Cardiolipin modulates the secondary structure of the presequence peptide of cytochrome oxidase subunit IV: a 2D ^{1}H-NMR study

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Abstract The secondary structure of the presequence of cytochrome oxidase subunit IV (p25) was studied by circular dichroism and 2D nuclear magnetic resonance in micelles of dodecylphosphocholine (DPC) and mixed micelles of DPC and mitochondrial cardiolipin (CL). In both systems, α-helix formation was observed. The α-helix stretches from the N- to the C-terminus with a break at the proline residue at position 13. Upon introduction of CL in the DPC micellar system, an increased stability of the helix was observed around proline[13] and in the C-terminal half. This observation, together with reported results on specific interactions between CL and p25, led to the proposal of a two-state equilibrium of the α-helical conformation of p25, modulated by CL.

Key words: Mitochondrial presequence; 2D NMR; Micelle; Cardiolipin; Conformational flexibility; Lipid–protein interaction

1. Introduction

Nuclearly encoded proteins which are destined for the mitochondria, are synthesized in the cytosol as precursors carrying an amino terminal extension, the presequence (for a review on mitochondrial protein import, see [1]). These presequences contain the information to target the precursors into the mitochondria and are proteolytically removed once they have reached the mitochondrial matrix. It is assumed that presequences interact with both protein and lipid components of the mitochondrial membranes during protein import. Although hardly any sequence homology exists among presequences from different proteins, they are generally enriched in positively charged, hydroxylated and apolar amino acid residues [2]. This suggests that some similarity in the secondary structure must be more important for their function than a specific sequence. It has been suggested that presequences have the potential to form an amphiphilic α-helix [2]. Support for this comes from (i) statistical analysis of mitochondrial presequences [2]. (ii) Analysis of the import competence of both artificial peptides that were positively charged amphiphiles, which indeed were able to target attached proteins into the mitochondrial matrix [3]; and of hybrid proteins encoded by clones of random fragments of *E. coli* DNA and the yeast coxIV gene, lacking its own presequence [4]. (iii) CD observations that synthetic presequence peptides from different precursors can adopt an α-helical conformation upon interaction with phospholipid vesicles [5], micelles [6] and in bulk solvent mixtures that also mimic a membrane environment [7]. More detailed information on the conformation of several synthetic mitochondrial presequence peptides in membrane-like environments was obtained using high-resolution 2D NMR. The peptide, corresponding to the presequence of yeast cytochrome oxidase subunit IV, also denoted as p25 [8], as well as the presequence peptide of rat liver aldehyde dehydrogenase [9] and a mutant peptide lacking the flexible linker comprising residues 11–13 [10], were investigated in the presence of DPC micelles. The presequence peptide of F_1-ATPase β-subunit [11] and the non-cleavable N-terminal targeting extensions of rat rhodanese, rat 3-oxalyl-CoA thiolase [12] and rat chaperonin 10 [13] were analysed in aqueous trifluorethanol solutions. The results showed that the rat liver aldehyde dehydrogenase and F_1-ATPase β-subunit presequences contain two α-helices separated by a flexible 'linker' region. The helix-break-helix motif has not been observed for the mutant peptide of the rat liver aldehyde dehydrogenase presequence lacking residues 11–13, nor for the N-terminal targeting sequences of rat rhodanese and rat 3-oxalyl-CoA thiolase. These peptides, which are not processed upon protein import, form one α-helix which runs from the N- to the C-terminus. Surprisingly, the non-cleavable targeting signal of rat chaperonin 10 does contain the helix-turn-helix motif, although it is not processed upon import. P25 bound to DPC micelles was reported to contain one helix at the N-terminus followed by a less structured region, of which the precise secondary structure remained to be defined.

It is unknown whether the location and size of the α-helices induced in presequence peptides depend on the type of lipids present. This question is particularly important because presequences strongly interact with lipid membranes, with a preference for anionic lipids (see for review [14]). Of special interest is CL, an anionic phospholipid which in the eukaryotic cell is only synthesized [15] and found [16,17] in mitochondria. Recently, a number of studies revealed specific interactions between the presequence peptide p25 and CL. It was shown that p25 is able to induce intermembrane contacts between model membranes with a remarkable specificity for CL [18–21]. More-

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Abbreviations: CD, circular dichroism; CL, cardiolipin; 2D, two-dimensional; 3D, three-dimensional; DPC, dodecylphosphocholine; DPC-d_{38}, [$^2H_{38}$]dodecylphosphocholine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, 2D nuclear Overhauser enhancement spectroscopy; p25, peptide corresponding to the presequence of cytochrome oxidase subunit IV; PC, phosphatidylcholine; TOCSY, 2D total correlation spectroscopy; TPPI, time-proportional phase increment.

