Secondary structure analysis of the putative membrane-associated domains of the inward rectifier K⁺ channel ROMK1

Stephen P. BRAZIER*, Bala. RAMESH*, Parvez I. HARIS†, David C. LEE‡ and Surjit K. S. SRAI*¹

*Department of Biochemistry and Molecular Biology, Royal Free Hospital School of Medicine, Rowland Hill Street, Hampstead, London NW3 2PF, U.K., †Department of Biological Sciences, De Montfort University, Leicester LE1 9BH, U.K., and ‡Department of Spectral Analysis, SmithKline Beecham, New Frontiers Science Park, Third Avenue, Harlow, U.K.

The inward rectifier K⁺ channels contain two putative membrane-spanning domains per subunit (M1, M2) and a 'pore' (P) region, which is similar to the H5 domain of voltage-gated K⁺ channels. Here we have used Fourier transform infrared (FTIR) and CD spectroscopy to analyse the secondary structures of synthetic peptides corresponding to the M1, M2 and P regions of ROMK1 in aqueous solution, in organic solvents and in phospholipid membranes. A previous CD study was unable to provide any structural data on a similar P peptide [Ben-Efraim and Shai (1997) *Biophys. J.* **72**, 85–96]. However, our FTIR and CD spectroscopic analyses indicate that this peptide adopts an α -helical structure when reconstituted into dimyristoyl phosphatidylcholine vesicles and lysophosphatidyl choline (LPC) micelles as well as in trifluoroethanol (TFE) solvent. This result is in good agreement with a previous study on a peptide corresponding to

the pore domain of a voltage-gated K⁺ channel [Haris, Ramesh, Sansom, Kerr, Srai and Chapman (1994) *Protein Eng.* **7**, 255–262]. FTIR spectra of the M1 peptide in LPC micelles displayed a strong absorbance characteristic of an intermolecular β -sheet structure, suggesting aggregation of the M1 peptide. Sucrose gradient centrifugation was used to separate aggregated peptide from peptide incorporated into micelles in an unaggregated manner; subsequent analysis by FTIR suggested that the M1 peptide adopted an α -helical structure when incorporated into phospholipid membranes. FTIR and CD spectra of the M2 peptide in phospholipids and high concentrations of TFE suggest that this peptide adopts an α -helical structure. The structural data obtained in these experiments have been used to propose a model for the structure of the membrane-associated core (M1-P-M2) of the inward rectifier K⁺ channel protein.

INTRODUCTION

Inward rectifiers permit a large inward conduction of K⁺ ions; this rectification property is largely due to a voltage-dependent blockade of the channel by intracellular Mg²⁺ ions [1]. Each functional channel consists of four similar subunits [2], each subunit containing two membrane-spanning regions (M1, M2), which flank a region that is similar to the H5 region of voltage-gated K⁺ channels known as the P region.

Mutagenesis and electrophysiological studies have indicated that the H5 region forms the outer part of the channel pore and is particularly important in conferring ion conduction characteristics [3] and ion selectivity [4,5]. The P region of many cloned inward rectifier channels contains the TXXTXGYG sequence (X is equivalent to any amino acid), which has been found in most voltage-gated K⁺ channel pore regions [6]. This similarity led to the hypothesis that the P region of inward rectifiers formed the pore of the channel.

Two inward rectifiers, the ROMK1 and IRK1 channels, have different conduction and rectification characteristics: ROMK1 has a low conductance and a low affinity for Mg²⁺ blockade, whereas IRK1 has a high conductance and a high affinity for Mg²⁺ blockade. Taglialetta et al. [7] produced chimaeric channels where the P regions, the N-terminus and the C-terminus, the N-terminus only and the C-terminus only were exchanged between the two channels [7]. The results indicated that the P region does not determine the extent of rectification and con-

duction. Furthermore certain residues within the C-terminus have been shown to affect ion conduction and rectification.

A single asparagine residue at position 171 of the ROMK1 channel, located within the M2 membrane-spanning segment has been exchanged for an aspartic residue that is found at position 171 of the IRK1 channel [8,9]. The resulting channel showed a strong affinity for Mg²⁺ blockade, suggesting that part of the M2 region is also in contact with the pore lumen.

As the voltage-gated K⁺ channel pore comprises the H5 and also elements of the S6 [10] and S4–S5 linker [11] regions it is quite possible that the P region of the inward rectifier channels forms an ion-selective filter with regions within the C-terminus and the M2 region forming the lower vestibule of the pore.

Until recently very little information was available on the conformation of these channel proteins involved in pore formation. This is primarily due to the absence of sufficient quantities of this protein for biophysical studies. Synthetic peptides provide an alternative approach for structural investigation of the structure of ion channel proteins [12,13]. We have synthesized peptides corresponding to the P domain, the M1 domain and M2 membrane-spanning segments of the ROMK1 channel. These peptides have been characterized by Fourier transform infrared (FTIR) and CD spectroscopy. The secondary structures of these peptides were characterized in an aqueous environment, in organic solvent and in phospholipid membranes. The findings are of interest for comparison with structural studies performed on similar peptides corresponding to transmembrane domains of

Abbreviations used: DMPC, dimyristoyl-L- α -phosphatidylcholine; FTIR, Fourier transform infrared; LPC, lysophosphatidyl choline; TFA, trifluoroacetic acid; TFE, trifluoroethanol.

