Synthetic putative transmembrane region of minimal potassium channel protein (minK) adopts an α -helical conformation in phospholipid membranes

Eric A. J. MERCER, Geoffrey W. ABBOTT, Stephen P. BRAZIER, Bala RAMESH, Parvez I. HARIS and Surjit K. S. SRAI*

Department of Biochemistry and Molecular Biology, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

Minimal potassium channel protein (minK) is a potassium channel protein consisting of 130 amino acids, possessing just one putative transmembrane domain. In this study we have synthesized a peptide with the amino acid sequence RDDSKLE-ALYILMVLGFFGFFTLGIMLSYI, containing the putative transmembrane region of minK, and analysed its secondary structure by using Fourier-transform IR and CD spectroscopy. The peptide was virtually insoluble in aqueous buffer, forming intermolecular β -sheet aggregates. On attempted incorporation of the peptide into phospholipid membranes with a method involving dialysis, the peptide adopted a predominantly

intermolecular β -sheet conformation identical with that of the peptide in aqueous buffer, in agreement with a previous report [Horváth, Heimburg, Kovachev, Findlay, Hideg and Marsh, (1995) *Biochemistry* **34**, 3893–3898]. However, by using an alternative method of incorporating the peptide into phospholipid membranes we found that the peptide adopted a predominantly α -helical conformation, a finding consistent with various proposed structural models. These observed differences in secondary structure are due to artifacts of aggregation of the peptide before incorporation into lipid.

INTRODUCTION

Minimal potassium channel protein (minK) is a novel potassium channel protein expressed in epithelial cells of the kidney [1], heart myocytes [2] and the endometrium and myometrium of the uterus [3]. When expressed in *Xenopus* oocytes [4] and HEK cells [2], minK induces very slowly activating, voltage-dependent potassium currents, very reminiscent of those of the slow component of the cardiac delayed rectifier [2]. MinK is composed of 130 amino acid residues and, in contrast with other multi-transmembrane domain potassium channel proteins, it possesses only one putative transmembrane domain with an extracellular N-terminal domain and therefore an intracellular C-terminal domain [4]. Evidence suggests that the N- and C-terminal domains serve to regulate the minK channel [5]. Point mutations in the putative transmembrane domain of minK resulting in altered gating and ion selectivity strongly suggest that minK forms an integral part of the channel [6].

More recently, studies have indicated that minK protein co-assembles with the voltage-gated heart channel K_v LQT1 to form the channel responsible for the I_{Ks} cardiac current [7,8], the major repolarizing current in the heart. This finding suggests that *in vivo* the minK protein associates with other proteins to form functional channels.

To ascertain the molecular mechanism by which the minK channel conducts K^+ ions specifically across the plasma membrane, it is necessary to determine the structure of the channel pore. To address this problem we have synthesized a peptide corresponding to the amino acid sequence of the putative transmembrane region of minK protein involved in ion conductance, and determined its secondary structure after membrane incorporation by using Fourier transform IR (FTIR) and CD spectroscopy. This approach has been used successfully for the pore region (H5) of the *Shaker* A voltage-dependent potassium channel [9].

CD spectra of a synthetic peptide corresponding to the transmembrane domain of minK protein in methanol reveal an α -helical conformation [10]. Furthermore our preliminary FTIR data indicated an α -helical conformation for the peptide incorporated into phospholipid bilayers [11]. However, the findings of a recent FTIR study of a similar peptide in dimyristoyl L- α -phosphatidylcholine (DMPC) vesicles suggested a β -sheet structure [12].

The conflicting results of the different studies suggest that the method of sample preparation might influence the conformation adopted by the hydrophobic synthetic peptides, a criterion known to influence the structure of the M13 coat protein [13,14]. Furthermore the interaction of amphipathic α -helical peptides with lipids is affected by sample preparation [15]. To investigate these discrepancies we have synthesized a peptide corresponding to the transmembrane domain of minK and analysed its secondary structure by FTIR and CD spectroscopy, in aqueous solution and in phospholipid membranes with different sample preparation methods.

EXPERIMENTAL

Peptide synthesis

The peptide was synthesized with an automated peptide synthesizer Rainin PS3 (Protein Technologies, Woburn, MA, U.S.A.) by a stepwise solid-phase procedure [16] with fluoren-9-ylmethoxycarbonyl (Fmoc) protecting group strategy [17]. The polypeptide was cleaved from the resin by using 95% (v/v) trifluoroacetic acid (TFA) with scavengers and was purified isocratically on a reverse-phase HPLC column (250 mm $\times$ 10 mm internal diam.) with 0.1% (v/v) TFA and acetonitrile as eluents. One major peak was observed when the eluate was monitored at 220 nm. The peptide peak was freeze-dried in the presence of 0.1 M HCl to remove the TFA. The sequence of the 30-residue polypeptide corresponding to the putative transmembrane region

Abbreviations used: DMPC, dimyristoyl L- α -phosphatidylcholine; FTIR, Fourier-transform IR; LPC, lysophosphatidylcholine; minK, minimal potassium channel protein; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

* To whom correspondence should be addressed.

of the minK protein was $\text{CH}_3\text{CO-RDDSKLEALYILMVLGF-FGFFTLGIMLSYI-CONH}_2$. The amino acid composition of the synthetic peptide was confirmed by amino acid analysis.

