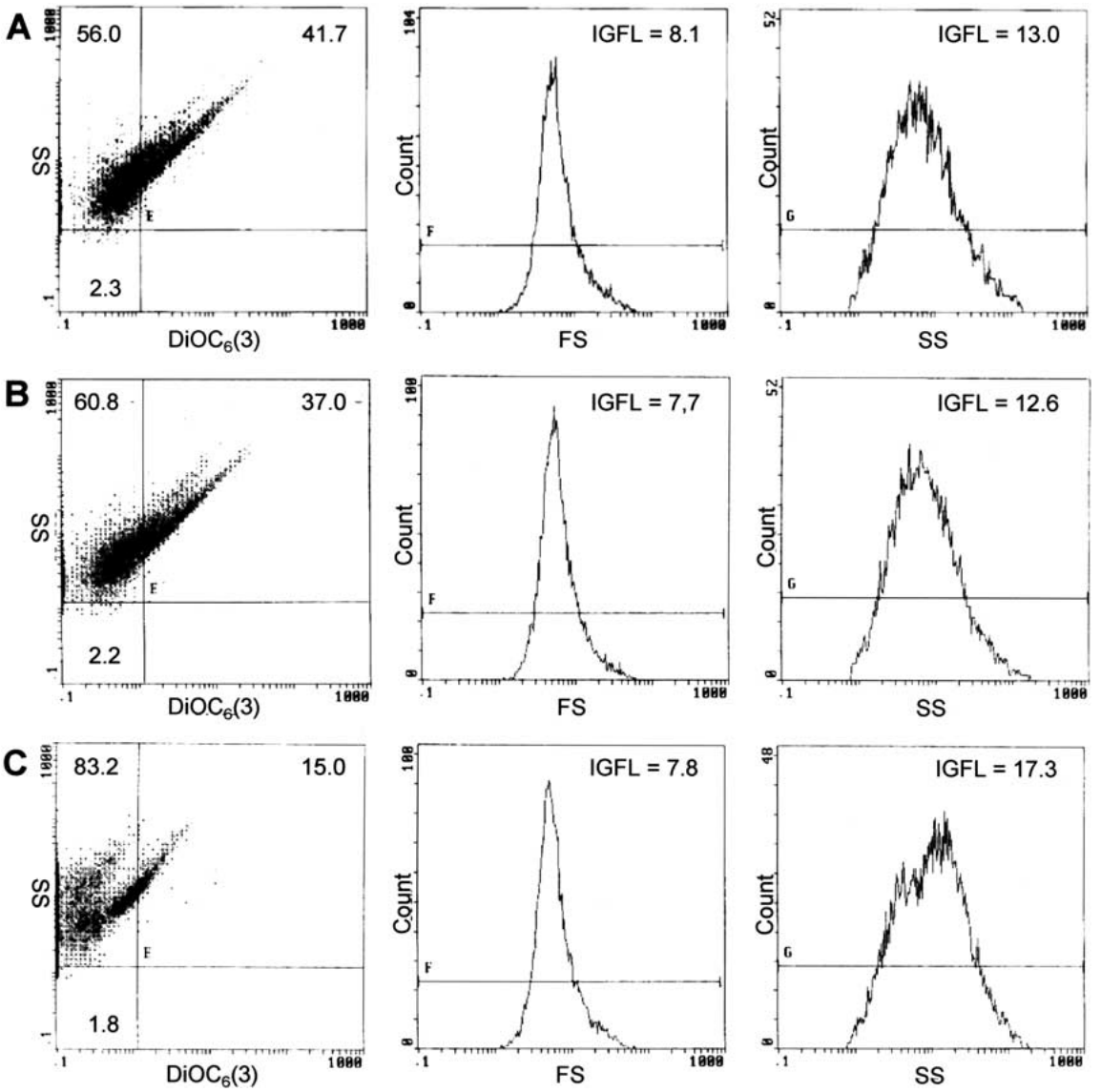


Fig. 8. Light-scattering properties of cytochrome c-enriched mitochondria: *FS*, size and *SS*, granularity. Mitochondria (0.3 mg of protein) not pre-incubated (*A*) or pre-incubated 15 min at 37°C in de-energized state (*B* and *C*) were added with respiratory substrates and 5 mM potassium phosphate in the absence (*A* and *B*) or in the presence (*C*) of cytochrome c (1 nmol). Samples were then loaded with $DiOC_6(3)$ (0.03 μM) and incubated for 5 min at room temperature before light-scattering analysis. A representative experiment of three is shown.

