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Secondary structure of a mitochondrial signal peptide in lipid bilayer membranes

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The secondary structure of the synthetic signal peptide of cytochrome *c* oxidase subunit IV (coxIV-25) has been measured by circular dichroism spectroscopy in different lipid environments. CoxIV-25 is polymorphic in membranes. It forms an amphiphilic α-helix both in negatively charged lipid bilayers (up to 49% helix) and in detergent micelles (up to 42% helix). In associaton with bilayers of the zwitterionic lipid phosphatidylcholine, coxIV-25 takes an aperiodic, unidentified structure. CoxIV-25 is also partially α-helical in bilayers of cardiolipin, mitochondrial lipid extracts and mixtures of synthetic phosphatidylcholine and phosphatidylglycerol.

Mitochondrial signal peptide; Circular dichroism; Protein-lipid interaction; Secondary structure; Amphiphilic helix

1. INTRODUCTION

There is much interest in trying to understand the mechanism of protein translocation across membranes. Proteins to be imported into mitochondria are usually synthesized as larger precursors with N-terminal signal sequences [1]. Most mitochondrial signal sequences are removed from the mature protein by specific signal peptidases, which reside in the mitochondrial matrix or in the inter-membrane space [2]. The information contained in the mitochondrial signal sequences is necessary and, in most cases, sufficient to target proteins to the mitochondrial membranes, but not to other membranes within the eucaryotic cell. Several distinct proteinaceous receptors that can recognize mitochondrial protein precursors are known to exist on the outer membranes of mitochondria, and different mitochondrial precursors utilize different mitochondrial receptors on their route into mitochondria [3]. In the past, most of the evidence for these receptors came from (competitive) binding and antibody blocking experiments with whole mitochondria. Only recently, a candidate for a mitochondrial precursor receptor, mom19, could be identified [4]. After binding, the protein precursors are translocated into mitochondria in the contact sites between the inner and outer mitochondrial membranes [5,6]. A 42 kDa polypeptide, which crosslinks to an artificial precursor on its way into mitochondria, has recently been found in mitochondrial contact sites [7]. These findings will certainly facilitate the search for more components of the mitochondrial protein translocation machinery. Nevertheless, despite these major recent advances, the molecular details of the translocation mechanism have remained elusive.

Apart from binding to receptors and to proteins which catalyse the insertion of polypeptides into membranes and their translocation across mitochondrial membranes, mitochondrial signal peptides also exhibit high affinities for mitochondrial and synthetic phospholipids [8,9]. Practically all mitochondrial signal peptides are highly positively charged, and negatively charged lipids strongly promote their insertion into lipid model membranes [9,10]. Recent binding and lateral diffusion experiments of the 25-residue signal peptide of cytochrome *c* oxidase subunit IV (coxIV-25) have revealed that this peptide partitions into the surface region of uncharged bilayers, but becomes more deeply inserted into bilayers which contain negatively charged lipids [10]. As has been proposed for a large number of mitochondrial matrix targeting peptides [11], coxIV-25 adopts an amphiphilic, partially α-helical structure in detergent micelles [8]. In the present work, we extend these structural studies to phospholipid bilayers of different lipid compositions. In particular, we show that this signal peptide can assume different secondary structures depending on the lipid composition of the host membrane.

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Abbreviations: POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol

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2. MATERIALS AND METHODS

The sequence of coxIV-25 is:

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-$CONH_2$

This peptide was custom synthesized by Novabiochem AG (Switzerland). It eluted as a single peak from a reversed-phase HPLC column [10], and the amino acid composition deviated from the expected composition by less than 5%. All synthetic lipids were from Avanti Polar Lipids (USA). Cardiolipin of *Escherichia coli* was from Sigma (FRG).

