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The effects of ATP and sodium chloride on the cytochrome *c*–cardiolipin interaction: The contrasting behavior of the horse heart and yeast proteins

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ABSTRACT

In cells a portion of cytochrome *c* (cyt *c*) (15–20%) is tightly bound to cardiolipin (CL), one of the phospholipids constituting the mitochondrial membrane. The CL-bound protein, which has nonnative tertiary structure, altered heme pocket, and disrupted Fe(III)-M80 axial bond, is thought to play a role in the apoptotic process. This has attracted considerable interest in order to clarify the mechanisms governing the cyt *c*–CL interaction. Herein we have investigated the binding reaction of CL with the *c*-type cytochromes from horse heart and yeast. Although the two proteins possess a similar tertiary architecture, yeast cyt *c* displays lower stability and, contrary to the equine protein, it does not bind ATP and lacks pro-apoptotic activity. The study has been performed in the absence and in the presence of ATP and NaCl, two compounds that influence the (horse cyt *c*)-CL binding process and, thus, the pro-apoptotic activity of the protein. The two proteins behave differently: while CL interaction with horse cyt *c* is strongly influenced by the two effectors, no effect is observed for yeast cyt *c*. It is noteworthy that NaCl induces dissociation of the (horse cyt *c*)–CL complex but has no influence on that of yeast cyt *c*. The differences found for the two proteins highlight that specific structural factors, such as the different local structure conformation of the regions involved in the interactions with either CL or ATP, can significantly affect the behavior of cyt *c* in its reaction with liposomes and the subsequent pro-apoptotic action of the protein.

1. Introduction

Cytochrome *c* (cyt *c*) is a single-chain hemoprotein acting as electron carrier in mitochondria. It is composed of 104 amino acids (12.4 kDa) and the prosthetic group is heme. In the protein the heme is covalently bound to the polypeptide chain by two thioether bridges formed with residues C14 and C17, while H18 and M80 are the two axial ligands of the heme iron. In the cellular environment the protein functions between the inner and the outer membrane of the mitochondrion, mediating electron transfer between different proteins of the respiratory chain. In healthy cells a portion of cyt *c* (15–20%) remains tightly bound to cardiolipin (CL), one of the phospholipids constituting the mitochondrial membrane [1,2]. CL-bound cyt *c* shows tertiary structural rearrangements, which include alteration of the heme pocket region and detachment of M80 from the sixth coordination position of the heme iron [3–6]. The CL-specific peroxidase action acquired by membrane-bound cyt *c* in the early stages of apoptosis, which initiates the protein pro-apoptotic activity, is critical for cells; CL peroxidation induces cyt *c* release into the cytosol and favors the accumulation of products releasing pro-apoptotic factors [7–10]. This explains why considerable effort has been made over recent years to clarify the mechanisms governing the cyt *c*–CL interaction and the (cyt *c*–CL) complex stability.

Two models have been proposed to describe the process leading to (cyt *c*–CL) complex formation. Both consider that, upon binding, one acyl chain of CL protrudes in the protein interior. However, one model identifies the hosting region in the hydrophobic channel close to the invariant residue N52 [3], whereas the other in the M80-containing loop [4]. The fact that the CL liposomes bind cyt *c* at two distinct binding sites of the protein [5,11] led to the proposal that two acyl chains of CL (instead of one, as suggested by the two above mentioned models) may protrude into cyt *c* [11]. This event is sterically permitted in view of the unique structure of CL which, contrary to the other phospholipids constituting the mitochondrial membrane, possesses four (instead of two) acyl chains [6]. This hypothesis is supported by the fact that CL is

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the only phospholipid of the mitochondrial membrane which binds the protein firmly [12,13].

In this paper we investigate the interaction of CL liposomes with ferric cyt *c* obtained from two distinct sources, horse heart and yeast. The two proteins have a similar tertiary architecture [14–17], but yeast cyt *c* displays a significantly lower stability than the protein from horse, it does not bind ATP and, contrary to horse cyt *c*, lacks pro-apoptotic activity [18,19]. The CL-cyt *c* binding reaction has been investigated in the absence and in the presence of ATP and sodium chloride, two compounds that significantly influence the (horse cyt *c*)–CL binding process [5]. The data clearly show that the two proteins behave differently. Although the horse cyt *c*–CL interaction is strongly influenced by the two effectors, no effect is observed for the binding reaction of CL liposomes with yeast cyt *c*. In particular, the fact that sodium chloride dissociates the (horse cyt *c*)–CL complex but exerts no influence on the (yeast cyt *c*)–CL complex, suggests that the complex behavior strictly depends on the type of protein utilized. The different behavior shown by the two cytochromes is explained on the basis of the different local structure of the regions involved in the interactions with either CL or ATP.

2. Materials and methods

2.1. Materials

Horse heart cyt *c* (type VI, oxidized form) and cardiolipin, as sodium salt from bovine heart (approx 98% purity, lyophilized powder), were obtained from Sigma Chemical Co (St. Louis, MO, USA) and used without further purification. All reagents were of analytical grade.

2.2. Liposome preparation

Aqueous dispersions of CL liposomes were prepared according to a procedure described previously [20]. Briefly, a film of lipid was prepared on the inside wall of a round bottom flask by evaporation of a chloroform solution containing the proper amounts of lipid. The films obtained were stored in a dessicator overnight under reduced pressure and then 1 mL of a 20 mM Hepes buffer solution was added in order to obtain a 2.5 mM lipid dispersion. Solutions were vortex-mixed and then freeze-thawed six times from liquid nitrogen to 30 °C. Dispersions were then extruded (10 times) through a 100 nm polycarbonate membrane. Extrusions were carried out at 30 °C. The vesicle size was determined by light scattering measurements. In the buffer, the vesicle diameter is approximately 130 nm; the diameter slightly increases to 140 nm in the presence of 0.5 M NaCl.

