Interaction between cardiolipin and the mitochondrial presequence of cytochrome *c* oxidase subunit IV favours lipid mixing without destabilizing the bilayer structure

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Abstract **We demonstrate the ability of a peptide corresponding to the presequence of the cytochrome *c* oxidase subunit IV to induce lipid mixing between large unilamellar liposomes. This lipid mixing requires the presence of CL or PE, lipids able to form non-bilayer structures, and is not observed with other negatively charged lipids. However, the fact that this mixing occurs without mixing of the liposome aqueous phases and without destabilizing the lipid organisation is unusual and has not been observed for other amphiphilic peptides. This observation supports the idea that the presequence could play a role in the formation of translocation contact sites between the two mitochondrial membranes and facilitate the structural rearrangements of the outer and inner membrane proteins involved in the two import machineries in a way to permit the formation of a continuous import channel through the two mitochondrial membranes without mixing the cytoplasmic and mitochondrial aqueous contents.**

Key words: Mitochondrial presequence; Contact site; Lipid mixing; Cardiolipin

1. Introduction

Most mitochondrial proteins are synthesised in the cytosol as precursors carrying an amino-terminal extension, the presequence [1]. This presequence is required to import proteins to their intramitochondrial location and is proteolytically cleaved in the mitochondrial matrix [2–4].

The import mechanism requires several steps as binding on the external mitochondrial surface and translocation across one or two membranes with the help of the mitochondrial machineries (for review see [5]). Although the molecular mechanism of this crossing is incompletely understood, it occurs via contact sites between outer and inner mitochondrial membranes [6]. These contact sites are believed to be not permanent but dynamic structures [7–9]. Mitochondria contain two distinct import sites, one in the outer and one in the inner membrane [7,10,11]. The transport machineries of both membranes can function independently of each other but cooperate during the translocation of matrix proteins in the intact mitochondria [5,11,12]. An important question is how are these translocation sites formed. Their formation could be mediated by interaction between proteins of both membranes or it is the precursor that induces their formation by interacting with lipids or proteins of both membranes. This second hypothesis is supported by the work of Török et al. [13] and Leenhouts et al. [14] who have provided evidence that the presequence of the cytochrome *c* oxidase subunit IV induces cardiolipin specific interbilayer contacts which are dissociated by a transmembrane potential.

The aim of this paper is to provide evidence that this presequence not only does induce interbilayer contacts sites but also may favour lipid mixing of the inner and outer mitochondrial membranes. For this purpose, we studied the ability of the monomeric form of cytochrome *c* oxidase subunit IV presequence (p25) to promote lipid mixing of large unilamellar liposomes of different lipid compositions.

We show that this presequence induces the lipid mixing between vesicles without significant destabilization of the bilayer structure in the presence of CL and PE. This last observation was not expected since most amphiphilic fusogenic peptides strongly destabilize the lipid organisation [15]. This finding supports the idea of a presequence-induced semi-fusion process in which the two lipid bilayers mix their lipids and eventually favour structural rearrangements of the proteins involved in the two import machineries in a way to permit the formation of a continuous import channel through the two mitochondrial membranes without mixing the cytoplasmic and mitochondrial aqueous contents.

2. Materials and methods

2.1. Materials

Bovine heart cardiolipin (CL), *Escherichia coli* cardiolipin (*E. coli* CL), L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylglycerol (PG), L-α-phosphatidylinositol (PI), L-α-phosphatidylserine (PS) and Triton X100 were purchased from SIGMA (St. Louis, MO, USA).

N-(Nitrobenzo-2-oxa-1,3-diazol)-PE (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-PE (Rh-PE) were obtained from Avanti Polar Lipids (Birmingham, AL, USA). 8-Aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS) and *N,-N'-p*-xylylenebis(pyridinium)bromide (DPX) were from Molecular Probes (Junction City, OR). The synthetic peptide P25 ($^{+}NH_3$-Met-Leu-Ser-Leu-Arg-Gln-Ser-Ile-Arg-Phe-Phe-Lys-Pro-Ala-Thr-Arg-Thr-Leu-Cys-Ser-Ser-Arg-Tyr-Leu-Leu-COO^{-} has been prepared by solid phase synthesis by Dr. T. Saemark (University of Copenhagen, Denmark). The sequence was verified by protein sequencing and confirmed by mass spectroscopy and HPLC. The peptide was solubilized in water to a final concentration of 1 mg/ml and aliquots were stored at −20°C. Peptide concentration was determined

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Abbreviations: ANTS, 8-aminonaphtalene-1,3,6-trisulfonic acid sodium salt; CL, bovine heart cardiolipin; DPX, *N,N'-p*-xylylenebis(pyridinium) bromide; DTT, dithiothreitol; *E. coli* CL, cardiolipin from *Escherichia coli*; EDTA, (ethylenedinitrilo)tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; LUVs, large unilamellar vesicles; NBD-PE, *N*-(nitrobenzo-2-oxa-1,3-diazol)phosphatidylethanolamine; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PG, L-α-phosphatidylglycerol; PI, L-α-phosphatidylinositol; PS, L-α-phosphatidylserine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine.