Mitochondrial lipids were prepared from beef heart mitochondria [12] by solvent extraction [13]. Briefly, 100 ml of mitochondrial suspension in 0.01 M Tris buffer (pH 7.8) was extracted with 375 ml chloroform/methanol (2:1). 180 ml of each, Tris buffer and chloroform, were added and the two resulting phases were separated. The organic phase was washed with an equal volume of Tris buffer and subsequently removed by evaporation under nitrogen. To remove the non-polar lipids, the phospholipds were precipitated in a 50-fold excess of cold (−20°C) acetone [14] and redissolved in chloroform. 19.1 mg of lipid and 12.9 μmol of lipid phosphorus were recovered as determined by weighing and by quantitative phosphorus determination [15], respectively. Silica gel thin-layer chromatography with a mobile phase of chloroform/methanol/acetone/acetic acid/water (6:2:8:2:1) yielded the expected proportions of phosphatidylcholine, phosphatidylethanolamine and cardiolipin (approx. 4:4:2) [16,17].

Circular dichroism (CD) spectra were recorded as described previously [18]. Since coxIV-25 strongly adsorbs to quartz, separate samples were prepared at each lipid/peptide ratio, rather than titrating a fixed amount of peptide with lipid in the CD cuvette. Typically, 5–30 nmol of coxIV-25 in water were mixed with the appropriate amounts of lipid in chloroform. Methanol was added until a clear solution was obtained. The mixtures were dried under a flow of nitrogen followed by high vacuum for 2 h. 0.5 ml of aqueous 0.1 M NaF was added, and the samples were sonicated with a tip sonicator for 20–30 min at 10°C under nitrogen. They were used immediately for CD spectroscopy. After CD measurements, the samples were centrifuged at low speed (approx. 2500 × *g*) to eliminate possible large aggregates. The concentrations of peptide and lipid in each sample were determined subsequently by quantitative amino acid analysis and the organic phosphorus assay [15], respectively. Typically, 80–90% of the input concentrations of peptide and lipid were recovered by this procedure. The CD spectra were corrected for the actual peptide concentrations. Normally, peptide concentrations of 30 μM were used at low lipid/peptide ratios and 9 μM at higher ratios.

CD spectra of coxIV-25 in lyso-lecithin were obtained by adding the detergent directly to the peptide solution. To improve the signal/noise in the spectra with mitochondrial lipids, three spectra of individually prepared samples were averaged at each concentration. For the calculation of the contents of α-helix, θ_{222} = 30000 deg·cm^2/dmol was taken as 100% α-helix [19]. Spectra which extended over the full range between 190–250 nm were analysed for the contents of the different secondary structures as described [20,21].

3. RESULTS AND DISCUSSION

Circular dichroism (CD) spectra of coxIV-25 in various environments are shown in Fig. 1. The spectrum in 0.1 M NaF and in the absence of any added detergent or phospholipid does not show any features characteristic for an α-helix which is in agreement with previous studies [8]. A fit of this spectrum to a set of reference spectra [21] reveals a composition of spectral components corresponding to 51% β-strand, 15% random coil and 34% turns and unidentified structures. Changing the concentration of coxIV-25 in the range between 9–60 μM does not alter the solution spectrum of this peptide. When an excess of the detergent lyso-lecithin is added, 42% α-helix is induced. 7% is β-strand and 51% are turns and unidentified structures. Also shown in Fig. 1 are the CD spectra of this peptide in association with small unilamellar vesicles of 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) and of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC). The spectrum in POPG is qualitatively very similar to that in lyso-lecithin, although the absolute mean residue molar ellipticities are somewhat larger. The content of α-helix in this spectrum is determined to be 49% from the mean residue molar ellipticity at 222 nm.