produced a significant decrease of $DiOC_6(3)$ fluorescence. The high increase of $\Delta\psi_m$ produced by heart CL, compared to brain CL, can be specifically ascribed to the high 18:2n-6 content. In the metabolic pathway of CL synthesis, the precursor PG contains low levels of unsaturated acyl chains. On the other hand, CL synthase is highly specific for unsaturated PG substrates (Ostrander et al., 2001a). In heart mitochondria, the existence of distinct PG pools was observed (Hatch, Cao & Angel, 1995) but only the newly synthesized pool is used for CL synthesis (Hatch, 1996). In addition, newly-formed CL may be remodeled by a deacylation-reacylation pathway (Hatch, 1998; Schlame & Rustow, 1990). If an 18:2n-6 pool is available, it is reasonable to argue that the remodeling pathway is operating also in the brain. In this regard, it has been shown that, despite the high baseline levels of 18:2n-6 in heart mitochondrial CL, compared to brain, CL 18:2n-6 levels increased also in the brain in proportion to dietary 18:2n-6 supply (McGee, Lieberman & Greenwood, 1996).

Exogenous cytochrome c crosses the outer mitochondrial membrane without requiring protease-sensitive components or membrane potential (Fig. 7). This finding is in agreement with mitochondrial apocytochrome c importation, which requires the protease-resistant component (TOM40) of the translocase of the outer membrane (TOM) complex (Mayer,

Neupert & Lill, 1995; Diekert et al., 2001). TOM machinery is also involved in cytochrome c importation through TOM22 in yeast mitochondria (Wiedemann et al., 2003). Exogenously added cytochrome c was retained in mitochondria even after permeabilization of the outer mitochondrial membrane with digitonin. It has been demonstrated that the association of cytochrome c to the membrane is a cooperative process (Nantes et al., 2001). This may justify our finding that the inner membrane is able to bind large amounts of exogenous cytochrome c. We found that exogenous cytochrome c favored the drop of $\Delta\psi_m$ in response to respiratory substrates and ADP, as previously reported for liver mitochondria (Mootha et al., 2001). In liver mitochondria, a concomitant increase in oxygen consumption was observed, suggesting that added cytochrome c actively participates in the recovery of respiratory control. A further contribution to elucidate the role of exogenous cytochrome c in respiratory control comes from the analysis of FS and SS. First, the incorporation of exogenous cytochrome c was confirmed by the high complexity values evidenced by SS. Second, mitochondrial heterogeneity with respect to $DiOC_6(3)$ fluorescence associated to a single particle showed that $\Delta\psi_m$ decrease could be ascribed to a selected mitochondrial population containing the incorporated cytochrome c (Fig. 8).

Mitochondria defects occur in a wide variety of neurodegenerative disorders and increasing evidence implicates apoptosis-mediated cell death in the pathogenesis of neurodegenerative diseases. Release of cytochrome c is a necessary first step for initiation of apoptosis. A recent report strongly supports a relationship between decreased CL synthesis and cytochrome c release, correlating the concept of altered lipid metabolism and cardiac cell death (Ostrander et al., 2001a). Depletion of CL accompanies ischemia in heart and brain (Nakahara et al., 1991; Lesnefsky et al., 2001). In glutamate-induced neuron death and in staurosporine-treated cultured cerebrocortical neurons a release of cytochrome c was ascertained (Budd et al., 2000; Luetjens et al., 2000). Neuronal cytochrome c immunoreactivity increased in Huntington's disease, with a shift in the distribution of the protein from mitochondria to the cytosolic fraction (Kiechle et al., 2002). Since release of cytochrome c appears to be a common feature in many events leading to cell suffering, studies on mechanisms regulating the interaction between CL and cytochrome c in the inner membrane of brain mitochondria may be relevant.

