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The N-terminal half of a mitochondrial presequence peptide inserts into cardiolipin-containing membranes

Consequences for the action of a transmembrane potential

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Abstract The orientation of a mitochondrial presequence peptide, associated with anionic lipid-containing model membranes, was investigated. The peptide inserts with its N-terminal α-helical part into cardiolipin (CL) monolayers so that the N-terminal 14 residues are protected from proteinase K. In phosphatidylglycerol (PG) monolayers the inserted peptide was fully accessible to the protease. A consequence of the different orientations of the peptide was that membrane potential-dependent protection from trypsin was much faster for the peptide bound to PG-containing vesicles compared to CL-containing membranes, suggesting that in the mitochondrial protein import process other components of the import apparatus are involved in the efficient potential-driven translocation of presequences across the inner mitochondrial membrane.

Key words: Mitochondrial presequence; Peptide topology; Monolayer insertion; Amino acid sequence analysis; Membrane potential

1. Introduction

Proteins, synthesized in the cytosol as precursors, and destined for the mitochondria, generally carry an amino terminal extra sequence, the presequence. This presequence is required for the import of these proteins into mitochondria and is proteolytically removed once it has reached the mitochondrial matrix. The import process involves binding to the mitochondrial outer surface, and translocation across one or two membranes with the help of mitochondrial import machineries (for reviews, see [1,2]). Translocation of the presequence across the inner mitochondrial membrane requires a membrane potential ($\Delta\psi$) across that membrane [3,4].

Although the amount of information about the import process and components involved is still growing, the molecular mechanism of the protein translocation process is largely unknown. This applies in particular to the molecular interactions the presequence is involved in. The presequence is assumed to interact with different proteinaceous components of the import apparatus, as well as with membrane lipids (cf. [5,6]). Of special interest is cardiolipin (CL), a phospholipid with a unique chemical structure, which in the eukaryotic cell is only synthesized [7] and found in mitochondria [8,9]. Several observations suggest that presequences have a special interaction with this lipid. Different mitochondrial proteins bind specifically to CL-containing liposomes [10,11]. CL can unfold precursor proteins [12], and synthetic presequence peptides can induce intermembrane contacts which depend on the presence of CL in the membranes [13–15]. Recently, a 2D ^{1}H-NMR study revealed that CL can modulate the secondary structure of the peptide, corresponding to the presequence of cytochrome oxidase subunit IV from yeast (p25) [16]. In that study, it was observed that, associated with dodecylphosphocholine micelles, there was α-helix formation in p25 from the N- to the C-terminus, with a break at the proline residue at position 13. Upon introduction of CL, an increased stability of the α-helix was observed around proline^{13} and in the C-terminal half. This suggests that the presequence–CL interaction results in a specific mode of insertion of the peptide in CL-containing membranes. This question is addressed in this study. It is demonstrated that in the presence of CL the N-terminal α-helical half is deeply embedded in the hydrophobic core of CL-containing membranes. The consequences of this CL-specific orientation of p25 for action of a transmembrane potential on the peptide are also investigated.

2. Materials and methods

2.1. Materials

Cardiolipin from bovine heart, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), and phosphatidylcholine from egg yolk (egg PC) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL, USA).

The peptide corresponding to the presequence of yeast cytochrome oxidase subunit IV (p25, H_3N^+-Met^1-Leu-Ser-Leu-Arg^5-Gln-Ser-Ile-

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Abbreviations: ATR, attenuated total reflection; CL, cardiolipin; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; egg PC, phosphatidylcholine from egg yolk; EDTA, (ethylenedinitrilo)tetraacetic acid; FTIR, Fourier transform infrared; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; π_i, initial surface pressure; $\Delta\pi$, surface pressure increase; $\Delta\psi$, membrane potential; LUVs, large unilamellar vesicles; p25, peptide corresponding to the presequence of cytochrome oxidase subunit IV; p25NBD, p25 labeled with 7-nitro-2,1,3-benzoxadiazol-4-yl; PK, proteinase K; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol

