Mol Cell Biochem (2010) 341:149–157
DOI 10.1007/s11010-010-0446-1

Cytochrome c redox state influences the binding and release of cytochrome c in model membranes and in brain mitochondria

Lara Macchioni · Teresa Corazzi · Magdalena Davidescu · Ermelinda Francescangeli · Rita Roberti · Lanfranco Corazzi

Received: 25 December 2009 / Accepted: 16 March 2010 / Published online: 30 March 2010

Abstract Cytochrome c (cyt c), a component of the respiratory chain, promotes apoptosis when released into the cytosol. Cyt c anchorage within mitochondria depends on cardiolipin (CL). Detachment and release have been related to CL loss and peroxidation. We report that NaN_3-dependent complex IV inhibition, accompanied by impairment of respiration, resulted in cyt c release. Contrarily, inhibition of respiration upstream cyt c with complex I and III inhibitors was not accompanied by the release of the protein, despite CL decrease and monolyso-CL increase. No CL changes and H_2O_2 formation were observed by inhibiting complex IV. In cyt c–CL liposomes, breaching cyt c–CL hydrophilic interactions produced a higher release of the reduced, compared to the oxidized form, suggesting that the hydrophobic component of cyt c–CL binding is prevalent in the oxidized form. Free or liposome-reconstituted cyt c was able to form fatty acid–protein complexes (palmitate < linoleate < oleate) only in its reduced form. We hypothesize that reduced cyt c–fatty acid binding favors the dislocation of the protein from anchoring CL. A mechanism for cyt c release independent of CL peroxidation by H_2O_2 is feasible. It could weaken the hydrophobic component of cyt c–CL interactions and might function following complex IV inhibition or in oxygen lack, both conditions producing accumulation of reduced cyt c and free fatty acids.

Keywords Cytochrome c · Brain mitochondria · Liposomes · Cardiolipin · Monolyso-cardiolipin · Fatty acids

L. Macchioni · T. Corazzi · M. Davidescu · E. Francescangeli · R. Roberti · L. Corazzi (✉)
Department of Internal Medicine, Laboratory of Biochemistry, University of Perugia, via del Giochetto, 06122 Perugia, Italy
e-mail: corazzi@unipg.it

Abbreviations

Cyt c	Cytochrome c
S/H buffer	10 mM HEPES (pH 7.4)
4 mM KCl	0.1 mM EDTA and 0.30 M sucrose
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
$\Delta\psi_m$	Mitochondrial membrane potential
CL	Cardiolipin
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PG	Phosphatidylglycerol

Introduction

Cytochrome c (cyt c) is a single-chain hemoprotein of 104 amino acids known for its function as a key component of electron carriers in mitochondria. In addition, when a cell receives an apoptotic stimulus cyt c is detached and released into the cytosol. In this compartment the protein is instrumental in the early stages of apoptosis [1]. It has been proposed that cyt c release requires a two-step process consisting in the detachment of the protein from the membrane-anchoring lipid, followed by permeabilization of the outer mitochondrial membrane. The first step regulates the concentration of free cyt c in the intermembrane space of mitochondria. In turn, the concentration of free cyt c regulates the second step, i.e., the transfer rate of the protein outside the inner mitochondrial membrane, by stimulating MPT or triggering Bcl-2/Bax-mediated mechanism [2].

Cyt c binds to membranes containing acidic phospholipids, particularly cardiolipin (CL) either as such or complexed with cyt c oxidase [3–5]. Two different types of

interactions have been characterized for binding of cyt c to the membrane through distinct sites in the protein, the A-site for electrostatic and 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 cyt c, whereas the other chains remain in the lipid bilayer [5, 6]. Studies of the interaction of reduced cyt c with long-chain fatty acids indicated that acyl chain penetration produced perturbation of cyt c interior, with loosening of its tertiary structure, alteration in the heme environment, and loss of the Met80–Fe coordination [7]. It has been also reported that the association of cyt c with different liposomes is sensitive to the heme iron valence state [8]. Therefore, the different extent of binding of the two cyt c redox states with CL and other anionic phospholipids should influence the hydrophobic/hydrophilic interaction balance. In the cell, this could modulate the equilibrium between bound and unbound cyt c.

In this study, cyt c release in the intermembrane space of mitochondria and outside the outer membrane has been studied in different respiratory conditions. Our results suggest that conditions favouring reduced cyt c and free fatty acids accumulation in mitochondria cause the dislodging of the protein from the inner membrane and the increase of the unbound cyt c pool. This hypothesis was supported by experiments performed with soluble or liposome-reconstituted cyt c, demonstrating that only the reduced form of cyt c is able to bind free fatty acids. The same form is more prone to be detached from model membranes following the breaching of the hydrophilic component of cyt c–phospholipid interactions.

Materials and methods

Chemicals

Digitonin, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), rotenone, antimycin A, HEPES, and cyt c were from Fluka. ADP (K^+ salt), pyruvic acid, malic acid, succinate, glycerol-3-phosphate, CL, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), Na-palmitate, Na-oleate, Na-linoleate, horse radish peroxidase, and phospholipase A_2 from *Najam mossambica mossambica* were obtained from Sigma Chemicals. Amplex Ultra Red reagent was from Molecular Probes (Invitrogen). Pyruvic acid and malic acid solutions were adjusted to pH 7.0 with KOH. Mouse anti-cyt c monoclonal IgG and goat anti-mouse HRP-conjugated IgG were from Santa Cruz Biotechnology.