Sample preparation for spectroscopic analysis

Peptide in an aqueous buffer was prepared by suspending the peptide in $^2\text{H}_2\text{O}$ PBS (pH 7.4) buffer at 10 mg/ml by vortex mixing.

Peptide in phospholipid membranes was prepared by the following methods.

Dialysis method

The peptide (3 mM) and either lysophosphatidylcholine (LPC) (150 mM) or DMPC (150 mM), both purchased from Sigma Chemicals (Poole, Dorset, U.K.), were dissolved together in 2-chloroethanol. Samples were then dialysed against PBS buffer or phosphate buffer [80 mM Na_2HPO_4 /20 mM NaH_2PO_4 (pH 7.4)]. The dialysate was subsequently freeze-dried. It was resuspended into $^2\text{H}_2\text{O}$ or H_2O for FTIR and CD spectroscopy respectively, giving a molar ratio of peptide to phospholipid of approx. 1:50.

Film method

The peptide was dissolved in TFA and a thin film of the peptide was formed on the base of a glass Quickfit round-bottomed flask by rotary evaporation of the organic solvent. Residual traces of TFA, which can interfere in the amide I region of the peptide, were removed by freeze-drying in the presence of 0.1 M HCl. Phospholipid dissolved in CHCl_3 was then added to the flask and the organic solvent was removed by rotary evaporation to form a second film on top of the original peptide film. Further drying was performed under vacuum for 24 h. Finally, either $^2\text{H}_2\text{O}$ PBS for FTIR or H_2O phosphate buffer for CD was added to the dried films and mixed thoroughly with a Vortex mixer. This method of membrane constitution has previously been successfully employed in the structural analysis of peptide in phospholipid membranes [9,18]. As in the dialysis method, the film method used a molar ratio of peptide to phospholipid of approx. 1:50.

As an alternative reconstitution method, further samples were dissolved in trifluoroethanol (TFE) and a thin film was formed as previously described. The dried sample was treated with 0.1 M HCl to remove any traces of TFA remaining from peptide synthesis. The peptide was then resuspended into LPC micelles that had previously been dissolved in 30 % (v/v) TFE/ $^2\text{H}_2\text{O}$. The solution was thoroughly mixed with a vortex before FTIR measurements were made.

FTIR spectroscopy

IR spectra were recorded at 30 °C with a 1750 Perkin-Elmer FTIR spectrometer continuously purged with dry air. Samples were placed in a 10 μl volume gas-tight CaF_2 cell (path length 6 μm). For each sample 100 scans were signal averaged at a resolution of 4 cm^{-1} . The peptide concentration used for the FTIR measurements was 10 mg/ml. Absorbance spectra of the peptide were obtained by digital subtraction of a spectrum of $^2\text{H}_2\text{O}$ -containing buffer from the sample spectrum so as to give a straight baseline in the 2100–1800 cm^{-1} region [19]. The spectra of the aqueous buffer and the sample were recorded under identical conditions. Detailed analysis of the amide I band was performed with a second-derivative procedure. Second-derivative spectra were calculated with GRAMS Derivative function with a 13 data point Savitzky–Golay smoothing window.

CD spectroscopy

Far-UV CD spectra of the peptide at 0.5 mg/ml in LPC micelles were recorded with a JASCO 600 CD spectrometer. The spectra were measured at wavelengths of 260–185 nm at 22 °C with a 0.02 cm pathlength cell. A scan rate of 10 nm/min was employed. Spectra of LPC micelles in phosphate buffer were subtracted from their corresponding peptide spectra to remove any of the effects of buffer absorbance. Spectra were subsequently corrected for concentration and their $\Delta\epsilon$ values per residue calculated.

RESULTS

FTIR and CD spectroscopy are now widely used in the structural analysis of peptides and proteins [19,20]. Information about the secondary structure of a peptide investigated by FTIR spectroscopy can be discerned by analysis of the amide I band (1600–1700 cm^{-1}) [19]. With CD spectroscopy, it is the shape, intensity and position of the maxima and minima of the recorded spectrum that reflect and allow estimation of the secondary structure content of the peptide [21].

FTIR spectra

Peptide in aqueous buffer

Owing to its hydrophobic nature the transmembrane peptide was insoluble in PBS buffer. A suspension of the peptide was therefore used for FTIR analysis. The FTIR second-derivative spectrum of the peptide suspension is presented in Figure 1.