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over, the mode of insertion of p25 into the lipid phase is strongly and specifically modulated by the presence of CL [22,23].

In this study, we analyse by CD and 2D NMR the structure of p25 associated with DPC micelles. Because of their small size, micelles undergo rapid isotropic motion, yielding a high-resolution NMR spectrum for any associated peptide. The effect of CL on the secondary structure of p25 was investigated by incorporation of approximately two molecules of non-deuterated CL per DPC micelle. The results indicate that in the micelle-bound form p25 contains two helices separated by proline[13]. The helical content is higher at the N-terminal part. The presence of CL increases the helical content around Pro[13] and at the C-terminus. This study allowed us to propose a two-state equilibrium of the α-helical structure of the presequence peptide, modulated by CL.

2. Materials and methods

2.1. Materials

Perdeuterated DPC-d_{38} was obtained from MSD Isotope (Montreal, Canada). Sodium acetate-d_3 and ^2H_2O were from Isotec (Miamisburg, USA). 3-(Trimethylsilyl)-propionate-d_4 was from Merck (Germany). CL from bovine heart was obtained from Avanti Polar Lipids (Birmingham, 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-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 as described previously [24].

2.2. NMR measurements

For the preparation of the NMR samples, p25 was dissolved in buffer, containing 20 mM sodium acetate-d_3, 3 mM dithiothreitol, 0.1 mM (ethylenedinitrilo)tetraacetate, 0.1 mM 3-(trimethylsilyl)-propionate-d_4 in either 90% H_2O/10 %^2H_2O or ^2H_2O, pH 4.6 (direct reading), and mixed with a micelle solution of DPC or DPC/CL (30/1, mol/mol) in the same buffer. The final concentration of p25 was 3 mM (based on weight). The molar ratio p25/detergent of 1/60 was used to obtain an approximate occurrence of 1 peptide molecule per micelle. At higher pH values, the quality of the NMR spectra in the NH region was significantly reduced due to line broadening. All NMR experiments were performed at 25°C in an argon atmosphere to avoid oxidation. NMR spectra were recorded on Bruker AMX spectrometers operating at 500 or 600 MHz proton frequencies (the latter at the SON hf-NMR facility, Nijmegen University, The Netherlands). Chemical shifts were measured relative to 3-(trimethylsilyl)-propionate-d_4. TOCSY spectra were recorded using a clean MLEV-17 mixing sequence [25,26] with total mixing times of 30–50 ms which include the delays of the clean-TOCSY pulse scheme. NOESY spectra [27,28] were recorded with a mixing time of 100, 200 and 300 ms. All 2D spectra were recorded in a phase-sensitive absorption mode using TPPI in t_1 [29]. The cross-peaks around 4.6 p.p.m., which are normally irradiated together with the water line were made observable using the SCUBA method [30]. In order to assess the Hα–Hβ cross-peaks under the water line, spectra were recorded in ^2H_2O. 2D spectra were collected as a 400–512 (t_1) real and 1024 (t_2) complex point time domain matrix with a spectral width of 5000 Hz (^1H = 500 MHz) or 6000 Hz (^1H = 600 MHz) in both dimensions and 80–160 scans per t_1 increment. Data were transformed after zero-filling in the F1 dimension, into 1024 and 1024 real points in the F1 and F2 dimension frequency-domain spectra. Sixth-order polynomial baseline corrections in each domain were applied after the double Fourier transformation was completed [31]. The data were processed with the 'Triton' software library (Bijvoet Center for Biomolecular Research, Utrecht University, The Netherlands). 3D structures were build using the program Insight II on an Indy workstation (Silicon Graphics, Mountain View, USA).