Incubation of mitochondria

Highly pure mitochondria were prepared from rat brain cortex (CD, 2 months old, Charles River, Italy) as previously reported [9]. Mitochondria were resuspended in 10 mM HEPES (pH 7.4), 4 mM KCl, 0.1 mM EDTA, and 0.30 M sucrose (S/H buffer), and their functionality was checked by monitoring the sensitivity of $\Delta\psi_m$ to different metabolic conditions [10]. The S/H buffer makes the system sensitive to changes in ionic strength and then suitable to study the hydrophilic interaction component responsible for cyt c binding to the inner membrane of mitochondria.

Mitochondria (usually 0.4 mg protein in 0.4 ml S/H buffer) were incubated in the absence (resting) or in the presence of respiratory substrates (1.5 mM pyruvate/3.0 mM malate, or 5 mM succinate, or 5 mM glycerol-3-phosphate), 5 mM potassium phosphate, and 0.8 mM ADP (state 3). In some experiments mitochondria were incubated in the presence of substrates in deoxygenated S/H buffer (state 5). Inhibitors were used at the following concentrations: up to 3.5 mM NaN_3, 2 μM rotenone, 2 μM antimycin A, 1 μM CCCP. Routinely, mitochondria were incubated at 37°C for 10 min in a closed system (Eppendorf microtubes, 1.5 ml). In some experiments, 30 mM phosphate and/or 100 μM fatty acids (palmitate or oleate) were present during the incubation of mitochondria.

Oxygen was determined using a fiber optic oxygen monitor (Instech, USA) equipped with a probe fitted into a 400 μl capacity thermostatic water-jacketed chamber. An electromagnetic stirrer and a bar flea were used to mix the incubation medium. Oxygen was sensed by fluorescence quenching of an indicator dye trapped in a matrix at the tip of the probe.

To measure cyt c released in the incubation medium, mitochondria were centrifuged at 9,000×*g* for 10 min and supernatant was analyzed by Western blot. For the determination of free cyt c in the intermembrane space, the mitochondrial pellets were resuspended in S/H buffer and the outer mitochondrial membrane was permeabilized by digitonin treatment (0.3 mg/mg mitochondrial protein, 10 min at 0°C) [9]. After centrifugation for 10 min at 9,000×*g* the post-digitonin supernatant was recovered and cyt c analyzed. In the experimental conditions used, digitonin permeabilized the outer mitochondrial membrane without significantly affecting the inside of mitochondria. Indeed, 14–15% of total mitochondrial phospholipids, but no CL, were solubilized and recovered in the post-digitonin supernatant. Respiratory activity was maintained and mitochondria were sensitive to respiratory substrates.

Complex IV activity was determined in mitochondria that had been incubated for 10 min at 37°C in respiratory state 3 from succinate in the absence or in the presence of NaN_3. Mitochondrial pellets were resuspended in S/H

buffer and aliquots (15 μg protein) were incubated in 1 ml of hypotonic buffer containing 80 mM sucrose, 5 mM HEPES (pH 7.4), and 25 μM reduced cyt c. The activity was measured by following the decrease of reduced cyt c absorbance at 550 nm.

Measurement of H_2O_2 formation

Generation of H_2O_2 in mitochondria was measured by a continuous monitoring of resorufin fluorescence (λ_{ex} 550 nm, λ_{em} 585 nm) produced by following H_2O_2-dependent Amplex Red oxidation, under the catalysis of horseradish peroxidase. Mitochondria (0.1 mg/ml) were incubated at 37°C in S/H buffer plus horseradish peroxidase (2.5 U/ml) and Amplex Red reagent (1 μM). Respiratory substrates and inhibitors were added at the concentrations indicated above and incubation was carried out in a Shimadzu RF-5000 spectrofluorometer equipped with temperature control and magnetic stirrer device. Calibration signals were generated with known amounts of H_2O_2 during each experiment. Inhibitors did not interfere with peroxidase activity.

Mitochondrial lipid analysis

Lipids were extracted from purified mitochondria as described [11]. For the efficient recovery of CL, solvent mixtures were added with HCl (5 mM). After phase separation, the chloroform phase was collected and the hydromethanolic phase and the interphase were extracted two additional times with chloroform. The combined extracts were evaporated to dryness and lipids were dissolved in chloroform. Phospholipid classes were separated by two-dimensional TLC (10 × 10 cm, PE SIL G 250 μm, Whatman) with (i) chloroform/methanol/1.6 M ammonia (70:30:5, by volume) and (ii) chloroform/methanol/acetone/acetic acid/water (75:15:30:15:7.5, by volume). Phospholipid spots were made evident by brief exposure to iodine and identified with pure reference standard. For detection of monolyso-CL a standard was prepared by incubating 2 mg CL in 10 mM Tris–HCl (pH 7.4), 0.1 mM $CaCl_2$, and 1 nmol phospholipase A_2. Reaction was carried out for 4 h at 37°C and monolyso-CL recovered and utilized as reference. Lipids were quantified by phosphorus assay after digestion with 70% perchloric acid [11]. Free fatty acids were separated from total lipid extract by monodimensional TLC with *n*-exane/ethyl ether/acetic acid (70:30:1, by volume) and visualized with Cu-acetate reagent [12]. Band densities were quantified as described for Western blotting. Pure oleate standard was run on the same plate as the samples to construct calibration curves. Lipid peroxidation was estimated by the appearance of conjugate dienes, registering absorption spectra between 210 and 310 nm of mitochondrial lipids dissolved in chloroform:methanol (1:1), as described [13].