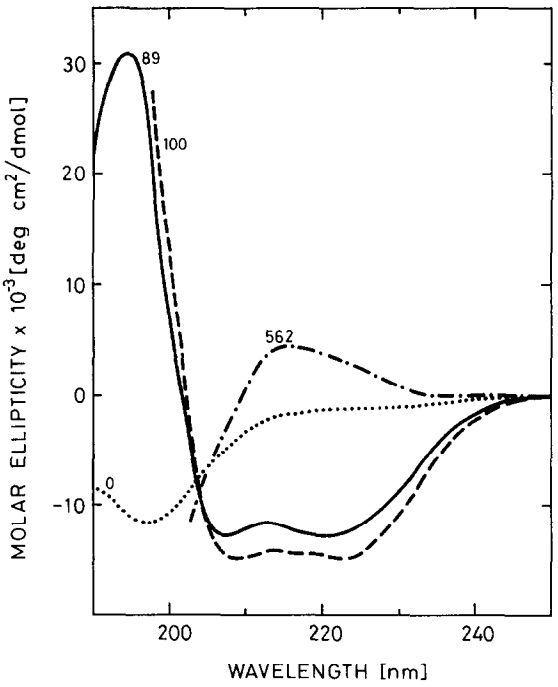

Fig. 1. Circular dichroism spectra of coxIV-25 in 0.1 M NaF (···), in association with lyso-lecithin micelles (——), and in association with bilayers of POPG (– – –) and POPC (-·-·-). For each spectrum, the respective molar ratios of detergent/peptide or lipid/peptide are indicated.

A totally different spectrum is observed when coxIV-25 is associated with bilayers of POPC (Fig. 1). No characteristic features of an α-helical structure are evident. The spectrum is also distinct from that recorded in solution. Unfortunately, the far UV part of this spectrum could not be measured because of light scattering of the lightly turbid solution. Due to the lack of spectral information between 190 and 204 nm, this spectrum could not be fitted unambiguously to the reference spectra. Nevertheless, the spectrum above 204 nm closely resembles reference spectra for unordered structures [20,22]. Maxima around 220 nm are common in polypeptide structures which are not ex-

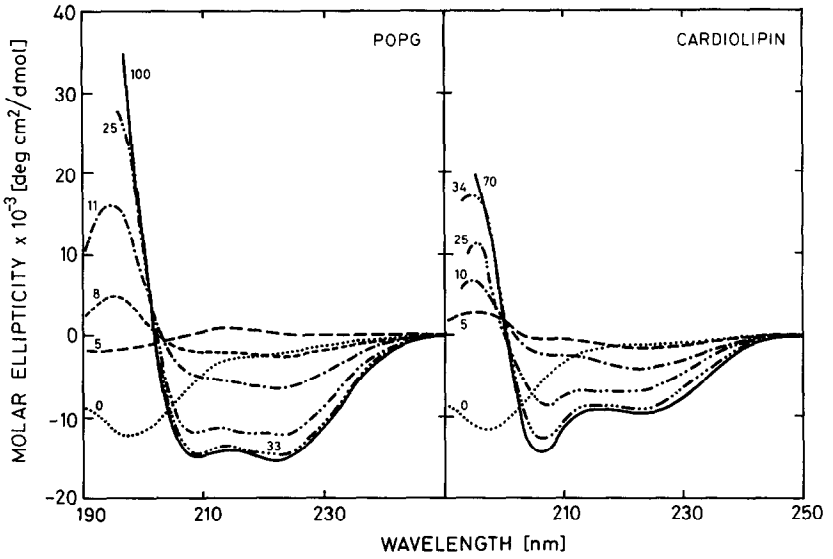

Fig. 2. Comparison of the ability of POPG and cardiolipin to induce an α-helical structure in coxIV-25. Circular dichroism spectra of coxIV-25 in bilayers of the two phospholipids are shown at various lipid/peptide ratios as indicated.

tensively hydrogen-bonded such as, for example, polylysine at pH 7, polyproline I and collagen [23,24]. Thus, coxIV-25 in association with POPC takes an unordered, non-α-helical and extended structure which is different from its solution structure and which cannot be precisely determined from the present CD data.

With a partition constant of 3900 M^{-1} [10], it can be estimated that 95% of all coxIV-25 is bound to POPC under the conditions of the CD experiment of Fig. 1. Furthermore, our lateral diffusion experiments [10] have suggested that coxIV-25 partitions into the headgroup region of POPC bilayers without penetrating much into the hydrophobic interior of the bilayer. Therefore, the spectrum in POPC must represent a conformation of coxIV-25 in the headgroup region of this lipid bilayer. A deeper insertion of coxIV-25, to the point of being partially in the hydrophobic region of negatively charged bilayers, has been previously deduced from lateral diffusion experiments in POPG [10]. The present results now suggest that only in this deeper state of association does the membrane provide enough hydrophobic surface to induce a substantial amount of α-helix in this peptide.