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by the micro-BCA assay (Pierce, Rockford, IL) using bovine serum albumin as a standard.

2.2. Methods

2.2.1. Vesicles preparation: large unilamellar vesicles (LUV). Dry lipid films were hydrated in appropriate buffers by vortexing. The solutions were frozen and thawed five times and extruded (Extruder Liposofast, Avestin Inc., Ottawa, Canada) 25 times through two 0.1 mm pore filters as described by MacDonald et al. [16]. The phosphatidylcholine content was estimated by the enzymatic colorimetric test of Boehringer-Mannheim.

2.2.2. Lipid mixing assay. The percentage of lipid mixing was determined by the resonance energy transfer between two probes NBD-PE and Rh-PE as described by Struck et al. [17].

Both fluorescent phospholipids were added to the lipid film and LUV were prepared as described above using a 10 mM HEPES–150 mM NaCl–0.1 mM EDTA and 0.02% NaN_3, pH 7.4, buffer. Labelled vesicles were prepared with 0.6% (w/w) NBD-PE and Rh-PE. Those vesicles were mixed with unlabelled ones at a 1:9 ratio at a final lipid concentration of 1.33×10^{-4} M. The residual fluorescence of this mixture before addition of the peptides was taken as 0% of fluorescence. A small volume of peptide solution (1 mg/ml) was added to the mixture at time zero, and the increase in fluorescence of NBD was monitored at 530 nm ($\lambda_{excitation}$ 470 nm) using a SLM 8000C spectrofluorometer with excitation and emission slits of 4 nm. The 100% fluorescence was determinated using an equivalent concentration of vesicles containing 0.06% (w/w) of each fluorescent phospholipids or by lysis of the liposomes suspension (20 μl of 10% Triton X-100).

2.2.3. Leakage assay and aqueous contents mixing assay. The leakage and mixing of aqueous contents of vesicles were measured using the ANTS/DPX assay according to Ellens et al. [18]. The assay is based on the quenching of ANTS by DPX.

Leakage of lipidic vesicles aqueous contents was followed by measuring the release of ANTS into the external medium. The LUV were prepared in buffer containing 12.5 mM ANTS, 45 mM DPX, 65 mM NaCl and 10 mM HEPES (pH 7.4). Liposomes containing ANTS and DPX were separated from unencapsulated material on Sephadex G75 using 10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3 (pH 7.4) as the elution buffer. The fluorescence of the suspension of liposomes before addition of the peptide corresponds to the 0% leakage. 100% leakage was obtained by lysis of the liposome suspension (20 μl of 10% Triton X-100).

Mixing of aqueous contents was followed as a decrease in ANTS fluorescence due to quenching of ANTS by DPX. LUV containing ANTS or DPX were prepared as mentioned above in buffer containing either 25 mM ANTS, 89 mM NaCl and 10 mM HEPES or 90 mM DPX, 39 mM NaCl and 10 mM HEPES. Liposomes were separated from unencapsulated material on Sephadex G75 using 10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 0.02% NaN_3 (pH 7.4) as the elution buffer. The fluorescence of a 1:1 mixture of ANTS and DPX liposomes corresponds to 0% fusion. The 100% fusion corresponds to the residual fluorescence of liposomes containing 12.5 mM ANTS, 45 mM DPX, 65 mM NaCl and 10 mM HEPES. For the two assays, samples were irradiated at 360 nm, emission was measured at 520 nm using a SLM 8000 spectrofluorometer with excitation and emission slits of 4 nm.