Preparation of cyt c solutions

Oxidized cyt c was dissolved in a degassed solution containing 10 mM HEPES (pH 7.4), 4 mM KCl, 0.1 mM EDTA, and 0.30 M sucrose (S/H buffer). This solution was carefully divided into two equal portions and one of them added with a semi stoichiometric amount of ascorbic acid to obtain complete cyt c reduction. Oxidized and reduced cyt c were considered to contain the same amount of cyt c and utilized for reconstitution of cyt c in liposomes. The concentration of oxidized and reduced cyt c solutions was evaluated by using the absorbance of γ band at 416 nm (ε_{mM} 129) for the reduced form and the absorbance of γ band at 410 nm (ε_{mM} 106) for the oxidized form.

Preparation of liposomes

Pre-fixed aliquots of PC, PE, PG, and CL, dissolved in chloroform/methanol (2:1, v/v), were combined and the solvent removed under a nitrogen flux. The lipid film was hydrated with degassed S/H buffer to a total lipid concentration of approximately 0.5 μmol lipid/ml. Multilamellar vesicles were prepared resuspending the lipid by shaking in a shaker-incubator. Unilamellar vesicles were formed by sonication with a MSE tip sonicator, to clearing. Metal particles from the sonicator tip and multilamellar liposomes were removed from the preparation by centrifugation. Liposomes were successively sized by extrusion with polycarbonate Unipore membranes (0.1 μm pore size, Millipore). Accurate phospholipid concentration of liposomal preparations was determined by phosphate analysis [11].

In cyt c reconstitution experiments, liposomes (10 nmol lipid in 200 μl of S/H buffer) were mixed with cyt c (oxidized or reduced form, 1 nmol). The mixtures were incubated 10 min at room temperature, then added with 30 mM phosphate and incubated for additional 10 min. Samples were filtered through Microcon YM-100 (Amicon–Millipore, Italy) by centrifuging at 10,000×*g* for 10 min and filtered free cyt c was detected by SDS-PAGE and silver staining. Individual band densities were integrated using Quantity One software (Bio-Rad, Milan, Italy).

Fatty acid–cyt c binding

Reduced or oxidized cyt c (2.7 nmol) in 0.3 ml S/H buffer was treated with fatty acids in 1:6 stoichiometric ratio. Fatty acids were given as ethanolic solution at 54 μM final

concentration, a value below the critical micellar concentration of the individual fatty acids. After equilibration for 10 min at room temperature the mixtures were filtered through Microcon YM-100. Filtered free cyt c and not filtered cyt c–fatty acid complexes were analyzed by SDS-PAGE as described above.

Binding assays of cyt c and spectral change determination

Samples containing 9 μM reduced cyt c were incubated in 1 ml S/H buffer in the presence of 0–100 μM palmitate, or oleate, or linoleate for 30 min (a time for which the spectral change reached a constant value) and the decrease of absorbance at 550 nm due to α band coalescence was measured. To measure the effect caused by the spin state change, absorbance value at 526.5 nm was also measured and subtracted from the 550 value. To assess the influence of extended lipid anchorage of reduced cyt c to phospholipids, increasing amounts of liposomes made of CL, or PG, or PC:PE:CL (1:1:1, mol) were added to cyt c solutions. Fatty acids (100 μM) were added to cyt c–liposome mixtures. The degree of spectral changes, ranging between 0 and 1, was calculated using the following equation: spectral change = $\{A_{(550–526.5)30'} - A_{(550–526.5)0'}\}/A_{(550–526.5)0'}$. Fatty acids (100 μM) were added to 9 μM oxidized cyt c solutions and the absorption spectra were registered in the 600–800 nm range.

Results

Incubation of mitochondria and oxygen consumption

Isolated mitochondria started respiration only after addition of respiratory substrates, ADP, and Pi (state 3), as detected by fluorescence quenching. Figure 1a shows typical fluorescence traces of oxygen concentration in mitochondrial suspensions respiring from different substrates in a closed system. Figure 1b reports oxygen levels at 7 and 15 min incubation time. Initial oxygen content was about 85 nmols/0.4 ml incubation medium, at 37°C. After 15 min incubation O_2 decreased to 60% with malate/pyruvate and to 30% with succinate. Rotenone blocked oxygen consumption from malate/pyruvate but increased that from succinate (Fig. 1b).

Respiration, inhibitors, and cyt c release

Mitochondria were incubated for 10 min at 37°C in respiratory state 3 from succinate, in a closed incubation system. Respiration was permitted in the presence of increasing NaN_3 concentrations and oxygen levels were determined at the end of incubation. Samples were

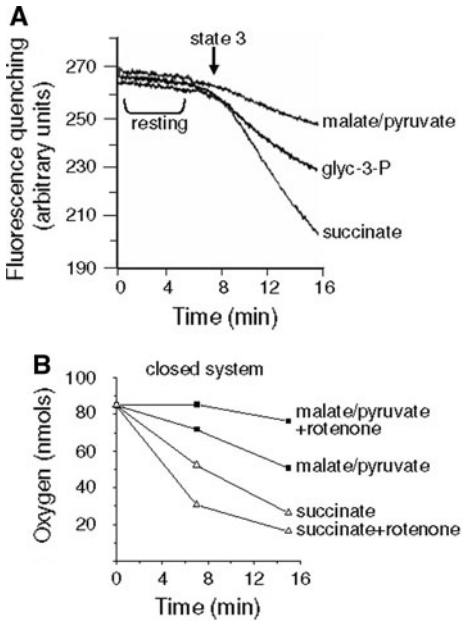

Fig. 1 Oxygen consumption of mitochondrial suspensions. **a** Fluorescence traces of oxygen concentration in mitochondrial suspensions respiring from different substrates in a closed system. **b** Oxygen levels after 7 and 15 min incubation of mitochondria in the presence of respiratory substrates in state 3 in a closed system. Initial oxygen content in the incubation medium was about 85 nmols in 0.4 ml at 37°C. Representative experiments of three are shown