Table 1
Chemical shifts of p25 in different environments at pH 4.6 and 25°C

	water		DPC		DPC/CL	
Residue	NH	αH	NH	αH	NH	αH
Met1		4.15		4.14		4.12
Leu2	8.71	4.46	8.50	4.40	8.41	4.40
Ser3	8.50	4.49	8.35	4.70	8.33	4.69
Leu4	8.40	4.38	9.26	4.14	9.27	4.14
Arg5	8.36	4.29	8.66	3.92	8.70	3.92
Gln6	8.35	4.36	7.86	4.14	7.87	4.14
Ser7	8.34	4.45	8.10	4.31	8.07	4.28
Ile8	8.13	4.15	7.75	3.91	7.73	3.90
Arg9	8.23	4.25	7.68	4.08	7.67	4.08
Phe10	8.13	4.62	7.75	4.43	7.76	4.41
Phe11	8.07	4.59	7.76	4.44	7.77	4.40
Lys12	8.17	4.51	8.22	4.33	8.32	4.31
Pro13		4.35		4.37		4.37
Ala14	8.49	4.37	8.29	4.27	8.20	4.23
Thr15	8.15	4.32	8.08	4.23	8.10	4.22
Arg16	8.44	4.43	8.14	4.19	8.14	4.16
Thr17	8.25	4.32	7.93	4.24	7.94	4.23
Leu18	8.40	4.41	8.14	4.19	8.14	4.16
Cys19	8.42	4.45	8.14	4.34	8.14	4.32
Ser20	8.45	4.49	8.10	4.47	8.05	4.47
Ser21	8.39	4.47	8.21	4.37	8.20	4.35
Arg22	8.24	4.25	8.22	4.08	8.20	4.07
Tyr23	8.14	4.58	7.86	4.37	7.84	4.37
Leu24	8.03	4.32	7.69	4.15	7.67	4.15
Leu25	7.93	4.30	7.64	4.22	7.61	4.22

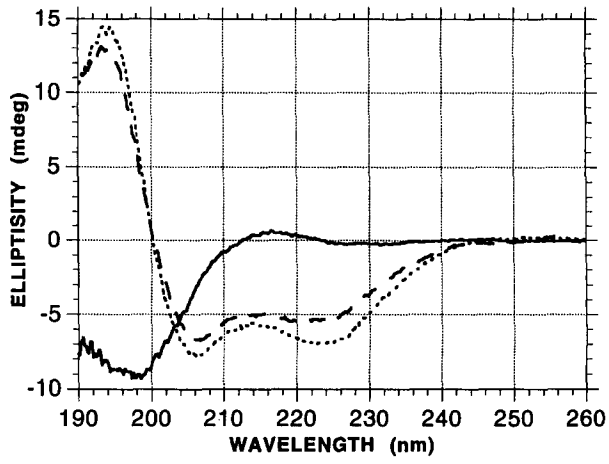

Fig. 1. CD spectra of p25 in water (solid line) and upon interaction with DPC (– – –) and DPC/CL (.....) micelles at 25°C.

2.3. CD

CD measurements were carried out on a JASCO 600 spectropolarimeter, using a 0.2-mm path length cell, with a 1-nm bandwidth, 0.1-nm resolution, 1-s response time and a scan speed of 20 nm/min. Typically, 8 scans were added and averaged, followed by subtraction of the CD signal of peptide-free micelle solutions recorded under the same conditions. Spectra shown are from NMR samples diluted to a final peptide concentration of 100 μM. Identical results were obtained when samples were prepared freshly. The helix content was estimated by the method of Greenfield and Fasman [32].

3. Results

CD was used to get a first indication about the secondary structure induced in the peptide in the presence of the different detergent systems (Fig. 1). The CD spectrum of p25 in aqueous solution is consistent with that of a random coil and does not show any features characteristic for an α-helix. In micelle envi-

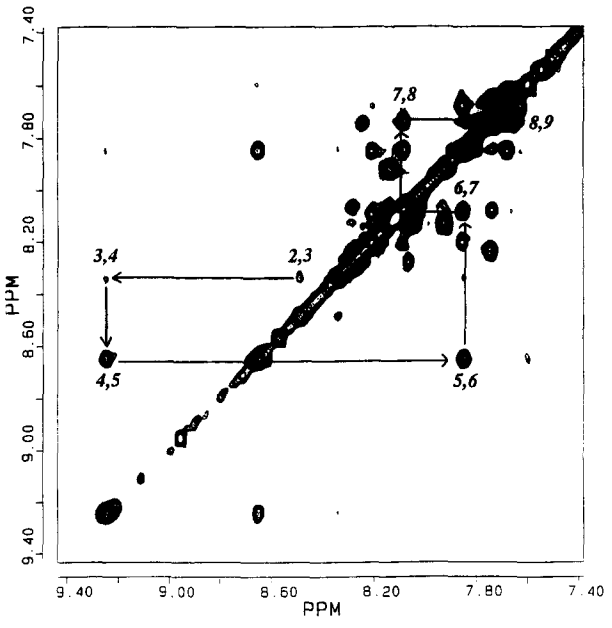

Fig. 2. Part of NH–NH region of a 300-ms NOESY spectrum of p25 in DPC micelles showing sequential connectivities.

r onments, the CD spectra are typical of a highly α-helical con-f rmation. From computer deconvolutions of the CD spectra, the helical content of the peptide was estimated to be 65 and 75% in the presence of DPC and DPC/CL micelles, respectively.