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Arg-Phe[10]-Phe-Lys-Pro-Ala-Thr[15]-Arg-Thr-Leu-Cys-Ser[20]-Ser-Arg-Tyr-Leu-Leu[25]-$CONH_2$) was prepared by Dr. A.I.P.M. de Kroon as described previously [17]. The peptide was purified and identified as described previously [14]. P25 was *S*-alkylated with *N,N'*-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-ethylene-diamide (IANBD amide) (Molecular Probes, Eugene, OR, USA) at Cys[19] (p25NBD) and purified by M. Maduke as described [18].

p25 peptide solutions were prepared at concentrations between 0.2 and 1 mM in water and stored at −20°C. p25NBD was dissolved at a concentration of 0.2 mM in 50% ethanol and stored at −20°C.

2.2. Monolayer experiments

All monolayer experiments were carried out in Teflon dishes at 22°C as described by Török et al. [15]. The experiments using p25 were performed in a 6 ml dish with a surface area of 8.8 cm^2. Monolayers of DOPG or CL were spread on a subphase of 150 mM Na_2SO_4, 20 mM HEPES (pH 7.0) and 2 mM EDTA from $CHCl_3$:CH_3OH (4:1, v/v) to give the desired initial surface pressure. Peptide and proteinase K (PK, Boehringer Mannheim GmbH, Mannheim, Germany) were added through a hole in the Teflon chamber connected to the stirred subphase.

In experiments aimed at identifying the protected presequence peptide fragment, the subphase was washed by injecting and ejecting buffer solution and, subsequently, water, at opposite sides of the dish at a rate of 13 ml/min. After washing of the subphase, monolayers were collected in a vial as described by Rietsch et al. [19] and lyophilized for sequence analysis.

2.3. Sequence analysis

Samples were redissolved in 50 μl H_2O, and subsequently analyzed for N-terminal amino acid sequence of the peptide (fragments) by the Sequentiecentrum (Utrecht, The Netherlands). The analysis was carried out on an Applied Biosystems Protein Sequencing system (model 476A), using the Edman degradation procedure, followed by 'on-line' microbore HPLC identification of the phenylthiohydantoin derivatives of the amino acids. The samples were analyzed for 20 subsequent steps. For comparison of the repetitive yield, p25 was also analyzed.

2.4. Vesicle preparation

Dry lipid films were hydrated by manual shaking or vortexing in buffer consisting of 50 mM K_2SO_4, 10 mM HEPES (pH 7.0) and 1 mM EDTA (fluorescence spectroscopy) or in water (infrared (IR) spectroscopy), and subsequently frozen and thawed 10 times. Large unilamellar vesicles (LUVs) were prepared by the extrusion technique [20] using 100 nm polycarbonate filters.

2.5. IR spectroscopy

Samples were prepared by addition of 18.4 nmol p25, preincubated with 50 mM dithiothreitol for 30 min at room temperature, to 0.184 μmol LUVs (phosphorus) consisting of either CL/DOPC (2/8, w/w) or DOPG/DOPC (2/8, w/w), in the presence of 50 mM dithiothreitol. The lipid/peptide mixture was incubated for 30 min at 37°C, after which LUVs with bound p25 were isolated by sucrose gradient centrifugation, as described in Vandenbussche et al. [21]. The LUVs/peptide complex was washed 3 times by centrifugation (35 min, 300 000 × *g*), dried on a clean Ge internal reflection element by slow evaporation at room temperature under a N_2 stream, and rehydrated under a N_2/D_2O flow for 2 h.

Fourier transform attenuated total reflection (FT-ATR) infrared spectra were recorded at room temperature on a Perkin-Elmer 1720X FTIR spectrophotometer, equipped with a mercury-cadmium-telluride detector. Spectra were recorded at a nominal resolution of 4 cm^{-1} and encoded every 1 cm^{-1}.