centrifuged and supernatant was analyzed for released cyt c. Mitochondrial pellets were tested for complex IV activity. The effect of NaN_3 on rates of state 3 respiration and on complex IV activity is shown in Fig. 2. NaN_3 caused a decrease of complex IV activity, which reached a value of about 30% of control at 3.4 mM NaN_3. The rate of state 3 respiration decreased to about 60% at 30% complex IV inhibition (about 0.5 mM NaN_3) and remained fairly constant even at the highest inhibition (Fig. 2, insert a). At the same time, cyt c release reached a plateau at about 0.4 mM NaN_3 (Fig. 2, insert b). When mitochondria were incubated from malate/pyruvate in state 3, the amount of released cyt c (3.8 ± 0.1% in the control) increased to 6.2 ± 0.5% in the presence of NaN_3 and decreased to 0.7 ± 0.1% and 0.6 ± 0.1% after inhibition with rotenone and antimycin, respectively. Incubation of mitochondria in a buffer deprived of oxygen (state 5) or when respiratory chain was blocked with antimycin, NaN_3 did not cause cyt c release (not shown), indicating that NaN_3 itself does not influence cyt c binding.

Cyt c release: effect of fatty acids and phosphate

Fatty acids (100 μM), or phosphate (30 mM), or both were added to mitochondrial suspensions respiring in state 3 from malate/pyruvate and incubated for 10 min at 37°C. Oxygen consumption and cyt c released in the incubation

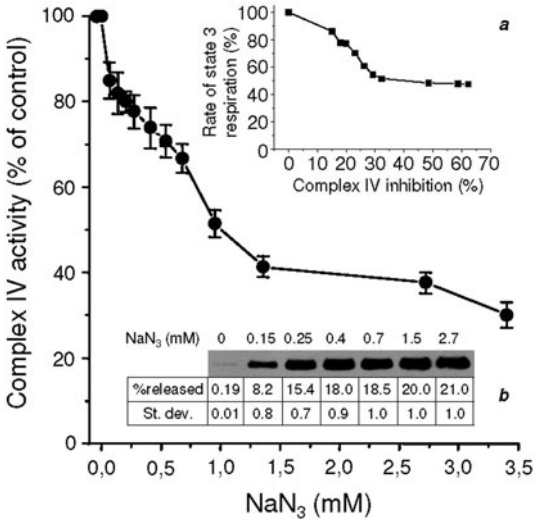

Fig. 2 NaN_3 titration of respiration and complex IV activity. Mitochondria (0.4 mg protein in 0.4 ml S/H buffer) were incubated for 10 min at 37°C (closed system), in respiratory state 3 from succinate, in the presence of increasing NaN_3 concentrations. Rate of state 3 respiration was determined by measuring oxygen levels at the end of incubation. Initial oxygen content was 85 nmols/0.4 ml at 37°C. Samples were centrifuged and supernatants were analyzed for released cyt c. Mitochondrial pellets were resuspended in S/H buffer. Aliquots (15 μg protein) were assayed for complex IV activity in a hypotonic medium (see "Materials and methods" section). Complex IV activity is expressed as percent of the control made omitting NaN_3 (423.6 ± 33.6 nmol oxidized cyt c/min/mg protein). Data are the mean ± SD (four determinations). *Insert a*, influence of complex IV inhibition on rate of state 3 respiration. *Insert b*, a representative blot of NaN_3-dependent cyt c release from mitochondria. Percent released cyt c is the mean ± SD of three experiments

medium and in the intermembrane space of mitochondria were evaluated. Treatment with phosphate released cyt c outside mitochondria (Table 1), confirming previous results [10]. Palmitate, and to a higher extent oleate, caused cyt c detachment and its accumulation in the intermembrane space of mitochondria (Table 1). In the presence of phosphate and fatty acids, released cyt c distributed in the intermembrane space and outside mitochondria.

Generation of H_2O_2: effect of substrates and inhibitors

Mitochondria were incubated in respiratory state 3 and the respiratory chain was fed with electrons derived from oxidation of either complex I substrates, pyruvate/malate, or complex II substrates, succinate and glycerophosphate. From malate/pyruvate, H_2O_2 production was about 70 pmol/min/mg protein and increased to 133 and 297 from succinate and glycerophosphate, respectively. The inhibition of complex III with antimycin A increased H_2O_2 formation from malate/pyruvate, from succinate, and from glycerophosphate to 136, 300, and 985 pmol/min/mg protein, respectively, whereas NaN_3-dependent inhibition of

Table 1 Cytochrome c release: effect of fatty acids and phosphate

	Cytochrome c released (%)	
	Supernatant (incubation medium)	Intermembrane space (post-digitonin supernatant)
Control	0	7.1 ± 1.1
Phosphate (Pi)	15.0 ± 1.4	8.5 ± 1.3
Palmitate	1.7 ± 0.3	11.2 ± 1.3
Palmitate + Pi	11.2 ± 1.2	9.0 ± 1.0
Oleate	1.6 ± 0.3	13.8 ± 1.5
Oleate + Pi	11.0 ± 1.3	13.4 ± 1.4

