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A novel property of a mitochondrial presequence

Its ability to induce cardiolipin-specific interbilayer contacts which are dissociated by a transmembrane potential

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A new property of the presequence of the mitochondrial precursor protein cytochrome oxidase subunit IV is presented. This mitochondrial presequence induces interbilayer contacts between large unilamellar vesicles consisting of phosphatidylcholine and cardiolipin. The presequence-vesicle aggregates can be dissociated by applying a membrane potential across the bilayers (negative inside). These effects require the presence of cardiolipin and are not observed for other negatively charged phospholipids. We propose a role for the presequence in the formation and dissociation of mitochondrial contact sites

Mitochondrial presequence; Membrane potential; Membrane contact; Cardiolipin

1. INTRODUCTION

Most mitochondrial proteins are synthesized in the cytosol as precursor proteins with an amino-terminal, 20–80 amino acid extension peptide, the presequence. Presequences do not share primary structure homology, but are amphipatic and contain several positively charged and few, if any, negatively charged residues [1,2]. Their common structural motive appears to be an amphiphilic α-helix [2–4]. They are required for the import of precursor proteins into mitochondria and are proposed to be involved in different steps in the process. Presequences contain the information to target attached proteins to mitochondria [5,6] where they are supposed to bind to the 'presequence receptor' MOM19 [7]. Presequence-specific cytosolic protein factors seem to participate in this process [8,9]. A 50 kDa protein, referred to as 'presequence binding factor' (PBF) [10] and a 28 kDa protein, designated 'targeting factor' [11] have been purified. For protein translocation across the inner mitochondrial membrane a membrane potential ($\Delta\Psi$) across the inner membrane is required [12–15]. It has been demonstrated that the membrane potential (negative inside) is needed for the translocation of only the presequence, and not the mature region, possibly via an electrophoretic effect [16–18]. Once the precursor protein has reached the mitochondrial matrix, the presequence is proteolytically removed [19]. Several biophysical studies revealed that presequences interact strongly with model membranes, with a preference for negatively charged phospholipids [3,20–25] and it has been suggested that these interactions are important for the import process.

Import into the mitochondrial matrix occurs at contact sites between the inner and outer membrane [16,26]. Recent studies suggest that mitochondrial contact sites are highly dynamic [27–30]. A model was proposed, involving the precursor protein reaching the intermembrane space, after passing the outer membrane, and subsequently engaging the inner membrane [29]. This raises the question of the mechanism of the dynamic contact formation. One possibility is that the presequence could induce membrane contacts and that the membrane potential plays a role in this process. To address this question, studies were carried out with a synthetic presequence, corresponding to the amino-terminal 25 residues of cytochrome oxidase subunit IV (pCoxIV), and lipid model membranes of varying compositions. This presequence is able to import attached proteins into mitochondria [5,6] and its interaction with model membranes is very well documented [3,20,22–

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Abbreviations: $\Delta\psi$, membrane potential; CL, cardiolipin; pCoxIV, presequence of cytochrome oxidase subunit IV; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PI, phosphatidylinositol; FCCP, carbonylcyanide-*p*-trifuoromethoxy-phenylhydrazone; LUVs, large unilamellar vesicles.

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24,31]. Moreover, these interactions were shown to be enhanced by a membrane potential [3,24].

We report here a novel property of the presequence: it induces cardiolipin (CL)-dependent interbilayer contacts, which can be reversed upon induction of a transmembrane potential (negative inside). The significance of these observations for the mitochondrial import process is indicated.

2. MATERIALS AND METHODS

Cardiolipin from bovine heart, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was synthesized as described [32]. Cardiolipin from *E coli* (strain AH930) was isolated and purified as will be described elsewhere (Killian et al., manuscript in preparation). Phosphatidylinositol (PI) from soybeans was obtained from Larodan Fine Chemicals AB (Malmö, Sweden).