Germanium crystals (50×20×2 mm, Harrick EJ 2121) were cleaned by washing with distilled water, methanol and chloroform, and subsequently placed for 5 min in a Plasma Cleaner PDC-23G (Harrick Scientific, Ossining, NY, USA). The crystals were placed under an aperture angle of 45°, yielding 25 internal reflections. A clean germanium plate was used for simultaneous background correction. Perkin-Elmer gold wire grid polarizers were placed in front of the sample and reference holder, making it possible to record spectra at 0° and 90° polarization of the incident light beam. The determination of the secondary structure and of the orientation of the α-helix with respect to a normal to the Ge crystal was performed as described [22].

2.6. Fluorescence spectroscopy

Fluorescence measurements were performed at 20°C on an SLM-Aminco SPF-500C fluorometer, using an excitation wavelength of 485 nm and an emission wavelength of 540 nm (5 nm band passes).

2.6.1. KI quenching

These experiments were performed under conditions that all peptide was bound to the LUVs consisting of CL/DOPC or DOPG/DOPC (both 2/8, molar ratio, based on phosphorus concentrations). p25NBD binding to the LUVs was determined as described [18]. For measuring the fluorescence quenching of free p25NBD in buffer (50 mM K_2SO_4, 10 mM HEPES (pH 7.0), 1 mM EDTA), a 100 nM peptide concentration was used. Quenching in the presence of LUVs was performed with 20 nM p25NBD and 200 μM LUVs (phosphorus concentration). To 1 ml of these solutions, KI was added in increasing amounts from a 4 M stock solution containing 1 mM $Na_2S_2O_3$ to prevent I_2 and I_3^- formation, and the fluorescence intensity was read at 540 nm. The values were corrected for dilution and vesicle scattering. Data were analyzed according to the Stern-Volmer equation for collisional quenching [23]: $F_0/F = 1 + K_{SV}.[KI]$, where K_{SV} is the Stern-Volmer quenching constant, and F_0 and F are the (corrected) fluorescence intensities in the absence and presence of quencher. To correct for a possible decrease in binding of p25NBD upon increasing the salt concentration, F_0 was determined in a similar titration experiment using KCl instead of KI.

2.6.2. Protease protection

Time- and potential-dependent protection of vesicle-bound p25NBD from added trypsin was measured essentially as described [18]. These experiments were also performed under conditions that all peptide was bound to the vesicles. LUVs (40 mM) were made in buffer containing 50 mM K_2SO_4, 10 mM HEPES (pH 7.0), and 1 mM EDTA, and consisted of CL/egg PC or POPG/egg PC (both 2/8 molar ratio, based on phosphorus). Valinomycin (Boehringer Mannheim; 9 μM final concentration) was added (from a 1 mg/ml stock solution in ethanol) directly to this lipid solution. LUVs were diluted 100-fold in 1 ml buffer, containing 50 mM Na_2SO_4, 10 mM HEPES (pH 7.0), and 1 mM EDTA, to generate a membrane potential. For control experiments, LUVs were diluted in K_2SO_4-containing buffer, so that no $\Delta\psi$ could be generated. To this solution, at t=0, p25NBD was added (20 nM final concentration). At different time points, trypsin (Sigma Chemical Co., St. Louis, MO, USA; 50 μg) was added, and the percentage protected peptide was determined from the fraction of the initial fluorescence of p25NBD bound to the LUVs remaining after trypsin treatment.

2.7. Other methods

p25 peptide concentrations were determined using a micro-BCA protein assay (Pierce, Rockford, IL, USA), using bovine serum albumin as a standard. p25NBD concentration was determined by amino acid analysis. Phosphorus concentrations were determined according to Rouser et al. [24]. CL concentrations are always given as phosphorus concentrations.