For 2D NMR data analysis, the standard method proposed by Wüthrich [33] was used to carry out the sequential assignment of all the proton resonances of the presequence peptide in different environments. TOCSY spectra were used to identify spin systems, and NOESY spectra were used to obtain interresidue connectivities and to distinguish equivalent spin systems. Unambiguous assignments were obtained with the help of sequential NH(i)–NH(i + 1), Hα(i)–NH(i + 1) and other NOE connectivities characteristic of α-helical conformation. Fig. 2 shows as example part of the NH-NH region of the NOESY spectrum of p25 in DPC micelles. In this spectrum, sequential connectivities from Leu2 to Arg9 are indicated. The assignment for all the protons of the peptide in DPC micelles was in agreement with Endo et al. [8]. In Table 1, the chemical shift values of the Ha and NH protons of the peptide are compared in water, DPC and DPC/CL micelles.

The determination of the secondary structure of p25 in the membrane-like environments involved identifying NOE cross-peaks between protons on non-neighboring residues and chemical shifts of backbone protons. The NOE connectivity pattern for an α-helix consists of medium Hα(i)–Hβ(i + 3) and weak Hα(i)–NH(i + 3) medium-range contacts. The medium-range Hα(i)–Hβ(i + 3), Hα(i)–NH(i + 3) and sequential NOE connectivities are summarized in Fig. 3 for the presequence peptide in DPC and DPC/CL micelles. Comparison of the conformation of the peptide in two different micelle systems by comparing the secondary structure-sensitive interresidue NOE cross-peaks was hampered by cross-peak overlap problems and by quantification difficulties due to dynamical effects. The intensities of the medium range NOEs for p25 are relatively low compared with those for the bacterial signal peptides of OmpA [34] or PhoE [35] associated with similar micelles. This can be explained by the fact that p25 is less hydrophobic than the bacterial signal peptides, resulting in a dynamical equilibrium between water-soluble (random coil) and micelle-bound (α-helical) forms of p25. The rate of exchange between these two conformations is fast on the NMR time scale as indicated by the presence of only one set of the resonances observed in NMR spectra. Consistent with this explanation, the ^1H–^2H backbone exchange was found to be much faster (data not shown) than previously reported for the PhoE signal peptide [35].

Therefore, Hα proton chemical shift values (see Table 1) were used as an additional tool for structural analysis. It has been shown that the difference between the Hα proton chemical shifts in a protein structure and the chemical shifts in a random coil also correlates with protein secondary structure. An α-helix

Fig. 3. Sequential and medium-range NOE contacts observed in the NOESY spectra of p25 in DPC and DPC/CL micelles. The thickness of the horizontal bars is a qualitative indication of the relative intensity of the sequential NOEs as observed in NOESY spectra. Solid bars indicate NOEs that have been identified, while asterisks indicate NOEs whose presence or absence cannot be assessed due to overlap.

structure is indicated by an upfield chemical shift of > 0.1 p.p.m. with respect to random coil chemical shift values [36]. For peptides that are visiting both random coil and α-helical conformations, one can expect that the chemical shift difference of Hα resonances between micelle and water solution is proportional to the amount of time the residues spend in an α-helical conformation. Thus, the chemical shifts of Hα protons can be used in a qualitative manner to analyze the conformation of peptides [34]. For p25 in the presence of DPC micelles, the differences between the Ha chemical shift values with respect to those in water, are given in Fig. 4 as a function of the residue position. The overall negative values reflect the α-helical conformation of the peptide. The reason why Ser^3 gives a positive instead of a negative difference value is not clear to us. In order to compare the peptide's secondary structure in the presence of either DPC or mixed DPC/CL micelles, the differences of the Hα proton chemical shift values of p25 between these two systems are given in Fig. 5.