Large unilamellar vesicles (LUVs) were prepared at 10 mM phospholipid concentration in 150 mM K_2SO_4, 20 mM HEPES (pH 7.0), 2 mM EDTA (K^+-buffer) by the extrusion technique [33], after 10 times freezing and thawing, using 400 nm polycarbonate filters. Ion gradients were applied to the vesicles by replacing the external K^+-buffer by Na^+-buffer by passing the LUVs through a Sephadex G 50 minicolumn (1 ml) [33], eluted with 150 mM Na_2SO_4, 20 mM HEPES (pH 7.0), 2 mM EDTA (Na^+-buffer). Concentrations of phosphorous were determined according to Rouser et al. [34]. CL concentrations are always given as phosphorous concentration. A membrane potential was induced by adding valinomycin (Boehringer, Mannheim, Germany; 10 μg/ml in ethanol), to a $1:10^4$ molar ratio with respect to phospholipid, to the LUVs exhibiting a K^+_{in}/Na^+_{out} ion gradient. When required, valinomycin and the uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; Sigma, St. Louis, MO; 10 μg/ml in ethanol), which was added in a $1{:}8{\cdot}10^3$ molar ratio with respect to phospholipid, were added to the LUVs in order to investigate the influence of the pH gradient resulting from the membrane potential in the observed effects [35]. Transmembrane Na^+_{in}/K^+_{out} gradients were applied by diluting LUVs prepared in Na^+-buffer into K^+-buffer.

The presequence of yeast cytochrome oxidase subunit IV (H_3N^+MLSLRQSIRFFKPATRTLCSSRYLLCOO$^-$) was prepared by solid phase synthesis by Mr. D. Olshevski (University of California, San Diego) and purified as described [24] The identity of the peptide was confirmed by analysis of the amino acid composition and sequence. Peptide solutions (1 mM in water) were always freshly prepared; concentration was determined according to Lowry et al [36]. The presequence was preincubated with 2% β-mercaptoethanol for 15 min to reduce dimer forms of the peptide.

The turbidity of the vesicle solutions was measured as the absorbance at 400 nm on a Hitachi U-3200 spectrophotometer. The experiments were carried out under continuous stirring at room temperature, using a 1 ml quartz cuvette with a light path length of 1 cm. All experiments were carried out at least two times and the standard deviations in all turbidity changes were less than 10%.

The presence of a membrane potential (negative inside) upon addition of valinomycin to vesicles, experiencing a K^+_{in}/Na^+_{out} ion gradient, was established using 3,3'-diethylthiadicarbocyamine iodide (diS-C_2-(S), Molecular Probes, Inc., Eugene, OR) as a membrane potential-sensitive fluorescent dye [37]. The presence of a membrane potential (positive inside) upon addition of valinomycin to LUVs with a Na^+_{in}/K^+_{out} ion gradient, was established by monitoring (3-propyl-5-oxoisoxazol-4-yl) pentamethine oxonol (oxonol VI, Molecular Probes, Inc., Eugene, OR) absorbance upon generation of the potential [38]. Under the experimental conditions employed, the addition of the presequence did not collapse the membrane potential (data not shown).

3. RESULTS AND DISCUSSION

In order to study the formation of interbilayer contacts, we analyzed the influence of the presequence on the turbidity of a solution of large unilamellar lipid vesicles. If such contacts are formed, the vesicle aggregates give rise to increased lightscattering, which can be measured as an increased absorbance. In this system the effect of a membrane potential can be studied by applying valinomycin to LUVs with a K^+_{in}/Na^+_{out} ion gradient.

Fig. 1 shows a typical experiment with LUVs of 30% CL and 70% PC, without or with a K^+_{in}/Na^+_{out} ion gradient. At $t = 0$, presequence was added to the solutions, resulting in both cases in a dramatic and rapid increase in turbidity. Without vesicles the presequence had no effect on the absorbance. The increased turbidity reveals the formation of larger sized structures. In principle this could be due to both vesicle aggregation and fusion, which were both reported by Roise et al. [3], who showed by electronmicroscopy in a similar system massive aggregation and increase in the size of vesicles.