Fatty acids (100 μM), or phosphate (30 mM), or both were added to mitochondrial suspensions (0.4 mg protein in 0.4 ml S/H buffer) respiring in state 3 from malate/pyruvate and incubation performed 10 min at 37°C. Mitochondria were centrifuged at 9,000×*g* for 10 min to recover the supernatant (incubation medium). For the determination of free cyt c in the intermembrane space, the mitochondrial pellets were resuspended in S/H buffer and the outer mitochondrial membrane was permeabilized by digitonin treatment (see "Materials and methods" section). After centrifugation for 10 min at 9,000×*g* the post-digitonin supernatant was recovered. Cyt c was analyzed by Western blot. Percent released cyt c is the mean ± SD of three experiments

complex IV did not result in an increase of basal H_2O_2 synthesis from any substrate. These data altogether confirm previous reports indicating that the site for H_2O_2 production should be localized within complex I, being sustained by the reversed electron transfer from succinate and glycerophosphate [14, 15]. Our results do not support the indication that complex IV is involved in ROS production.

Inhibition of respiratory complexes and CL alterations

Glycerophospholipids were extracted from mitochondria incubated in state 3 in the presence of inhibitors. No significant variations of composition were observed for all major phospholipids classes, except CL. Percent phospholipid composition was 34.9 ± 1.5, PE; 40.7 ± 1.7, PC; 11.0 ± 1.2, PS; 9.7 ± 1.2, other phospholipids (total phospholipids were 410 ± 10 nmol/mg protein). Values of CL and monolyso-CL are reported in Table 2. CL decreased after complex I inhibition or by uncoupling of respiratory chain with CCCP. The inhibition of complex III with antimycin produced CL decrease only from malate. Contrarily, no changes were observed upon complex IV inhibition. Monolyso-CL was detected in mitochondrial lipids. In accordance with CL decrease, monolyso-CL increased significantly after disturbing complex I and complex III, but not after inhibition of complex IV (Table 2). After incubation from succinate, free fatty acids increased from a basal value of 8.29 ± 1.1 to 17.1 ± 0.9 and 29.0 ± 1.5 nmol/mg protein in the absence or presence of NaN_3 plus rotenone, respectively. Similar values were

Table 2 Cardiolipin and lyso-cardiolipin levels

	CL	Mono-lyso CL
t_o	5.09 ± 0.81	0.51 ± 0.16
Resting	4.81 ± 0.80	Not determined
Malate/pyruvate	4.72 ± 0.37	0.79 ± 0.17
Malate/pyruvate + NaN_3	4.49 ± 0.64	0.62 ± 0.07
Malate/pyruvate + antimycin	3.87 ± 0.78*	1.60 ± 0.30*
Malate/pyruvate + rotenone	4.04 ± 0.54*	1.27 ± 0.40*
Malate/pyruvate + CCCP	3.99 ± 0.73*	Not determined
Succinate	4.88 ± 0.51	0.74 ± 0.22
Succinate + NaN_3	5.08 ± 0.74	0.43 ± 0.15
Succinate + antimycin	5.03 ± 0.61	0.95 ± 0.10
Succinate + rotenone	4.00 ± 0.92*	0.88 ± 0.15*
Succinate + CCCP	3.84 ± 0.84*	0.97 ± 0.15*

Glycerophospholipids extracted from mitochondria incubated in state 3 in the presence of inhibitors were separated and analyzed as described in "Materials and methods" section. Values of CL and of monolyso-CL are reported as mole percent of total phospholipids (410 ± 10 nmol/mg protein). Data are the mean ± SD (six determinations)

* Significance reached, $P < 0.05$

obtained when incubation was performed in the presence of malate/pyruvate as substrates. There was no evidence of conjugate dienes formation in any type of experiment performed.

Interaction of cyt c with fatty acids and phospholipids

To study the binding of fatty acids with cyt c, oxidized or reduced cyt c solutions were treated with different fatty acids and filtered through Microcon YM-100. In the absence of fatty acids, both cyt c forms were completely recovered in the filtrate. Binding of oxidized cyt c was poor with oleate and absent with palmitate and linoleate, as demonstrated by the high recovery of filtered protein (Fig. 3a). Higher propensity to form unfilterable fatty acid–protein complexes was observed for reduced cyt c. The affinity order for reduced cyt c was palmitate (69% filtered) < linoleate (45.6%) < oleate (38.5%).

Cyt c–fatty acid binding resulted in spectral changes, due to transition from low to high spin ferrous heme spectrum that follows displacement of Met80 side chain from coordination with iron [7]. Fatty acids effect was concentration-dependent and was exerted in the order palmitate < linoleate < oleate (Fig. 3b). Spectral changes of reduced cyt c were determined after binding of the protein with liposomes. The α band coalescence of reduced cyt c spectrum was noticeably influenced by pure CL and PG, whereas liposomes composed of equimolar amounts of PC, PE, and CL were less effective (Fig. 3c). When 100 μM fatty acids were added after cyt c binding to liposomes had occurred, spectral changes further increased. The effect of fatty acids was barely the same to that exerted towards pure protein (Fig. 3c) and was additive to that of phospholipids. In the oxidized cyt c, the absorbance peak at 695 nm, related to the ligand field of Met80 [16], was not significantly affected (Fig. 3d).