In this work, evidence was provided that the release of cytochrome c from the inner membrane of brain mitochondria is strictly related to the respiratory activity of mitochondria, the retention of cytochrome c being sustained by high $\Delta\psi_m$. Since hydrophobic interactions with CL 18:2n-6 acyl chains mainly support the binding of cytochrome c to the membrane, saturation of CL fatty acids during metabolic activity of mitochondria was responsible for weakening of protein anchorage to the membrane. While the externalization of the released protein is known to be a regulated process, exogenous cytochrome c was incorporated in mitochondria without the involvement of protease-sensitive factors or requirement of membrane potential. The internalized protein became anchored to the inner membrane and contributed to the recovery of respiratory control.

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References

Allman, R., Hann, A.C., Phillips, A.P., Martin, K.L., Lloyd, D. 1990. Growth of *azotobacter vinelandii* with correlation of Coulter cell size, flow cytometric parameters, and ultrastructure. *Cytometry* **11:**822–831

Andreyev, A., Fiskum, G. 1999. Calcium induced release of mitochondrial cytochrome c by different mechanism selective for brain versus liver. *Cell Death Differ.* **6:**825–832

Bartlett, G.R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:**466–468

Beal, M.F. 2000. Oxidative metabolism. *Ann. N. Y. Acad. Sci.* **924:** 164–169

Brustovetsky, N., Brustovetsky, T., Jemmerson, R., Dubinsky, J.M. 2002. Calcium-induced cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane. *J. Neurochem.* **80:**207–218

Budd, S.L., Tenneti, L., Lishnak, T., Lipton, S.A. 2000. Mitochondrial and extramitochondrial apoptotic signaling pathways in cerebrocortical neurons. *Proc. Natl. Acad. Sci. USA* **97:**6161–6166

Budihardjo, I., Oliver, H., Lutter, M., Luo, X., Wang, X. 1999. Biochemical pathways of caspase activation during apoptosis. *Annu. Rev. Cell. Dev. Biol.* **15:**269–290

Camici, O., Corazzi, L. 1995. Import of phosphatidylethanolamine for the assembly of rat brain mitochondrial membranes. *J. Membrane Biol.* **148:**169–176

Chinopoulos, C., Adam-Vizi, V. 2001. Mitochondria deficient in complex I activity are depolarized by hydrogen peroxide in nerve terminals: relevance to Parkinson's disease. *J. Neurochem.* **76:**302–306

Corazzi, L., Pistolesi, R., Carlini, E., Arienti, G. 1993. Transport of phosphatidylserine from microsomes to the inner mitochondrial membrane in brain tissue. *J. Neurochem.* **60:**50–56

Das, M.L., Hiratsuka, H., Machinist, J.M., Crane, F.L. 1962. The proteolipids of cytochrome c. *Biochim. Biophys. Acta* **60:**433–434

Diekert, K., de Kroon, A.I., Ahting, U., Niggemeyer, B., Neupert, W., de Kruijff, B., Lill, R. 2001. Apocytochrome c requires the TOM complex for translocation across the mitochondrial outer membrane. *EMBO J.* **20:**5626–5635

Folch, J., Lees, M., Sloane Stanley, G.H. 1957. A simplified method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:**497–509

Gogvadze, V., Robertson, J.D., Zhivotovsky, B., Orrenius, S. 2001. Cytochrome c release occurs via Ca^{2+}-dependent and Ca^{2+}-independent mechanisms that are regulated by Bax. *J. Biol. Chem.* **276:**19066–19071