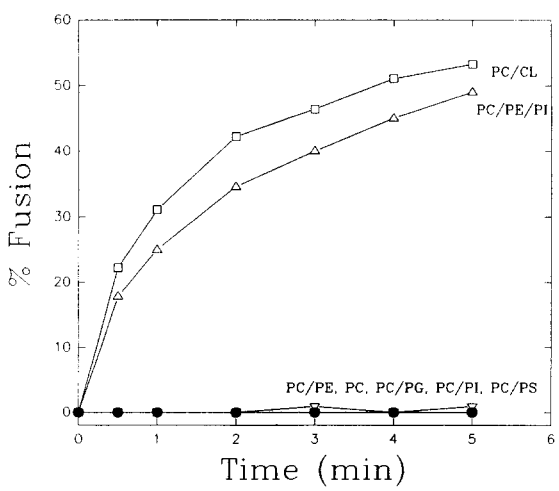

Fig. 1. p25-induced lipid mixing of large unilamellar vesicles (LUV) monitored by energy transfer between NBD-PE and Rh-PE at neutral pH, 37°C and in the presence of 50 mM DTT. At time 0, 10 μM of p25 was added to 133 mM liposome suspension. PC, PC/CL (8:2 molar ratio), PC/PI, PC/PS, PC/PG, PC/PE (8:4 molar ratio) and PC/PE/PI (4:4:4 molar ratio).

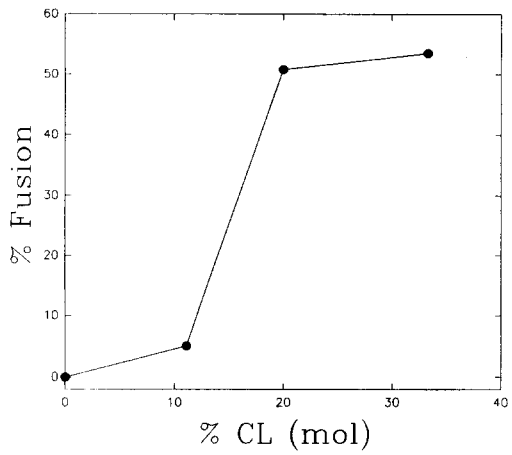

Fig. 2. Lipid mixing of PC/CL LUV induced by p25 peptide as a function of CL concentration. The percentage of fusion 5 min after addition of the peptide were represented versus the CL concentration. Experimental conditions were those described in Fig. 1.

3. Results and discussion

3.1. Lipid mixing assay

We have preliminarily verified by SDS-PAGE electrophoresis that addition of 50 mM DTT to p25 peptide solution eliminates the dimers resulting from the formation of a disulfide bond between cysteines-19 (data not shown). All experiments were therefore carried out in the presence of 50 mM DTT.

Addition of p25 induces a rapid and efficient lipid mixing of PC/CL LUV (8:2 molar ratio) (Fig. 1). The mixing of lipids is cardiolipin concentration dependent and increases abruptly around 20% in moles of CL (Fig. 2).

In order to demonstrate that the fusion observed is CL specific, presequence was added to LUVs containing other negatively charged phospholipids (PI, PS, PG). The PC/charged lipid ratio was chosen in such a way that the negative charge density was identical to that of the PC/CL liposomes. No significant lipid mixing was observed with these LUVs as well as with 100% PC LUV (Fig. 1).

This specificity for CL as compared with other negative phospholipids could be correlated with infra-red data [19,20] providing evidence that the α-helix domain of p25 is oriented parallel to the lipid acyl chains. This orientation is specific to CL and was never observed with liposomes containing PG instead of CL or 100% DOPC liposomes.

PE alone does not induce the lipid mixing but addition of PE to liposomes containing a negatively charged lipid (PI) strongly restores the fusion activity (Fig. 1).

These results suggest that the lipid mixing induced by the presequence required the presence of phospholipids able to form non-bilayer structures such as PE and CL. These two phospholipids have been shown to form HII-phases in the presence of fusogenic peptides or melittin [21,22,23]. Such non-bilayer structures are known to participate in the lipid fusion process. The fact that the lipid mixing decreases from 46% to 22% after replacement of CL from bovine heart with *E. coli* CL in PC/CL LUV (8:2 molar ratio) (data not shown) supports the participation of non-lamellar structures in the lipid mixing we observed. Indeed, cardiolipin from *Escherichia coli* contains a higher percentage of saturated fatty acid [24] and therefore prefers a bilayer organisation [25].

It is important to mention that CL, a lipid specific of the mitochondria, is essentially abundant (~20%) in the inner membrane [26] but is also present in the outer leaflet of the outer membrane of rat liver mitochondria [27]. Moreover, the outer mitochondrial membrane is enriched in PI and contains as the inner membrane about 30% of PE. Interesting, contact sites have been shown to be enriched in phospholipids (PE, CL) able to form inverted micellar structures [28].

3.2. *Is the lipid mixing accompanied by a mixing of the aqueous phases?*

A central question is to know whether the lipid mixing is accompanied by mixing and leakage of the aqueous phases as it has been described in virus-cell fusion. For instance, the fusogenic NH_2-terminal domain of gp41 HIV and gp32 SIV catalyses the lipid mixing but strongly destabilized the lipid organisation [15,29].