Release of cyt c from cyt c reconstituted liposomes: influence of redox state

In a model system of cyt c reconstituted in liposomes, phosphate was used to breach the hydrophilic lipid–protein interactions. After phosphate treatment (30 mM), samples of reconstituted liposomes were filtered through Microcon YM-100 and released cyt c was analyzed by Western blotting. In the absence of phosphate, cyt c found in the filtrate from mixed PC:PE:CL liposomes (1:1:1, molar ratio) was 3 and 45% of the oxidized and reduced protein, respectively, indicating that only oxidized cyt c was almost completely bound to liposomes (Fig. 4a). In the presence of 30 mM phosphate, the extent of released protein was higher for the reduced form (Fig. 4a). Both oxidized and reduced cyt c were bound to pure CL liposomes, as they were not found in the filtrate (Fig. 4b). The treatment with 30 mM phosphate produced a higher release of the reduced (about 35%) compared to the oxidized form (12%) (Fig. 4b). No cyt c detachment occurred with up to 10 mM NaN_3 (not shown). Phospholipid mixtures enriched in PC and PE, compared to CL, did not retain either cyt c form (not shown). These experiments confirm that CL is essential for cyt c binding and indicate that the hydrophobic component of cyt c interaction in model membranes is prevalent in the oxidized form of the protein.

Discussion

This study provides evidence that blocking respiration at the level of complex IV promotes detachment and release of cyt c from brain mitochondria. The process involves the interaction of the reduced form of cyt c with free fatty acids and is not mediated by CL decrease and hydrogen peroxide formation. Blocking the respiratory chain upstream cyt c with complexes I and III inhibitors, does not result in cyt c detachment and release.

Titration of complex IV activity with NaN_3 generates a curve correlating complex IV inhibition and impairment of mitochondrial respiration. Differently from synaptic mitochondria [17], in cortex brain mitochondria, impairment of state 3 respiration already occurred at low NaN_3-dependent complex IV inhibition (Fig. 2). It can be inferred that the gradual inhibition of complex IV produces an upstream accumulation of reduced electron carriers, including cyt c.

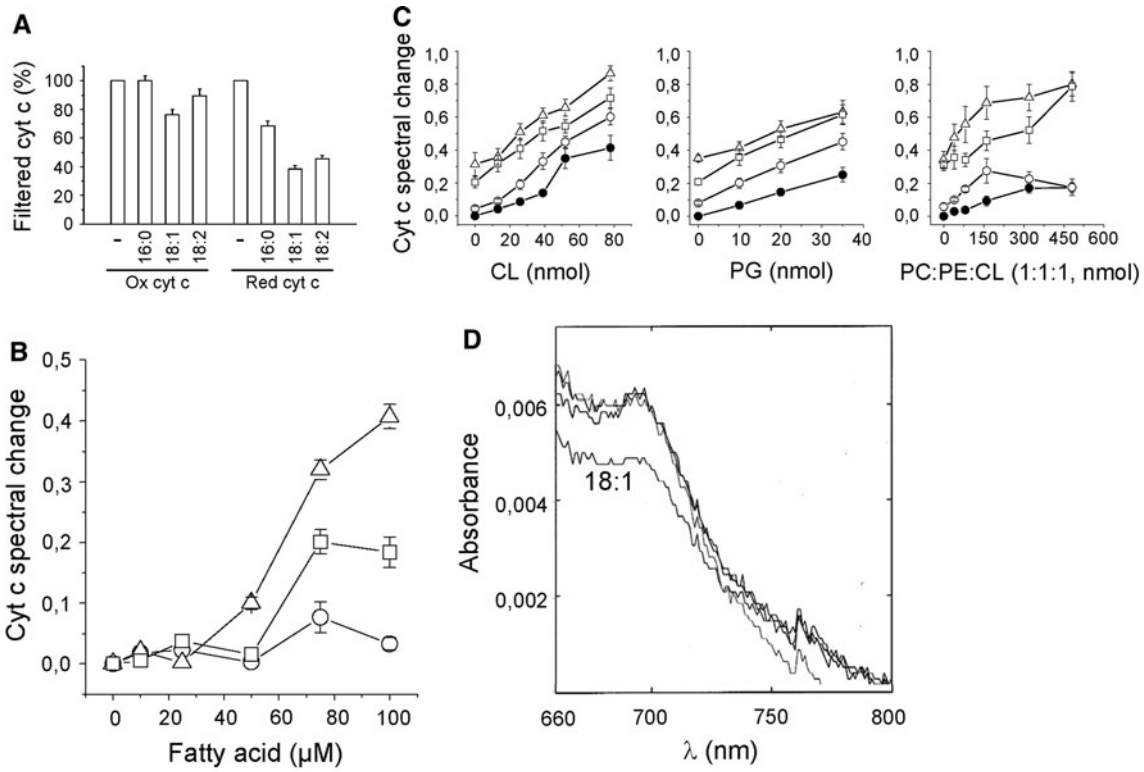

Fig. 3 Interaction of cytochrome c with fatty acids and phospholipids. **a** Cyt c–fatty acid binding. Oxidized or reduced cyt c (2.7 nmol) in 0.3 ml S/H buffer was treated with fatty acids at 54 μM final concentration (1:6, molar ratio). After equilibration for 10 min at room temperature the mixtures were filtered through Microcon YM-100. Fatty acid–free cyt c recovered in the filtrate was analyzed by SDS-PAGE and individual band densities were determined as described in "Materials and methods" section. Data are the mean ± SD of four experiments and are expressed as percent of cyt c filtered in the absence of fatty acids. **b**, **c** Spectral changes of reduced cyt c (9 nmol, 1 ml S/H buffer) were incubated for 30 min in the presence of 0−100 μM fatty acids (**b**) or increasing amounts of different liposomes followed by 100 μM fatty acids (**c**). Spectral changes were determined as described in "Materials and methods" section. Control (*filled circle*), palmitate (*circle*), oleate (*upright triangle*), linoleate (*square*). Data are the mean ± SD (four determinations). **d** Spectral changes of oxidized cyt c. Fatty acids (100 μM) were added to 9 μM oxidized cyt c solutions and absorption spectra were registered in the 600–800 nm range. Superimposed spectra were registered for control and fatty acids, except oleate. A representative experiment of three is shown