2.3. Yeast iso-1-cyt c expression and purification

The expression plasmid pBTRI harboring both the yeast iso-1-cyt *c* gene (*CYC1*) and heme lyase gene (*CYC3*), was a kind gift from A. Grant Mauk of British Columbia University, Vancouver. The *CYC1* gene encodes the Cys102Thr variant of yeast iso-1-cyt *c*. In this variant protein dimerization is precluded and the ferricytochrome autoreduction rate is greatly diminished. The optical and electrochemical properties of the variant are indistinguishable from those of the wt protein [21]. Bacterial expression and purification of the recombinant proteins were conducted essentially as described in [22] with minor modifications.

Escherichia coli strain JM 109 containing the pBTRI plasmid was grown at 37 °C in 2 L of SB medium containing 100 μg/mL ampicillin until an absorbance of 0.3 at 600 nm was achieved. Induction was accomplished by adding IPTG (isopropyl-ß-D-thiogalactopyranoside) to a final concentration of 0.75 mM. Cells were incubated at 37 °C overnight, harvested by centrifugation and frozen at −80 °C. After thawing, the reddish pellets were re-suspended in 50 mM Tris pH 8.0 (3–4 mL per gram of wet cells). Lysozyme (1 mg/mL) and DNase (5 μg/mL) were added to the homogenized cells. The suspension was left in ice for 1 h and then sonicated for 1 min, at medium intensity and 50% duty cycle. After centrifugation for 30 min at 10,000 g to remove cell debris, the clarified supernatant was dialyzed overnight against 10 mM phosphate buffer pH 6.2 and then loaded on a CM 52 column (40 mL bed volume) equilibrated with the same buffer. Purification was performed by washing the column with one volume of 45 mM phosphate pH 6.8, then one volume 45 mM phosphate pH 6.8, 75 mM NaCl and by eluting the protein with one volume of 45 mM phosphate pH 6.8, 250 mM NaCl. After purification, the recombinant C102T variant of yeast iso-1-cyt *c* (indicated herein as 'wt' protein) was promptly oxidized by adding a few grains of potassium ferricyanide and then subjected to extensive dialysis against 100 mM phosphate buffer pH 7.0. The recombinant protein (~500 μM) was more than 98% pure (from SDS-PAGE analysis and reverse phase HPLC, data not shown) and was stored at −80 °C in 200 μL aliquots.

2.4. Circular dichroism measurements

Circular dichroism (CD) measurements were carried out using a Jasco J-710 spectropolarimeter (Tokyo, Japan) equipped with a PC as data processor. The molar ellipticity, [Θ] (deg cm^2 $dmol^{-1}$), is expressed on a molar heme basis in the Soret (400–450 nm) region. In the far-UV region, measurements were carried out in the 215–250 nm range.

Binding of cyt *c* to CL liposomes (critical micelle concentration, cmc: approx. 1 μM [5], as determined by the spectroscopic method based on the fluorescence of 1,6-diphenylhexatriene [23]) was analyzed by following the changes induced in the Soret CD region (400–450 nm) of the protein by stepwise addition of a few microliters of 2.5 mM CL solution to a 10 μM cyt *c* solution ($V_i = 1.0$ mL; 0.5 cm pathlength cell). The buffer was 25 mM Hepes containing 0.1 mM EDTA, pH 7.0. Dichroic spectra were recorded 5 min after mixing. The same procedure was employed to follow the salt-induced (cyt *c*–CL) complex dissociation.

2.5. Electronic absorption measurements

Electronic absorption spectra were measured with a double-beam Cary 5 spectrophotometer (Varian, Palo Alto, CA, USA) at 25 °C using a 5-mm NMR tube and a 600 nm/min scan rate. Cyt *c* concentration was determined on the basis of the extinction coefficient $\varepsilon = 106$ mM^{-1} cm^{-1} at 408 nm.

2.6. Resonance Raman measurements

Resonance Raman (RR) spectra were recorded using a 5-mm NMR tube and by excitation with the 406.7-nm line of a Kr^+ laser (Innova 300 C, Coherent, Santa Clara, CA, USA). Backscattered light from a slowly rotating NMR tube was collected and focused into a triple spectrometer (consisting of two Acton Research SpectraPro 2300i and a SpectraPro 2500i in the final stage with a 3600 grooves per millimeter grating) working in the subtractive mode, equipped with a liquid-nitrogen-cooled CCD detector. It should be noted that the spectral resolution of the RR spectra cited in the figure captions is that calculated theoretically on the basis of the optical properties of the spectrometer. However, for the moderately broad experimental resonance Raman bands observed in the present study (about 10 cm^{-1}), the effective spectral resolution will in general be lower. RR spectra were calibrated with indene and CCl_4 as standards to an accuracy of 1 cm^{-1} for intense isolated bands. The complex between the CL liposomes and protein was prepared by stepwise addition of 1 μL aliquots of CL (2.5 mM) buffered solution to 50 μL of a 30 μM protein buffered solution until the desired final CL concentration was achieved giving a 7:1 CL/cyt *c* molar ratio. The aliquots were added at 20 min intervals and the spectrum recorded 20 min after the final addition of CL. The samples in the presence of NaCl were prepared by