Hartmann, A., Michel, P.P., Troadec, J.D., Mouatt-Prigent, A., Faucheux, B.A., Ruberg, M., Agid, Y., Hirsch, E.C. 2001. Is Bax a mitochondrial mediator in apoptotic death of dopaminergic neurons in Parkinson's disease? J. *Neurochem.* **76:**1785–1793

Hatch, G.M. 1996. Regulation of cardiolipin biosynthesis in the heart. *Mol. Cell. Biochem.* **159:**139–148

Hatch, G.M. 1998. Cardiolipin: biosynthesis, remodeling and trafficking in the heart and mammalian cells. *Int. J. Mol. Med.* **1:**33–41

Hatch, G.M., Cao, S.G., Angel, A. 1995. Decrease in cardiac phosphatidylglycerol in streptozotocin-induced diabetic rats does not affect cardiolipin biosynthesis: evidence for distinct pools of phosphatidylglycerol in the heart. *Biochem. J.* **306:**759–764

Hirsch, E.C., Hunot, S., Faucheux, B., Agid, Y., Mizuno, Y., Mochizuki, H., Tatton, W.G., Tatton, N., Olanow, W.C. 1999. Dopaminergic neurons degenerate by apoptosis in Parkinson's disease. *Mov. Disord.* **14:**383–385

Jiang, F., Ryan, M.T., Schlame, M., Zhao, M., Gu, Z., Klingenberg, M., Pfanner, N., Greenberg, M.L. 2000. Absence of cardiolipin in the *crd1* null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. *J. Biol. Chem.* **275:**22387–22394

Kiechle, T., Dedeoglu, A., Kubilus, J., Kowall, N.W., Beal, M.F., Friedlander, R., Hersch, S.M., Ferrante, R.J. 2002. Cytochrome c and caspase-9 expression in Huntington's disease. *Neuromolecolar Med.* **1:**183–195

Kinnunen, P.K.J., Koiv, A., Lehtonen, J.Y.A., Rytömaa, M., Mustonen, P. 1994. Lipid dynamics and peripheral interactions of proteins with membrane surfaces. *Chem. Phys. Lipids* **73:**181–207

Koshkin, V., Greenberg, M.L. 2002. Cardiolipin prevents rate-dependent uncoupling and provides osmotic stability in yeast mitochondria. *Biochem. J.* **364:**317–322

Lai, J.C.K., Rex Sheu, K.F., Carlson, K.C. Jr. 1985. Difference in some of the metabolic properties of mitochondria isolated from cerebral cortex and olfactory bulb of the rat. *Brain Res.* **343:**52–59

Lesnefsky, E.J., Slabe, T.J., Stoll, M.S.K., Minkler, P.E., Hoppel, C.L. 2001. Myocardial ischemia selectively depletes cardiolipin in rabbit heart subsarcolemmal mitochondria. *Am. J. Physiol.* **280:**H2770–H2778

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., Wang, X. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86:**147–157

Luetjens, C.M., Bui, N.T., Sengpiel, B., Münsterman, G., Poppe, M., Krohn, A.J., Bauerbach, E., Krieglstein, J., Prehn, J.H.M. 2000. Delayed mitochondrial dysfunction in excitotoxic neuron death: cytochrome c release and a secondary increase in superoxide production. *J. Neurosci.* **20:**5715–5723

Manfredi, G., Beal, M.F. 2000. The role of mitochondria in the pathogenesis of neurodegenerative diseases. *Brain Pathol.* **10:**462–472

Mayer, A., Neupert, W., Lill, R. 1995. Translocation of apocytochrome c across the outer membrane of mitochondria. *J. Biol. Chem.* **270:**12390–12397

McGee, C.D., Lieberman, P., Greenwood, C.E. 1996. Dietary fatty acid composition induces comparable changes in cardiolipin fatty acid profile of heart and brain mitochondria. *Lipids* **31:**611–616

Monni, M., Corazzi, L., Migliorati, G., Roberti, R. 2000. Respiratory rate and phosphatidylserine import in brain mitochondria in vitro. *J. Membrane Biol.* **173:**97–105