This event is accompanied by the release of cyt c outside mitochondria (Fig. 2), suggesting that cyt c redox state may influence the detachment of the protein. From malate/pyruvate cyt c release is decreased by rotenone and antimycin that prevent cyt c reduction by blocking the electron flux. Indeed, the results obtained in model liposomes indicate that cyt c is released more easily when in reduced form (Fig. 4). The stability of cyt c anchorage to the inner mitochondrial membrane when mitochondria were incubated in a medium deprived of oxygen (state 5) reinforces this hypothesis that cyt c is released in its reduced state. In fact, in state 5 the respiratory chain is blocked, substrates are not oxidized, and reduced cyt c does not accumulate. Altogether our results suggest that in brain cortex mitochondria complex IV activity, respiration, and cyt c detachment are coupled. CL is involved in the equilibrium between free and bound cyt c. In liver mitochondria, loss of CL and cyt c release have been attributed to fatty acid peroxidation due to ROS production, as indicated by the appearance of conjugate dienes in lipid extracts [13, 18]. In our experiments, no correlation among CL alteration, H_2O_2 production, and cyt c detachment was observed. NaN_3-dependent complex IV inhibition shifted the equilibrium towards the free protein (Fig. 2), although CL was unchanged (Table 2) and H_2O_2 production was at basal level, indicating that the mechanism of ROS-induced cyt c release was not relevant. Indeed, dienes were never detected, even in conditions producing high H_2O_2 levels. It is worth noting that radical-mediated lipid peroxidation in brain mitochondria is significantly increased only during post-ischemic reperfusion [19]. Using the complex III inhibitor antimycin, CL was unchanged (from succinate) or decreased (from malate), nevertheless cyt c was not released, even in conditions of antimycin-dependent H_2O_2 formation. In the same conditions CL decreases and H_2O_2 is not formed. CL decrease was always accompanied by monolyso-CL increase (Table 2), suggesting the involvement of mitochondrial PLA_2 activity. Indeed, PLA_2 is

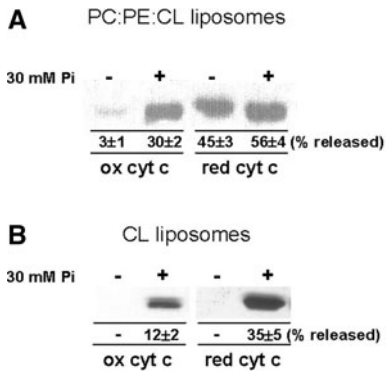

Fig. 4 Cyt c release from reconstituted liposomes: influence of cyt c redox state. Liposomes (10 nmol lipid in 200 µl of S/H buffer) made with PC:PE:CL (1:1:1, molar ratio) (**a**) or CL (**b**) were equilibrated with cyt c (oxidized or reduced form, 1 nmol) for 10 min at room temperature. Reconstituted liposomes were added with 2 mM NaN_3 or 30 mM phosphate and incubated for additional 10 min. Samples were filtered through Microcon YM-100 by centrifuging at 10,000×*g* for 10 min and cyt c in the filtrate was analyzed by SDS-PAGE and individual band densities were determined as described in "Materials and methods" section. A representative gel is shown. Data are the mean ± SD of three experiments and are expressed as percent of filtered cyt c

released from de-energized brain mitochondria [20]. Ca^{+2}-independent PLA_2 is involved in CL deacylation [21].

Previously, we found that exogenous CL reinforces cyt c binding in mitochondria [10]. In addition, cyt c release was observed only after deep alteration of CL synthesis [22] or after CL synthase silencing [23]. CL loss without cyt c release could be ascribed to CL organization in domains [24]. Indeed, cyt c pools that interact differently with the inner membrane of brain mitochondria were identified [25], suggesting that selected CL pools may be involved in cyt c binding.

Free fatty acids increase in mitochondria during cerebral ischemia [26, 27]. We found that free fatty acids promote cyt c detachment from the inner mitochondrial membrane and accumulation in the intermembrane space (Table 1). Interestingly, respiration of mitochondria in the presence of NaN_3 and rotenone resulted in free fatty acids accumulation and cyt c release. Soluble or liposome-reconstituted cyt c is able to bind fatty acids mainly in its reduced form (Fig. 3). In reduced cyt c, a putative binding channel for fatty acids was identified [7]. According to a proposed model, the crevice that may accommodate one of the CL acyl chains in reduced cyt c consists of two hydrophobic stretches with positively charged residues at either extremity [6]. Free fatty acids, mainly oleate and linoleate, may penetrate this crevice causing the release of the protein. This possibility should be hindered in the oxidized form of cyt c, due to its strong interaction with phospholipids [3, 8].

Complex mechanisms underlie cyt c release in mitochondria. These include lipid peroxide formation [13], modification of interactions with CL [11], and transient interactions with other components of the respiratory chain [28]. In this paper, we demonstrate that CL loss and peroxidation are not mandatory events for releasing cyt c from mitochondria. Another possible mechanism for dislodging cyt c from anchoring CL is proposed. It should act by weakening the hydrophobic component of cyt c–CL interactions and might be operative in oxygen shortage conditions that produce accumulation of reduced cyt c and free fatty acids. Based on model liposome studies we suggest that fatty acids, mainly oleate and linoleate, penetrate the cleft in reduced cyt c and displace it from the inner mitochondrial membrane as cyt c–fatty acid complexes, thus increasing the pool of unbound cyt c.