Four minutes after presequence addition, the turbidity level had nearly reached equilibrium (Fig. 1). At this moment, a membrane potential was induced by addition of valinomycin to the LUVs with a K^+_{in}/Na^+_{out} ion gradient (curve b). This gave rise to a rapid decrease in turbidity up to about 80% of the total increase. In the control situation, where no membrane potential could be induced (curve a), valinomycin had no effect on the turbidity of the solution showing that the membrane potential, and not valinomycin itself, caused the decrease in turbidity. If, together with valinomycin FCCP was added to the LUVs with a K^+_{in}/Na^+_{out} ion gradient, the resulting turbidity decrease was the same as when only valinomycin was added (not shown), indicating that the turbidity decrease is caused by $\Delta\Psi$, and not by the pH gradient which is induced by $\Delta\Psi$ [35]. The reversibility of the process indicates that the observed turbidity increase is, for at least 80%, due to vesicle aggregation and not to presequence-induced vesicle fusion. If valinomycin was added to the vesicles prior to presequence addition, there was a rapid turbidity increase, until a level lower than in the reverse experiment, because it was followed almost immediately by a decrease to approximately the same final level as in the reverse experiment, demonstrating the transient formation of interbilayer contacts in the presence of $\Delta\Psi$. A membrane potential with the opposite direction (positive inside) was not able to reverse the presequence-induced turbidity increase, as was revealed by employing valinomycin to LUVs with a Na^+_{in}/K^+_{out} ion gradient (not shown).

Fig. 2 shows the turbidity increase caused by addition of different amounts of presequence to LUVs. A threshold amount of presequence (corresponding to a peptide/lipid molar ratio of about 0.012) is required to give a

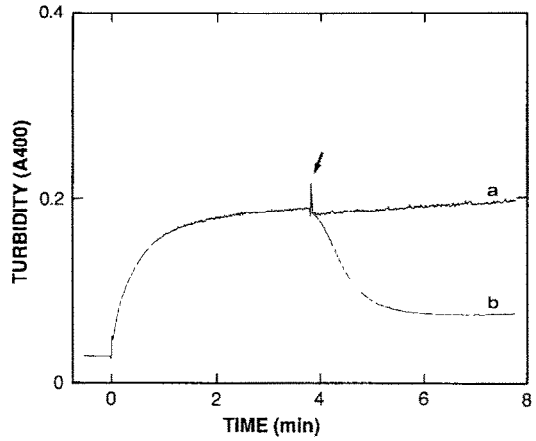

Fig. 1. Presequence-induced turbidity changes. At $t = 0$, 10 μM presequence is added to 300 μM DOPC/bovine heart CL (7/3 molar ratio) LUVs. At the arrow valinomycin is added to the vesicles (1:10^4 molar ratio with respect to total phospholipid) exhibiting a K^+_{in}/K^+_{out} (a) or a K^+_{in}/Na^+_{out} ionic distribution (b).

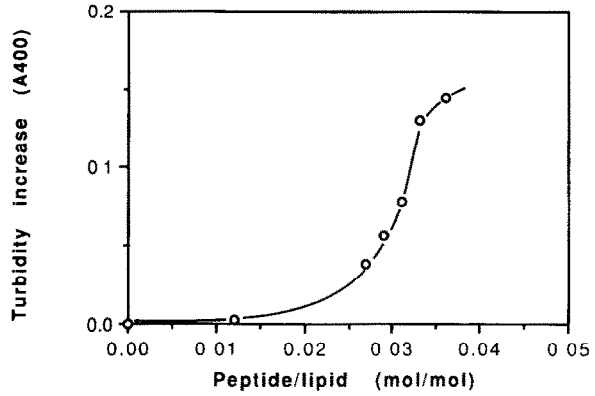

Fig. 2. Dependence of the presequence-induced turbidity increase of LUVs on the peptide to lipid ratio. Different amounts of presequence were added to 300 μM DOPC/bovine heart CL (7/3 molar ratio) LUVs. The turbidity increase 2 min after presequence addition is plotted against the peptide/lipid ratio.

detectable turbidity increase. Above a peptide/lipid ratio of approximately 0.025 the turbidity increases with the peptide /lipid ratio.

The interbilayer contact formation and the reversibility of the process were found to require the presence of bovine heart CL in the vesicles (Fig. 3). With 100% PC LUVs, no turbidity increase could be observed, even up to a peptide/lipid ratio of 0.08 (not shown). The presence of only 10% CL already gave rise to a presequence induced turbidity increase of the vesicle solution. In this respect it is important to notice that the mitochondrial membranes contain about 20% CL [39]. With higher contents of CL in the LUVs, the turbidity increase, caused by the presequence, was greater and approached a saturation level at approximately 30% CL (Fig. 3A). Parallel to this turbidity increase, the turbidity decrease after addition of valinomycin was larger with higher CL contents in the vesicles (Fig. 3B). The total turbidity decrease after induction of the membrane potential varied from 65% (10% CL) up to 95% (50% CL) of the total turbidity increase. Not only the total turbidity increase and decrease were affected, but also the initial rates of the turbidity changes, after the addition of presequence and valinomycin, respectively, were increased with higher CL content in the vesicles (data not shown).