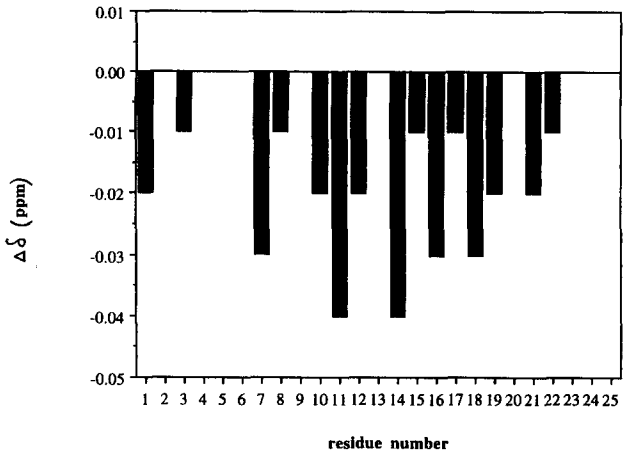

Fig. 5. The difference between the Hα chemical shifts observed in DPC/CL and DPC micelles as a function of residue position for p25.

4. Discussion

The aim of this work was to study the conformational behavior of p25 in micelles consisting of DPC or DPC/CL. Upon addition of DPC micelles to p25, both CD and NMR data demonstrate that the interaction of p25 with the micelles induces α-helix formation in the peptide. This is in agreement with earlier reports on the secondary structure of p25 [5,6]. The CD spectra of p25 in the presence of DPC micelles are typical for a highly α-helical conformation of the peptide (Fig. 1). Moreover, the medium range $H\alpha(i)$–$H\beta(i+3)$, $H\alpha(i)$–$NH(i+3)$ and sequential NOE connectivities (Fig. 3) reveal that the peptide adopts a largely α-helical conformation, starting at Ser^3 and present along the full length of the peptide. The number and intensities of the observed NOEs are higher for the N-terminal half of the peptide, compared with the C-terminal half, indicating that the C-terminal part of p25 has a less regular or more flexible structure compared with the N-terminus. Also the chemical shift differences of Ha resonances of the peptide in DPC solution, with respect to those in water (Fig. 4), show that the helix of the presequence peptide extends from Leu^4 to Leu^{25} and is destabilized at Pro^{13}, with the helical content being higher at the N-terminus and lower at the C-terminus of p25. The helix of the C-terminal half is destabilized at Ser^{20}, Ser^{21}. From these data, it appears that the peptide has a helix-break-helix motive, a motif that has been reported for other presequence peptides as well [9,11,13]. These conclusions extend a previous 2D NMR study on p25 [8], in which it was shown that p25 bound to DPC micelles has one helix at the N-terminus followed by a relatively structureless C-terminal half, of which the authors stated that the structure remained to be defined. The fact that we do see α-helical structure in the C-terminal half is most likely due to differences in the experimental conditions. Our experiments were carried out at pH 4.6 and at 25°C, while Endo et al. used pH 3.8 and temperature of 28°C. Both the temperature decrease and the pH increase promote helix formation. Additionally, our NMR measurements were carried out at a higher field, which results in a better resolution and allows a more sensitive detection of the relatively weak and overlapping interresidue NOEs.

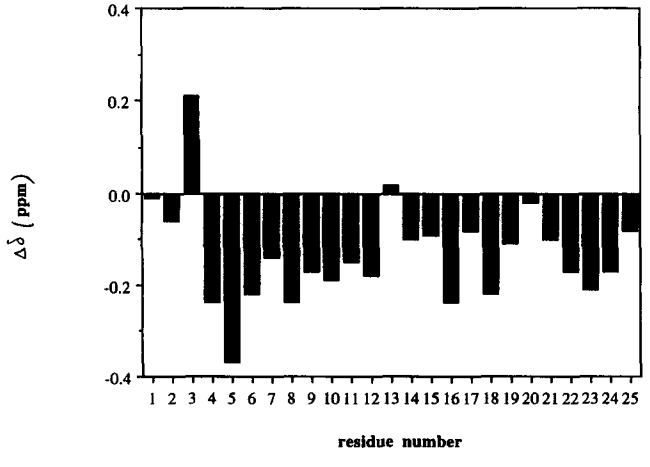

Fig. 4. The difference between the Hα chemical shifts observed in DPC micelles and the random coil chemical shifts determined in water solution is represented as a function of residue position for p25.