Acknowledgments This work was supported by grant from Fondazione Cassa di Risparmio di Perugia and from Fondazione Cassa di Risparmio di Terni, Italy. We thank Mr. Carlo Ricci for skillful technical assistance.

References

1. Wang X (2001) The expanding role of mitochondria in apoptosis. Genes Dev 15:2922–2933
2. Gogvadze V, Zhivotovsky B (2007) Alteration of mitochondrial function and cell sensitization to death. J Bioenerg Biomembr 39:23–30
3. 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
4. Rytömaa M, Kinnunen PK (1995) Reversibility of the binding of cytochrome c to liposomes. Implications for lipid–protein interactions. J Biol Chem 270:3197–3202
5. Tuominen EKJ, Wallace CJA, Kinnunen PK (2002) Phospholipid–cytochrome c interaction. Evidence for the extended lipid anchorage. J Biol Chem 277:8822–8826
6. Kalanxhi E, Wallace CJA (2007) Cytochrome c impaled: investigation of the extended lipid anchorage of a soluble protein to mitochondrial membrane models. Biochem J 407:179–187
7. Stewart JM, Blakely JA, Johnson MD (2000) The interaction of ferrocytochrome c with long-chain fatty acids and their CoA and carnitine esters. Biochem Cell Biol 78:675–681
8. Nantes IL, Zucchi MR, Nascimento OR, 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 investigation. J Biol Chem 276:153–158
9. Monni M, Corazzi L, Migliorati G, Roberti R (2000) Respiratory rate and phosphatidylserine import in brain mitochondria in vitro. J Membr Biol 173:97–105
10. 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
11. Piccotti L, Buratta M, Giannini S, Gresele P, Roberti R, Corazzi L (2004) Binding and release of cytochrome c in brain mitochondria is influenced by membrane potential and hydrophobic interactions with cardiolipin. J Membr Biol 198:43–53
12. Macala LJ, Yu RK, Ando S (1983) Analysis of brain lipids by high performance thin-layer chromatography and densitometry. J Lipid Res 24:1243–1250

13. Petrosillo G, Ruggiero FM, Paradies G (2003) Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. FASEB J 17:2202–2208
14. Liu Y, Fiskum G, Schubert D (2002) Generation of reactive oxygen species by the mitochondrial electron transport chain. J Neurochem 80:780–787
15. Tretter L, Takas K, Hegedus V, Adam-Vizi V (2007) Characteristics of α-glycerophosphate-evoked H_2O_2 generation in brain mitochondria. J Neurochem 100:50–663
16. Schejter A, Luntz TL, Koshy TI, Margoliash E (1992) Relationship between local and global stabilities of proteins: site-direct mutants and chemically-modified derivatives of cytochrome c. Biochemistry 35:8336–8343
17. Davey GP, Peuchen S, Clark JB (1998) Energy threshold in brain mitochondria. Potential involvement in neurodegeneration. J Biol Chem 273:12753–12757
18. Vladimirov YA, Proskurnina EV, Izmailov DY, Novikov AA, Brusnichkin AV, Osipov AN, Kagan VE (2006) Cardiolipin activates cytochrome c peroxidase activity since it facilitates H_2O_2 access to heme. Biochemistry (Mosc) 71:998–1005
19. Sun D, Gilboe DD (1994) Ischemia-induced changes in cerebral mitochondrial free fatty acids, phospholipids, and respiration in the rat. J Neurochem 62:1921–1928
20. Macchioni L, Corazzi L, Nardicchi V, Mannucci R, Arcuri C, Porcellati S, Sposini T, Donato R, Goracci G (2004) Rat brain cortex mitochondria release group II secretory phospholipase A_2 under reduced membrane potential. J Biol Chem 279:37860–37869
21. Malhotra A, Edelman-Novemsky I, Xu Y, Plesken H, Ma J, Schlame M, Ren M (2009) Role of calcium-independent phospholipase A_2 in the pathogenesis of Barth syndrome. Proc Natl Acad Sci USA 106:2337–2341
22. Buratta M, Castigli E, Sciaccaluga M, Pellegrino RM, Spinozzi F, Roberti R, Corazzi L (2008) Loss of cardiolipin in palmitate-treated GL15 glioblastoma cells favours cytochrome c release from mitochondria leading to apoptosis. J Neurochem 105:1019–1031
23. Choi S-Y, Gonzalvez F, Jenkins GM, Slomianny C, Chretien D, Arnoult D, Petit PX, Frohman MA (2007) Cardiolipin deficiency release cytochrome c from the inner mitochondrial membrane and accelerates stimuli-elicited apoptosis. Cell Death Differ 14:597–606
24. Mileykovskaya E, Dowhan W (2009) Cardiolipin membrane domains in prokaryotes and eukaryotes. Biochim Biophys Acta 1788:2084–2091
25. Buratta M, Piccotti L, Giannini S, Gresele P, Roberti R, Corazzi L (2006) Selective cytochrome c displacement by phosphate and Ca^{2+} in brain mitochondria. J Membr Biol 212:199–210
26. Crompton M (1999) The mitochondrial permeability transition pore and its role in cell death. Biochem J 341:233–249
27. 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
28. Solmaz SRN, Hunte C (2003) Structure of complex III with bound cytochrome c in reduced state and definition of a minimal core interface for electron transfer. J Biol Chem 283:17542–17549