To address the question whether the observed effects were specific for mitochondrial CL, a phospholipid which in the eukaryotic cell is only found in mitochondria [40], different negatively charged phospholipids were incorporated in PC vesicles, and the resulting turbidity increase and decrease upon addition of presequence and valinomycin, respectively, were compared to bovine heart CL-containing vesicles. The different negatively charged phospholipids that were examined are: DOPS, DOPG, DOPA, soybean PI, and *E. coli* CL. Presequence was added to LUVs, containing 20% negatively charged phospholipid and 80% DOPC, exhibiting a K^+_{in}/Na^+_{out} ion gradient. The resulting turbidity increases are shown in Fig. 4 (closed bars). No substantial turbidity increase could be observed for LUVs containing PI, PS or PG. This is very remarkable because in previous monolayer and conformational studies, no significant differences were observed between PG and CL in interaction with pCoxIV [20,23], demonstrating a unique lipid specificity in interbilayer contact formation. With CL from *E. coli*, only 40% of the effect which was obtained with CL from bovine heart was found. Vesicles containing PA gave the largest turbidity increase. However, when a membrane potential was induced upon addition of valinomycin, a decrease in turbidity (Fig. 4, open bars) could only be detected for the CL-containing vesicles, and hardly any decrease was observed for the vesicles containing DOPA. This suggests a different nature of the turbidity increase, for instance a more fusion-like, and thus irreversible, turbidity increase with the PA-containing vesicles. Alternatively, the membrane potential is not able to induce the same changes in the peptide–lipid aggregates as it does with the cardiolipin-containing vesicles.

The results obtained in the current study demonstrate the novel property of a mitochondrial presequence being able to induce interbilayer contacts, which can be reversed by the membrane potential. It is unclear how the interbilayer contacts are induced, but several mechanisms are possible, involving hydrophobic and hydrophilic interactions of the amphipathic presequence with the vesicles. Taking into account the direction of the potential (negative inside), the effect of $\Delta\Psi$ can be explained by a deeper insertion of the positively charged presequence into the bilayer, such that the contact with the opposing bilayer is lost. Most remarkable is the striking specificity in the observed effects for mitochondrial CL. No other negatively charged phospholipids

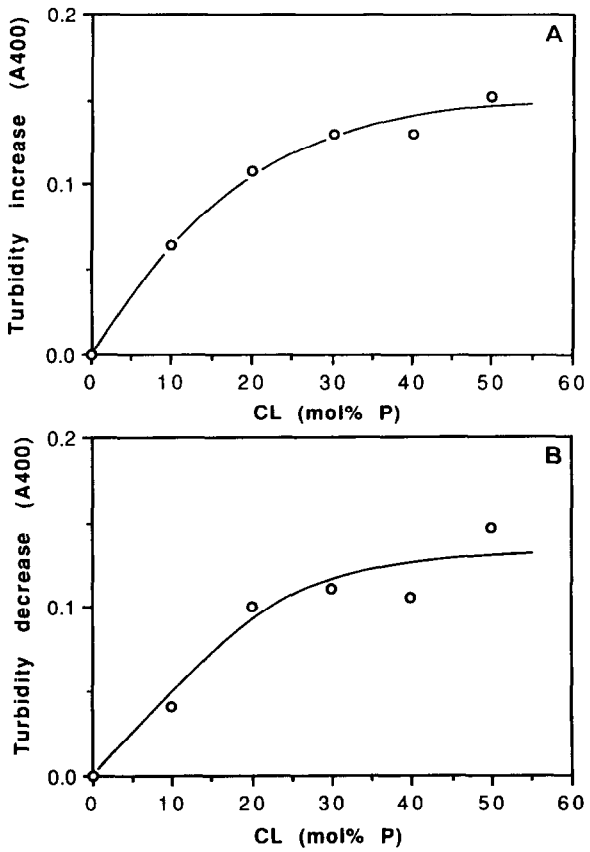

Fig. 3. The turbidity changes of LUVs of PC/CL, induced by the presequence and a membrane potential, are dependent on the CL content in the LUVs. The turbidity increase 2 min after addition of 10 μM presequence to 300 μM DOPC/bovine heart CL LUVs, exhibiting a K^+_{in}/Na^+_{out} ion gradient (A), and the turbidity decrease 3 min after valinomycin addition (B) are plotted against the CL content in the LUVs.