Cardiolipin is the major negatively charged lipid species in the outer and inner membranes of mitochondria [16,17]. Being the biosynthetic precursor of cardiolipin, negatively charged phosphatidylglycerol is also present in mitochondrial membranes. The synthetic phosphatidylglycerol POPG which resembles structurally half a cardiolipin molecule has been previously used as a model lipid in binding and lateral diffusion studies with coxIV-25 [9,10]. Fig. 2 shows a comparison of the ability of POPG and cardiolipin to induce an α-helical structure in coxIV-25. With POPG, the maximal amount of α-helix is induced at lipid/peptide (L/P) ratios of 33:1 or more. An almost featureless CD spectrum is observed at $L/P = 5$, where nearly all charges on the peptide are compensated by a lipid charge. The low intensity of this spectrum is not due to a precipitation of the complexes, because most of the peptide and lipid is recovered after low speed centrifugtion of the clear solution. All spectra with $L/P \geq 5$ ($L/P = 0$ excluded) intersect at an isodichroic point near 203 nm. This indicates (i) that there is a two-state equilibrium between the $L/P = 5$ and the $L/P = 33$ structures in this concentration range and (ii) that the solution structure ($L/P = 0$) does not take part in this equilibrium.

The spectra recorded in the presence of cardiolipin are similar to those recorded with POPG although there are some differences. The minimum at 206–208 nm is always more pronounced than that at 222 nm. An isodichroic point around 200 nm is again found and the spectral intensities increase in about the same relative proportions as in POPG with increasing L/P. However, although cardiolipin resembles structurally a dimer of POPG, it does not appear to be twice as potent as POPG in inducing the α-helix in coxIV-25.

CD spectra with increasing ratios of POPC:coxIV-25 are shown in Fig. 3. In this case, no isodichroic point is observed. In fact, the spectra change gradually from the predominantly β-strand/turn/random structure ($L/P = 0$), to a structure which appears to have some helical components at low L/P ratios (8 and 41) and then to the predominantly unordered structure at high L/P ratios (562 and 656). It is possible that coxIV-25 and POPC associate in micelle-like structures at low L/P and that POPC makes more hydrophobic surface available to coxIV-25 in these complexes than in lipid bilayers. This would ex-

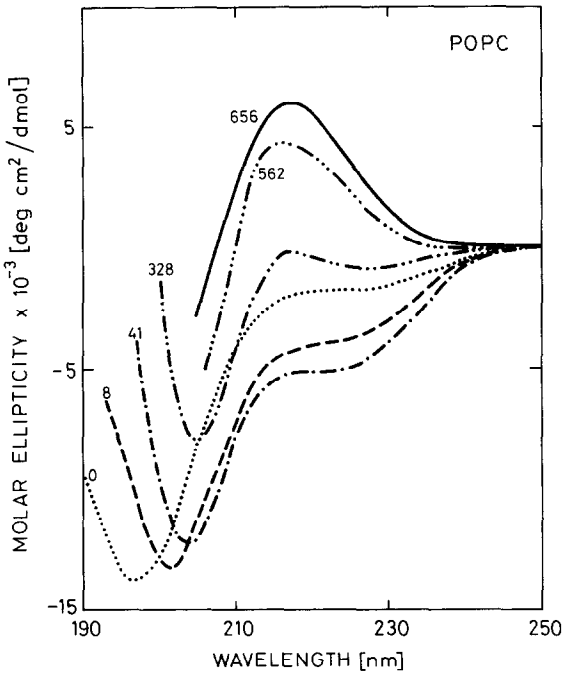

Fig. 3. A non-α-helical, aperiodic structure is induced in coxIV-25 when it associates with bilayers of POPC. Circular dichroism spectra of coxIV-25 in association with POPC are shown at various lipid/peptide ratios as indicated.