The second micelle system included in this study were mixed DPC/CL micelles. The CD analysis of the DPC and DPC/CL systems revealed that incorporation of only two molecules of CL per DPC micelle, i.e. two CL molecules per p25 molecule increased the helical content induced in the peptide by 10%. Comparison of the Ha proton chemical shift values between DPC and DPC/CL micelles (Fig. 5) leads to the conclusion that the increased helical content in the presence of CL is due to an increased helicity around Pro^{13} and at the C-terminal part of p25. This could result in a bended helix, due to the geometry of the proline. Proline-kinked helices appear to be a common structural element in membrane-spanning proteins [37]. Moreover, a bending of an α-helix, caused by proline, was observed for a number of proline-containing peptides (see for instance [38–41] and references cited therein). For p25, a bending of the peptide would result in a structure as depicted in Fig. 6B. In this structure, the α-helix runs from the N- to the C-terminus of p25, with only a break at Pro^{13}. The structure of p25 as depicted in Fig. 6B is consistent with many of the observed specific CL–p25 interactions (summarized in [42]). Among these observations, results indicated an orientation of the N-terminal part of the helix inserted into the lipid bilayer with its helix axis perpendicular to the membrane surface. As a result

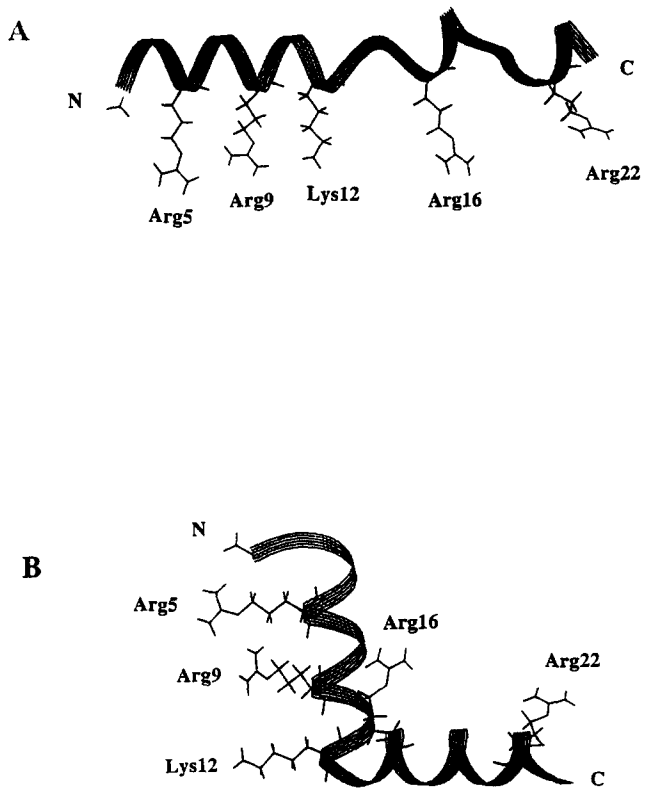

Fig. 6. Molecular model of the two proposed conformations of p25 bound to lipid membranes. For clarity, only the peptide backbone and positively charged side chains are depicted. See text for details.

of the bending, the C-terminal half then orients with positively charged residues towards the membrane surface, while some hydrophobic residues are directed towards the aqueous phase. We propose that the secondary structure of membrane-bound p25 is highly dynamical and consists of an equilibrium between two structures. One is as depicted in Fig. 6A, containing α-helical structure both in the N-terminal half and, somewhat weaker, in the C-terminal half. The peptide orients as a more or less linear amphiphilic α-helix, which lies flat on the membrane with the hydrophobic side chains embedded in the hydrophobic core of the bilayer and the basic residues directed towards the aqueous phase. In order to form this stretched amphiphilic helical structure, structural flexibility of the α-helix around Pro^{13} and Ser^{20} and Ser^{21} is required. The other conformation is as described above and depicted in Fig. 6B. The equilibrium between these two conformational states is shifted to the linear conformation in the absence of CL and to the bent structure in case of CL-containing membranes. This is consistent with the fact that the presence of CL enhances the helical content in the C-terminal half and around Pro^{13}. Since for all other presequences that are proteolytically processed after protein import studies by high-resolution 2D NMR so far, a helix-turn-helix motif has been reported, it can be hypothesized that the two-state equilibrium of the α-helical structure is a more general property of presequences. Presequences which form a continuous α-helix of > 11 residues, have been predicted not to be able to adopt a conformation necessary for proteolysis by the matrix located processing peptidase. Therefore, the consequences of the helix-turn-helix motif for the mitochondrial protein import process could be that the presequence inserts specifically into the CL-containing inner mitochondrial membrane, in such a way that it leads to a correct orientation for processing. Alternative implications of the CL-modulated conformation of p25 for the mitochondrial protein import pathway are suggested elsewhere [42].

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