It can be seen that the amide I band occurs at 1626 cm^{-1} . This frequency is too low to be due to α -helical structure. However, β -type structure is known to absorb in this region. Assignment of this band can be complicated by the overlapping of absorbance from different types of β -structure occurring in this frequency range. From previous studies it has been observed that a band frequency between 1610 and 1628 cm^{-1} can arise from aggregated polypeptide chains arranged in an intermolecular β -sheet conformation [22–26]. Absorbance near this frequency has also been observed for aggregated M13 coat protein [13,14]. It is therefore reasonable to assign the 1626 cm^{-1} band to intermolecular β -sheet structure, particularly as the sample was a suspension and visibly aggregated.

Figure 1 FTIR second-derivative spectrum of the peptide in $^2\text{H}_2\text{O}$ PBS

Figure 2 FTIR second-derivative spectra of the peptide in $^2\text{H}_2\text{O}$ PBS after incorporation by the dialysis method into (A) LPC micelles and (B) DMPC vesicles

Peptide in phospholipid membranes reconstituted by the dialysis method

FTIR second-derivative spectra of the peptide in LPC micelles and in DMPC vesicles prepared with the dialysis method are shown in Figure 2. The intense bands at 1725 cm^{-1} of the spectra in Figure 2(A) and at 1729 and 1743 cm^{-1} in Figure 2(B) arise from the vibration of phospholipid ester groups. The spectra of both samples reveal two components in the amide I region. The more prominent component is at $1625/1626\text{ cm}^{-1}$, indicating β -type structure, whereas the component at 1652 cm^{-1} corresponds to an α -helical conformation. The prominent $1625/1626\text{ cm}^{-1}$ component is at a frequency almost identical with that obtained when the peptide is dispersed in aqueous buffer, indicating that the type of β -structure adopted by the minK transmembrane peptide solubilized with LPC/DMPC with the dialysis method is similar to that which the peptide adopts in aqueous suspension.

Peptide in phospholipid membranes reconstituted by the film method

FTIR second-derivative spectra of the peptide in LPC micelles and in DMPC vesicles reconstituted with TFA by the film method are presented in Figure 3. The spectra differ from those obtained for the peptide in lipids prepared with the dialysis method in that their most prominent component is located at $1654/1655\text{ cm}^{-1}$, representing an α -helical conformation, with a

Figure 3 FTIR second-derivative spectra of the peptide in $^2\text{H}_2\text{O}$ PBS after incorporation by the film method into (A) LPC micelles and (B) DMPC vesicles

weak component at 1627 cm^{-1} indicating intermolecular β -sheet content.

The second-derivative spectrum of the peptide in LPC micelles reconstituted with TFE is shown in Figure 4. Absorbance in the amide I region is dominated by a sharp band located at 1653 cm^{-1} that can be attributed to an α -helical conformation. However, unlike the spectrum recorded from the peptide by the previous thin-film reconstitution method, negligible absorbance was seen in the $1627/1625\text{ cm}^{-1}$ region of the spectrum when TFE was used during sample preparation. This demonstrates that utilization of TFE during incorporation of the minK peptide into phospholipid micelles/vesicles minimizes the extent of adoption of intermolecular β -sheet type aggregation by the minK transmembrane peptide.

CD spectra

The hydrophobicity of the peptide prevented solubilization in phosphate buffer and consequently CD analysis could not be performed in this environment. In addition, the turbidity associated with DMPC vesicles causes light-scattering problems that impede CD spectroscopy in this media; spectra of the peptide were therefore only recorded in an LPC micelle environment. The shapes of the far-UV CD spectra of the peptide in LPC micelles prepared by the dialysis and film methods

Figure 4 FTIR second-derivative spectrum of the peptide in LPC micelles in 30% (v/v) TFE/ H_2O PBS after the formation of a thin film of peptide with TFE

Figure 5 CD spectra of peptide in phosphate buffer after incorporation into LPC micelles by (spectrum A) the dialysis method and (spectrum B) the film method

Abbreviation: V_L , wavelength.

presented in Figure 5 are clearly different. Spectrum A, corresponding to the peptide in phospholipid membranes prepared by the dialysis method, displays a minimum at 220 nm and becomes positive at 203 nm, reflecting predominantly β -type structures. It is, however, impossible to determine whether the β -structure is formed by intramolecular or intermolecular bonding. Spectrum B, representing the peptide in phospholipid membrane prepared by the film method, shows a minimum at 206 nm and becomes positive at 200 nm, characteristic of a predominantly α -helical conformation.

DISCUSSION

In this study we have investigated the secondary structure of a synthetic peptide corresponding to the putative transmembrane region of minK under different incorporation conditions by both FTIR and CD spectroscopic techniques. Measurements were

performed in aqueous suspension and after incorporation of the peptide into phospholipid membranes.