To study the aqueous contents mixing, one population of liposomes containing ANTS was mixed with one population containing DPX [18]. Mixing of the liposome contents is expected to cause a decrease in fluorescence intensity of ANTS.

No mixing of the aqueous contents was observed with PC/CL LUV (8:2 molar ratio), a composition which promotes the most efficient lipid mixing (Fig. 3). There are two ways to explain this observation. Either the lipid mixing does not induce the mixing of aqueous contents or the leakage due to the destabilization of the lipid vesicle is too fast to allow the detection of the aqueous content mixing. To investigate the rate of leakage induced by the presequence, ANTS and DPX were coencapsulated in one population of liposome, an increase of the

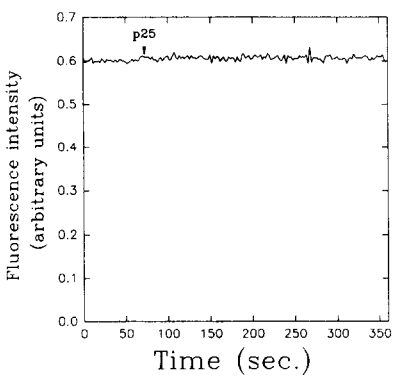

Fig. 3. p25-induced mixing of aqueous contents of large unilamellar vesicles (LUV) monitored by the ANTS/DPX assay at neutral pH, 37°C and in the presence of 50 mM DTT. At the arrow, 10 μM of p25 was added to 133 μM liposome suspension. PC/CL (8:2 molar ratio).

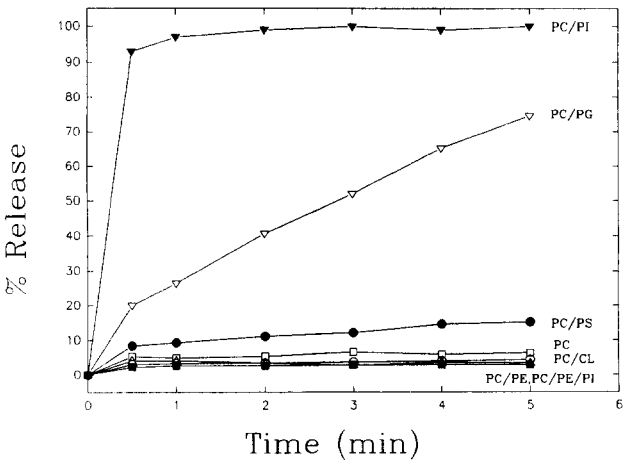

Fig. 4. p25-induced release of aqueous contents of large unilamellar vesicles (LUV) monitored by the ANTS/DPX assay at neutral pH, 37°C and in the presence of 50 mM DTT. At time 0, 10 μM of p25 was added to 133 mM liposome suspension. PC, PC/CL (8:2 molar ratio), PC/PI, PC/PS, PC/PG (8:4 molar ratio).

ANTS fluorescence was a measure of the destabilization of the LUV envelope. No significant leakage was associated to PC/CL LUV. The percentage of release was much higher for liposomes that do not induce lipid mixing but are however negatively charged such as PC/PI, PC/PG, PC/PS LUV (8:4 molar ratio) (Fig. 4).

These data strongly suggest that lipid mixing proceeds via a process of semi fusion which occurs through the formation of non-lamellar lipid structures between the lipid external monolayers of the LUV followed by a rearrangement in stable bilayers without a significant mixing of aqueous contents and without lysis [30].

Our data may suggest the following sequence of events for the import mechanism. After binding to the receptor on the cytoplasmic face of the mitochondrial outer membrane, the presequence penetrates in the outer membrane and induces the formation of non-bilayer structures between the two mitochondrial membranes. The semi-fusion process described above could facilitate the structural rearrangements of the outer (MOM 8, MOM 22, MOM 30 and MOM 38) [31] and inner (MIM 44, MAS6, MPI1) [32] membrane proteins involved in the two import machineries and allow the passage of the precursor protein to the matrix while avoiding a dramatic mixing of the mitochondrial and cytoplasmic aqueous contents. The process described above does certainly not explain the whole protein import into mitochondria but it may be important in our attempt to understand the mechanism of protein translocation to take into account the cytochrome *c* oxidase subunit IV presequence capacity, not reported for other amphiphilic peptides, to catalyse the mixing of the lipid components without affecting the lipid permeability.

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