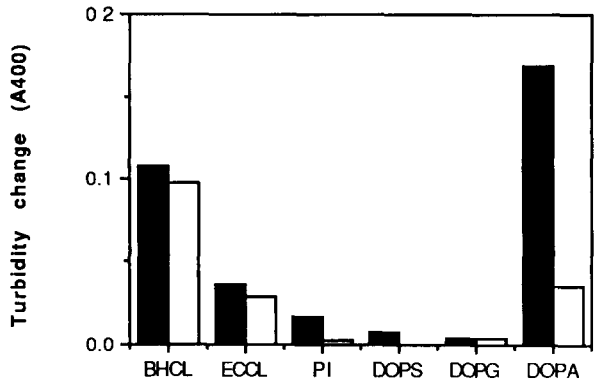

Fig. 4. Lipid specificity of the presequence and membrane potential modulated turbidity changes. LUVs were made of 80 mol% DOPC and 20 mol% negatively charged phospholipid. The different negatively charged phospholipids that were examined are: bovine heart CL (BHCL), *E. coli* CL (ECCL), soy bean PI, DOPS, DOPG and DOPA. The turbidity increase of 300 μM LUVs, exhibiting a K^+_{in}/Na^+_{out} ion gradient, 2 min after addition of 10 μM presequence, is given for the different negatively charged phospholipids containing LUVs (closed bars). The turbidity decrease 3 min after induction of a membrane potential is given as the open bars for the different negatively charged phospholipid-containing LUVs.

caused the same effects as bovine heart CL. Since the CL from *E. coli* gives only about 40% of the effect of the mitochondrial CL, the fatty acid composition plays a role in the formation of the interbilayer contacts. The fatty acid composition of bovine heart CL is unique in that it contains about 85% linoleic acid (18:2) [41] and therefore is much more unsaturated than *E. coli* CL (about 70% fully saturated; Killian et al., manuscript in preparation). A higher degree of unsaturation leads to a greater tendency to form aggregate structures with concave interfaces, such as the inverted H_{II} phase [42]. Such H_{II} phases can, among others, be induced by peptides [43]. For instance melittin, like pCoxIV, a positively charged amphiphilic peptide that forms an α-helix upon interaction with lipids, induces a H_{II}-phase in CL systems as a result of the deep penetration of the peptide inbetween the acyl chains [44,45]. Local formation of inverted non-bilayer structures have been proposed to play a role in intermembrane contact formation and protein transport [46]. It can be envisaged that the presequence bridges two bilayers by creating a local point semifusion contact between the opposing monolayers, which can be dissociated by the membrane potential because it pulls the presequence out of the contact area.

The fact that biological concentrations of CL in the vesicles is sufficient for the observed effects makes it interesting to speculate about the relevance for the mitochondrial import process. Our new finding of the presequence-induced CL-specific interbilayer contact formation supports the view that specific phospholipids play a role in the mitochondrial protein translocation process [47,48]. Previous studies demonstrated that CL participates, directly or indirectly, in the translocation of precursor proteins into mitochondria [47], and it was shown that the precursor proteins of P-450 (SCC), adrenodoxin and malate dehydrogenase have specific affinities for CL, which was due to presequence–cardiolipin interactions [48]. We argue yet another role for specific phospholipids in the import process. We propose that the presequence is involved in the formation and dissociation of mitochondrial contact sites. This proposal is supported by the growing evidence that contact sites are very dynamic structures [27–30,49]. Moreover, it has been demonstrated that CL is present in both mitochondrial membranes [39] and is enriched in the contact sites [50,51]. Our observations could be explained in terms of a model in which the presequence of a precursor protein, that is being imported into mitochondria, when it emerges from the outer membrane attaches to the inner membrane, thereby creating an intermembrane contact. After this contact has been formed the membrane potential across the inner mitochondrial membrane can act on the presequence, resulting in the subsequent translocation of the presequence

across the inner membrane. This $\Delta\Psi$ dependent translocation of the presequence could contribute to a dissociation of the inner and outer membrane contact.

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