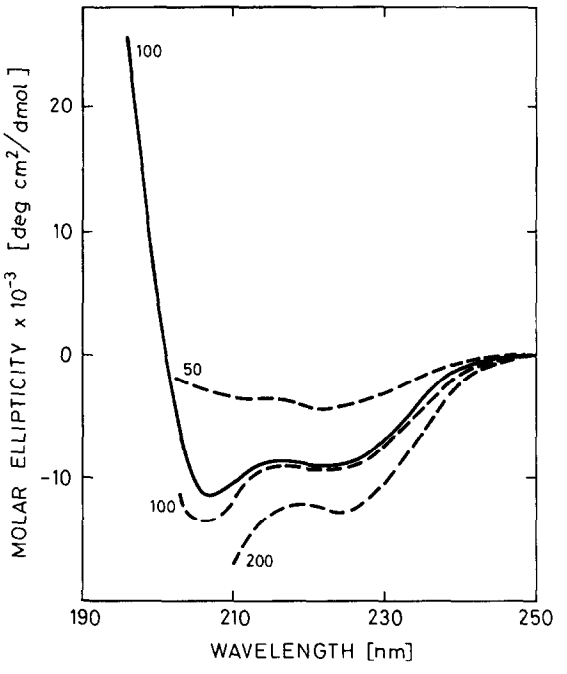

Fig. 4. The α-helix of coxIV-25 is also induced in bilayers of mitochondrial phospholipids and in synthetic bilayers composed of POPC and POPG. Circular dichroism spectra of coxIV-25 in mitochondrial phospholipids at three lipid/peptide ratios (broken lines) and one spectrum in POPC/POPG (7:3; L/P = 100; solid line) are shown.

plain the transient increase of α-helix of coxIV-25 in POPC and is in line with the high amount of α-helix found in micelles of lyso-lecithin (Fig. 1), which has the same headgroup structure as POPC.

Fig. 4 shows the structure of coxIV-25 in the phospholipids that were extracted from mitochondria. Apparently, the mitochondrial phospholipids, which contain about 20–30 mol% of negatively charged phospholipids (20–25 mol% cardiolipin) [16,17], can induce a substantial amount of α-helix (41% at L/P = 200) in this signal peptide. It is also evident from Fig. 4 that synthetic lipid mixtures of POPC/POPG (7:3) (or POPC/CL (7:3), data not shown) can mimic the effect of the natural lipid mixture quite well. Previously, we found that the lateral diffusion coefficient of coxIV-25 is reduced in bilayers of the synthetic lipid mixture of POPC/POPG (4:1) by about the same amount as in bilayers of pure POPG [10]. The present structural and the earlier dynamic investigations taken together show that 20–30 mol% of negatively charged lipids are sufficient to induce the amphiphilic helix in coxIV-25 and to pull it deeper into the membrane allowing for electrostatic and hydrophobic interactions of the signal peptide with the membrane lipids to occur.

Is the observed conformational polymorphism of coxIV-25 a general property of signal peptides in membranes and is it required for their function? Answers to these questions cannot yet be given on the basis of the presently available experimental data. Although it has been detected here for the first time in an amphiphilic signal sequence, there is evidence from other studies indicating that conformational polymorphism might be a common property of most signal sequences. For instance, there is the precedent of the 'hydrophobic' signal peptide which targets the *lamB* gene product across the cytoplasmic membrane to the outer membrane of *E. coli*. This peptide has been found to adopt either α- or β-structure, depending on the lateral pressure at which the phospholipid bilayers have been prepared on the solid substrate [25]. Circumstantial evidence for conformational flexibility comes also from (i) an artificial signal sequence which was designed for maximal helical amphiphilicity but which forms an amphiphilic β-strand in SDS micelles [26]; (ii) the ornithine carbamyl-transferase signal sequence in which all 27 residues will form an α-helix in membranes whereas the central 12 residues which from hydrophobic moment prediction [27] should fold into a highly amphiphilic α-helix actually forms a β-strand in membranes [28]; and (iii) the mannitol PTS permease signal sequence where also the 23 most N-terminal residues (rather than the terminal 15) are required for the induction of an amphiphilic helix in SDS or POPG ([18]; Tamm, L., Bartoldus, I., Tomich, J. and Saier Jr, M., unpublished results). Thus, it appears that many signal peptides can assume several structures in lipid bilayers and that parameters such as lipid charge, lipid pressure, lipid shape, and, perhaps, insertion pro-

teins can control the conformation of these sequences in membranes.

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