3. Results

3.1. The N-terminal half of the presequence peptide inserts specifically in CL monolayers

In the present study, PG was chosen for comparison with CL. PG can be considered to be a representative for other anionic phospholipids, like phosphatidylserine and phosphatidylinositol, which show a less specific interaction with presequences [10,13], and moreover, PG and CL are structurally related. To get insight into the differences in topology of the inserted presequence peptide in either CL- or PG-containing membranes, monolayer experiments were done, in which proteinase K was used as a tool to monitor the accessibility of the inserted peptide. This approach has the advantage that the interactions and their consequences for lipid packing can be continuously recorded. Typical experiments, using either a CL monolayer (A) or a PG monolayer (B), are shown in Fig. 1.

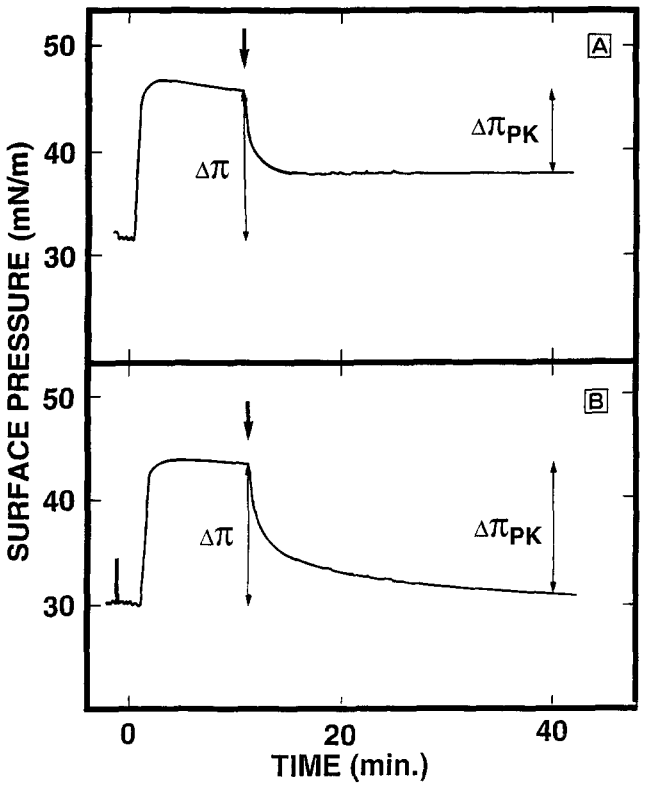

Fig. 1. Proteinase K digestion of p25, inserted into a monolayer of CL (A) or DOPG (B). p25 was injected to a subphase concentration of 1.5 μM underneath a lipid monolayer at t=0, resulting in a surface pressure increase (Δπ). At the points indicated with the arrows, PK (30 μg/ml) was added to the subphase, resulting in a decrease in surface pressure, defined at 30 min after PK addition as $\Delta\pi_{PK}$.

A monolayer was spread to an initial surface pressure (π_i) of 30–31 mN/m, and, at t=0, p25 was injected underneath it, resulting in a large surface pressure increase (Δπ), similar for both lipid systems, indicating the insertion of the presequence. After 10 min, PK was added to the subphase, resulting in a fast surface pressure decrease, which in the case of the PG monolayer continues until after approximately 30 min the surface pressure has reached the π_i value of the lipid monolayer without peptide. However, in the case of the CL monolayer, the surface pressure initially decreases rapidly, but then the decrease slows down or even stops, and a certain part of the p25-induced Δπ remains ($\Delta\pi-\Delta\pi_{PK}$). Addition of an extra amount of PK did not result in a further decrease of the surface pressure, demonstrating that the amount of protease was not the limiting factor. Mastoparan, which like p25 is a positively charged amphipathic α-helical peptide [25], did not display such CL-specific protection under similar conditions (data not shown), demonstrating the presequence specificity of this observation. These results indicate that in CL, and not in PG monolayers, part of the presequence peptide is deeply inserted and cannot be digested by PK. This part can either be a part of each peptide molecule, so that approximately half of the peptide is inserted in a way that it cannot be reached by the protease, while the other half of the molecule is readily digested. Or it could be that half of the total amount of peptide molecules is localized in such a way that it is not digested by PK, while there is a second population of molecules that is inserted, but accessible to the protease.