For the peptide in aqueous suspension the assignment of the amide I at frequency 1626 cm^{-1} is most consistent with an aggregated intermolecular β -sheet structure. Intermolecular β -sheet structure is a characteristic feature of aggregated and denatured polypeptides [13,14,22–26]. In this structure the polypeptide chains are in an extended form, allowing the close alignment of adjacent chains with correspondingly strong hydrogen bonding between chains [13,14,22–26]. This is reflected in low absorption frequencies ($1610\text{--}1628\text{ cm}^{-1}$) [13,14,22–26]. In such chains, intramolecular hydrogen bonding, and therefore intramolecular β -sheet, is not possible [25]. It is noteworthy that no α -helical structure was detected for the peptide in aqueous suspension.

On incorporation of the peptide into membrane from the helix-forming solvent 2-chloroethanol by the dialysis method, we found that the principal component was at a frequency almost identical with that recorded from the peptide in aqueous suspension, indicating that the peptide prepared by the dialysis method is in a predominantly intermolecular β -sheet conformation (Figures 2 and 5). These results suggest that peptide molecules prepared by the dialysis method aggregate before incorporation into the membrane. It is therefore not unreasonable to suggest that the absorbance band of the peptide at 1626 cm^{-1} , attributed to intermolecular β -sheet in a previous study [12], reflects aggregated intermolecular β -sheet structures and not the conformation adopted by this peptide in the lipid bilayer.

The film method, however, is designed to minimize peptide aggregation before incorporation into lipids and consequently favours peptide–lipid interactions as opposed to peptide–peptide interactions. Incorporation of the minK peptide with this approach resulted in the adoption of a predominantly α -helical structure by the peptide (Figures 3 and 5), unlike the predominantly β -structure observed on incorporation by the dialysis method. However, the FTIR spectrum contained weak absorbance associated with intermolecular β -sheet structures. We suggest that this β -sheet structure is due to aggregation of the peptide. This is supported by the absence of strong absorbance associated with intermolecular β -sheet when the transmembrane peptide was reconstituted in 30% TFE/lipid micelles (Figure 4). TFE increases the tendency of a protein to adopt the structure for which it has a propensity to form [27,28], while also decreasing the extent of protein aggregation. As a result TFE promotes hydrogen bonding and the formation of an α -helical structure in the minK peptide, thus allowing the presentation of the optimal peptide conformation to the lipid [15]. Our findings indicate that the minK transmembrane peptide adopts an α -helical conformation when reconstituted in phospholipids.

In conclusion, synthetic peptides corresponding to functional domains provide a model for studying membrane protein structure. However, if the functional domain is transmembranous, great care must be taken to ensure that its corresponding peptide is not aggregated before structural determination. This is of importance because hydrophobic peptides tend to aggregate to form intermolecular β -sheet structures leading to the incorrect assignment of peptide structure in membrane systems. There are several examples of this in the literature of proteins' being wrongly interpreted as adopting β -structure on the basis of incorrectly assigned spectroscopic data [29,30]. For example, the M13 coat protein was initially thought to undergo a conformational change from an α -helical structure to form large aggregated intermolecular β -sheet structures when inserted into the membrane [29,30]. These β -structures were, however, observed only under conditions that favoured or induced ag-

gregation and are considered to be a denatured form of the protein [13,14]. Subsequently it was shown that the coat protein remained in an α -helical conformation on membrane insertion [13,14]. We have also detected β -structure for the minK transmembrane peptide in a lipid environment, in agreement with other workers [12]. However, we have demonstrated that this β -structure is likely to be due to the formation of intermolecular β -sheets, which suggests that the peptide aggregates before incorporation into lipids. Reconstitution methods that either reduce or prevent peptide aggregation have shown that the minK transmembrane peptide adopts an α -helical conformation.

The demonstration of an α -helical conformation for the peptide in phospholipid membranes is in good agreement with a proposed model based on site-directed mutagenesis studies. Point mutations reveal the importance in activation gating of every third amino acid residue in the transmembrane region which, in an α -helical conformation, would align these amino acid residues along one face of the helix, forming one side of the pore as part of a multimer [6]. An α -helical conformation of the 28-residue transmembrane spanning region of minK would traverse the membrane once, placing the N-terminus on the extracellular side and the C-terminus on the intracellular side of the membrane.

Experimental evidence suggests that two minK monomers interact with one or more membrane-bound channel proteins to produce functional potassium channels [31]. Recently it has been shown that, in the heart, minK associates with the voltage-gated channel K_v LQT forming channels with properties identical with those of the endogenous I_{KS} channel [7,8]. The α -helical conformation of the transmembrane region of minK demonstrated in this study could contribute to the ion conductance pore in heteromultimeric channels.

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