Mootha, V.K., Wei, M.C., Buttle, K.F., Scorrano, L., Panoutsakopoulou, V., Mannella, C.A., Korsmeyer, S.J. 2001. A reversible component of mitochondrial respiratory dysfunction in apoptosis can be rescued by exogenous cytochrome c. *EMBO J.* **20:**661–671

Nakahara, I., Kikuchi, H., Taki, W., Nishi, S., Kito, M., Yonekawa, Y., Goto, Y., Ogata, N. 1991. Degradation of mitochondrial phospholipids during experimental cerebral ischemia in rats. *J. Neurochem.* **57:**839–844

Nantes, I.L., Zucchi, M.R., Nascimento, O.R., Faljoni-Alario, A. 2001. Effect of heme iron valence state on the conformation of cytochrome c and its association with membrane interfaces. A CD and EPR investigations. *J. Biol. Chem.* **276:**153–158

Nicholls, D.G., Budd, S.L. 2000. Mitochondria and neuronal survival. *Physiol. Rev.* **80:**315–360

Ostrander, D.B., Sparagna, G.C., Amoscato, A.A., McMillin, J.B., Dowhan, W. 2001a. Decreased cardiolipin synthesis corresponds with cytochrome c release in palmitate-induced cardiomyocyte apoptosis. *J. Biol. Chem.* **276:**38061–38067

Ostrander, D.B., Zhang, M., Mileykovskaya, E., Rho, M., Dowhan, W. 2001b. Lack of mitochondrial anionic phospholipids causes an inhibition of translation of protein components of the electron transport chain. A yeast genetic model system for the study of anionic phospholipid function in mitochondria. *J. Biol. Chem.* **276:**25262–25272

Ott, M., Robertson, J.D., Gogvadze, V., Zhivotovsky, B., Orrenius, S. 2002. Cytochrome c release from mitochondria proceeds by a two-step process. *Proc. Natl. Acad. Sci. USA* **99:**1259–1263

Piccotti, L., Marchetti, C., Migliorati, G., Roberti, R., Corazzi, L. 2002. Exogenous phospholipids specifically affect transmembrane potential of brain mitochondria and cytochrome c release. *J. Biol. Chem.* **277:**12075–12081

Rytömaa, M., Kinnunen, P.K.J. 1995. Reversibility of the binding of cytochrome c to liposomes. Implications for lipid-protein interactions. *J. Biol. Chem.* **270:**3197–3202

Schlame, M., Rustow, B. 1990. Lysocardiolipin formation and reacylation in isolated rat liver mitochondria. *Biochem. J.* **272:**589–595

Szoka, F. Jr., Papahadjopoulos, D. 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA* **75:**4194–4198

Tuominen, E.K.J., Wallace, C.J.A., Kinnunen, P.K.J. 2002. Phospholipid–cytochrome c interaction. Evidence for the extended lipid anchorage. *J. Biol. Chem.* **277:**8822–8826

Vander Heiden, M.G., Chandel, N.S., Williamson, E.K., Schumacker, P.T., Thompson, C.B. 1997. Bcl-x_L regulates the membrane potential and volume homeostasis of mitochondria. *Cell* **91:**627–637

Vanderkooi, J., Erecinska, M., Chance, B. 1973. Cytochrome c interaction with membranes. I. Use of a fluorescent chromophore in the study of cytochrome c interaction with artificial and mitochondrial membranes. *Arch. Biochem. Biophys.* **154:**219–229

Wiedemann, N., Kozjak, V., Prinz, T., Ryan, M.T., Meisinger, C., Pfanner, N., Truscott, K.N. 2003. Biogenesis of yeast mitochondrial cytochrome c: a unique relationship to the TOM machinery. *J. Mol. Biol.* **327:**465–474

Zhang, M., Mileykovskaya, E., Dowhan, W. 2002. Gluing the respiratory chain together: cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. *J. Biol Chem.* **277:**43553–43556