In order to distinguish between the two possibilities, the PK-protected part was identified. An experiment was done in which PK was added to the subphase of a CL monolayer with p25 inserted into the monolayer (as in Fig. 1A). After the surface pressure had stabilized, the subphase was washed with buffer, and subsequently with water to remove PK, digested p25 and salts. Next, the monolayer with the protected peptide was collected, lyophilized and dissolved in a small volume of water, and the sample was analyzed by N-terminal peptide sequence analysis. The result of the HPLC analysis of the first cycle is shown in Fig. 2. By far the most abundant residue present in this analysis was a methionine, in p25 only present as the first amino acid. With an estimate of the amount of inserted p25 in the monolayer, the yield of this first cycle of the amino acid sequence analysis is approximately 50%. The same yield was obtained when a known amount of intact p25 was analyzed by N-terminal sequence analysis. This demonstrates that the majority of the amount of peptide which was responsible for the induced surface pressure increase has an intact N-terminus, already indicating that a part of each peptide was digested by PK and not part of the total amount of inserted peptide. The analyses of the following 14 cycles each resulted in the expected subsequent residue of p25 being the main amino acid found by HPLC analysis (except for the serine residues, which are underestimated in this procedure; data not shown). For cycle 15, no distinct peak of threonine was detectable, confirming that part of p25 had indeed been removed by the proteinase. These results demonstrate that the N-terminal 14 residues of p25 were protected against PK treatment of p25 inserted into a CL monolayer.

3.2. *Lipid specificity of Δψ-driven movement of the presequence peptide*

In an earlier study, Maduke and Roise [18] concluded that upon application of a potential across the membrane of PG/PC LUVs, p25NBD could be translocated into the lumen of the vesicles. Because of the observed different mode of insertion of p25 with either PG- or CL-containing membranes, the consequences for the Δψ-driven translocation of p25NBD across the different bilayers were investigated. Therefore, the

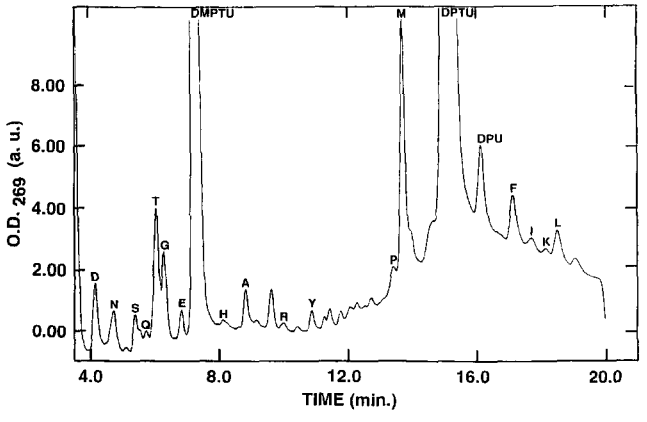

Fig. 2. HPLC analysis of the first cycle of the amino acid sequencing of a collected monolayer sample. To the subphase of a CL monolayer with p25 inserted, PK was added (see Fig. 1A) and, after 30 min, the subphase was washed with buffer and subsequently water. After this, the monolayer was collected, lyophilized, and subjected to amino acid analysis. The amino acids are indicated by their one-letter abbreviations. DMPTU, DPTU and DPU are by-products of the Edman degradation chemistry.

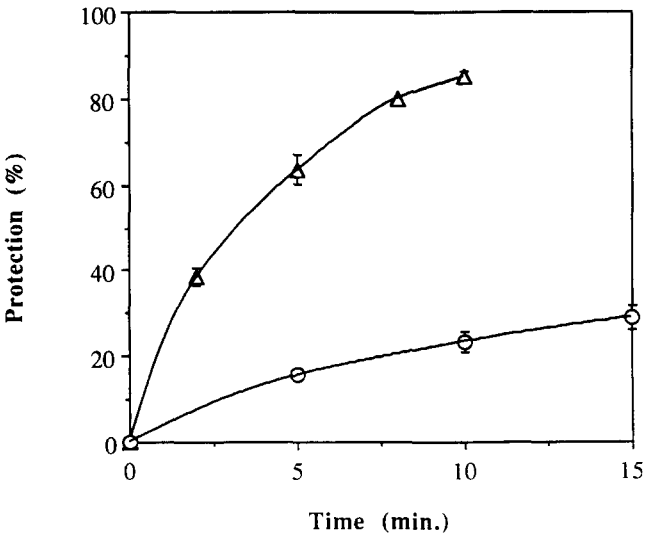

Fig. 3. Time-dependent protection of 20 nM p25NBD against added trypsin (50 μg). In the presence of 400 μM PG/PC (2/8 molar ratio, triangles) or CL/PC (2/8 phosphate molar ratio, circles) LUVs, experiencing a membrane potential. A membrane potential (negative inside) was generated by diluting LUVs, made in K^+-containing buffer, 100-fold in Na^+-containing buffer, in the presence of valinomycin. The percentage protection is calculated as the fraction of fluorescence remaining after trypsin treatment, corrected for vesicle scattering and for NBD fluoresence of p25NBD in the absence of vesicles, but in the presence of trypsin. The error bars indicate the standard deviations (n=4).

time- and $\Delta\psi$-dependent protection of p25NBD from digestion by externally added trypsin was investigated for the two lipid systems. These experiments were done under conditions that all p25NBD was bound to the LUVs. The results, shown in Fig. 3, confirm the earlier observation [18] that, in the presence of PG/PC LUVs, protection of p25NBD is fast, such that 80% of the p25NBD present is protected from trypsin digestion in 10 min. However, in the CL-containing system, time-dependent protection of that part of the peptide containing the NBD moiety is much slower. If PK was used instead of trypsin the results were identical. In all cases, protection was $\Delta\psi$-dependent, because in the absence of $\Delta\psi$ p25NBD was sensitive to digestion by the protease ($< 10\%$ protection). This suggests that the part of the peptide containing the NBD moiety is surface-localized in the case of both PG- and CL-containing LUVs. It can thus be concluded that the $\Delta\psi$-driven inward movement of the C-terminus of the presequence does occur in the case of CL-containing vesicles, but is very much slower than in the case of PG-containing vesicles.

4. Discussion

The results of this study demonstrate a different topology of a mitochondrial presequence peptide inserted into CL-containing membranes, compared to PG-containing membranes. This was concluded from a number of experiments. Firstly, the monolayer experiments demonstrate that only in the case of CL was a defined fragment of the peptide protected from protease digestion. Sequence analysis identified residues 1–14 as being protected in the monolayer. Secondly, from previous FTIR results [26] it can be concluded that the interaction of the presequence peptide with CL leads to an insertion of the N-terminal α-helix parallel to the acyl chains. This result was reproduced in our experimental system and compared with the peptide upon interaction with PG/PC membranes, for which no preferred orientation of the helical content of the peptide was found (not shown). Thirdly, despite the fact that upon binding both to CL- and to PG-containing LUVs, the part of peptide containing the NBD, attached to cysteine[19], could be readily digested by protease, the precise topology of the C-terminal half of the peptide appeared to be different in the two systems. Iodine quenching experiments revealed that the NBD was inserted more deeply in the case of PG/PC LUVs (K_{SV} =1.0 M^{-1}) than CL/PC LUVs (K_{SV} =1.7 M^{-1}; data not shown), which is in good agreement with the recently described results on p25, spin-labeled at cysteine[19], using electron spin resonance spectroscopy [27]. Fourthly, the different mode of insertion into the two lipid systems is supported by the molecular areas reported for the p25 in CL and PG monolayers. This area was determined to be 330 $Å^2$ [15] and 530 $Å^2$ [28] for CL and PG, respectively. The difference can now be explained by a different orientation of the N-terminal half of the peptide. In CL monolayers it inserts with the helix axis parallel to the acyl chains, which results in a protected localization for the N-terminal 14 residues and a small area for that part of the peptide, while the C-terminal half is oriented at the lipid-water interface, making the major contribution to the molecular area. In PG monolayers the amphipathic helix most probably lies parallel to the lipid-water interface, as proposed by Tamm [28], resulting in the larger molecular area. The proposed topology of the peptide at a PG-containing membrane surface is in agreement with the fact that the full length of the presequence was found to be accessible to the protease when inserted in a PG monolayer (this study) and with the recently determined topology of p25 bound to PG/PC (1/9 molar ratio) bilayers using electron paramagnetic resonance spectroscopy [29].

Taken together, these findings suggest a different behavior for the N- and the C-terminal halves of the presequence peptide in interaction with CL-containing membranes. In a previous study, the effect of CL on the secondary structure of p25 was investigated by 2D ^{1}H-NMR [16]. It was found that in the presence of CL there was an increased helical stability in the region around proline[13] and in the C-terminal half. Based on these findings, a structure of p25 was proposed in which the helix is bent due to specific interactions with CL. The data of the present study are consistent with such a structure. The amino terminal α-helical part inserts deeply into the hydrophobic core, oriented with its helix axis parallel to the lipid acyl chains. The C-terminal half is superfacially localized at the membrane surface, which might be involved in intermembrane contact formation, as also suggested by Snel et al. [27]. This presequence peptide-induced process was found to require the presence of CL in the membranes [13,15], further demonstrating the involvement of specific interactions between the peptide and CL. A molecular model of the topology of peptide in the two different systems has been described by Leenhouts et al. [30].

The unique mitochondrial localization of CL and the observed specific interactions between CL and mitochondrial presequences suggest a role of this lipid in the mitochondrial protein import pathway. Because of the localization of CL primarily in the inner mitochondrial membrane, it would be likely that CL is involved in the specific insertion of the pre-

sequence into the inner mitochondrial membrane. One possibility is that CL provides a correct orientation of the presequence to interact with the proteinaceous components of the import machinery. Another component that is required for presequence translocation across the inner mitochondrial membrane is the transmembrane electrical potential. It has been demonstrated that $\Delta\psi$ electrophoretically moves the presequence across the inner mitochondrial membrane in the protein import process [4], which has been proposed to drive the vectorial translocation of precursor proteins across the inner membrane to the next step of the protein import pathway, being the ATP-dependent binding to the MIM44/mt-hsp70 complex [31]. Maduke and Roise [18] have described that also in protein-free lipid vesicles p25NBD could be translocated into the lumen of PG/PC LUVs upon application of a transmembrane potential. In order to investigate whether the different topology of the presequence peptide affects this $\Delta\psi$-driven movement of the peptide into the membrane, the action of a membrane potential on p25NBD associated with PG/PC LUVs was compared to that on p25NBD bound to CL/PC LUVs. Upon application of a $\Delta\psi$ across the membranes (negative inside), the inward movement of the C-terminal half was much faster when the peptide was bound to PG-containing LUVs, compared to CL/PC LUVs. This suggests that the specific topology of p25 in the presence of CL results in a less efficient action of the membrane potential on the peptide. This can be explained by the fact that the C-terminal half, which contains the NBD label on cysteine[19], is localized more deeply in the case of the membrane orientation of p25 bound to PG/PC, compared to when the peptide is associated with CL/PC, so that the inward movement of this label is faster when p25NBD is bound to PG/PC LUVs. However, p25 associated with CL-containing LUVs could be pulled into the membrane by a transmembrane potential. The much slower time scale of this process suggests that in the mitochondria other components of the import apparatus are involved in the efficient translocation of presequences across the inner mitochondrial membrane.

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