¹ To whom correspondence should be addressed (e-mail Srai@rfhsm.ac.uk).

K⁺ channels [12–15] and on the recently resolved X-ray structure of a two-membrane-spanning K⁺ channel from *Streptomyces lividans* (KcsA) [16]. The results of our studies on the P domain of ROMK1 differ from those of Ben-Efraim and Shai [14]; an explanation for the discrepancy in the two studies is provided.

MATERIALS AND METHODS

Peptide synthesis

The peptides were synthesized with a Rainin PS3 automated peptide synthesizer (Protein Technologies, Woburn, MA, U.S.A.) by a stepwise solid procedure [17] with fluorenylmethoxycarbonyl protecting group strategy [18]. The polypeptides were cleaved from the resin with 95% (v/v) trifluoroacetic acid (TFA) with the use of scavengers, and purified isocratically on a reverse-phase C8 HPLC column (250 mm × 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 peptides were freeze-dried in the presence of 0.1 M HCl to remove the TFA. The amino acid sequence of the peptides corresponding to the P, M1 and M2 domains are shown in Figure 1. The purity of the polypeptides was confirmed by MS and amino acid analysis.

Sample preparation

Lysophosphatidylcholine (LPC), dimyristoyl-L- α -phosphatidylcholine (DMPC) and trifluoroethanol (TFE) were all purchased from Sigma Chemicals (Poole, Dorset, U.K.). The peptides were dissolved in TFA and a thin film of the peptide was formed at the base of a Quickfit glass round-bottomed flask by rotary evaporation of the organic solvent. Residual traces of TFA were eliminated by freeze-drying in the presence of 0.1 M HCl. The peptides were then either resuspended in TFE or reconstituted into LPC and DMPC. Phospholipid dissolved in chloroform was added to the flask and again the solvent was removed by rotary evaporation, thus forming a lipid film on top of the peptide film. Further drying was performed under vacuum for 24 h. The lipid and protein films were then resuspended in ²H₂O phosphate buffer for FTIR or ¹H₂O phosphate buffer for CD spectroscopic analysis.

Sucrose gradients

Linear 2–10% (w/v) sucrose gradients in PBS buffer were formed in Polallomer centrifuge tubes (Beckman). Stock ice-cold 2% and 10% (w/v) sucrose solutions were gradually mixed and layered slowly into centrifuge tubes to give a 2–10% linear gradient. A thin film of M1 peptide in LPC was prepared as described previously; the dried sample was resuspended in 2% (v/v) sucrose/PBS buffer and vortex-mixed. Approx. 0.5 mg of M1 peptide was added to the top of the sucrose gradient and the mixture was centrifuged at 201 000 g for 23 h at 4 °C. Fractions

were then collected from the top of the gradient by using a Gilson pipette, and analysed for the presence of protein with the Bradford assay. Fractions from individual peaks were pooled and the sucrose was removed by dialysis against deuterated PBS buffer; finally the samples were concentrated with an Amicon ultrafiltration apparatus.

FTIR spectroscopy

Infrared spectra were recorded at 30 °C with a 1750 Perkin-Elmer FTIR spectrometer continuously purged with dry air. Samples were placed in a 10 μ l volume gas-tight CaF₂ cell with a 6 μ m pathlength; 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 ²H₂O-containing buffer from the sample spectrum. Detailed analysis of the amide I band was performed with a second-derivative procedure. Second-derivative spectra were calculated by using GRAMS derivative function with a 13-results-point Savitzky-Golay smoothing window.

CD spectroscopy

Far-UV spectra (190–260 nm) of the peptide at 1 mg/ml in TFE and LPC were recorded at 22 °C in a 0.02 cm pathlength cell with a JASCO J-600 spectropolarimeter. A scan rate of 10 nm/min was employed. The instrument was purged continuously with nitrogen to prevent ozone accumulation. Spectra of the LPC micelles in phosphate buffer and TFE/¹H₂O buffer were subtracted from their corresponding peptide spectra to remove any of the effects of their absorbances. Spectra were subsequently corrected for concentration and their $\Delta\epsilon$ values per residue were calculated. The estimation of secondary structure content was determined by using a principal-component regression method (GRAMS/32 suite program) with a calibration data set of 16 proteins [19].

RESULTS

Structural studies of the P domain

P domain in ¹H₂O

The peptide was insoluble in aqueous solution. Nevertheless a FTIR spectrum of the peptide suspended in ²H₂O was obtained. Figure 2(A) shows the FTIR spectrum of the P peptide in ²H₂O. The amide I maxima at 1620 and 1694 cm⁻¹ are characteristic of an aggregated β sheet structure, which has been observed in the spectra of denatured proteins [20].

P domain in TFE

Samples of the P polypeptide were suspended in 50% and 95% (v/v) TFE; the resulting spectra are presented in Figure 2(A). The absorbance spectrum of P in 50% TFE shows the main peak centred at 1652 cm⁻¹; weaker absorbance is observable at 1620 and 1694 cm⁻¹ (Figure 2A). Absorbance in the 1649–1658 cm⁻¹ region of the amide I spectrum has been shown to correspond to α -helical secondary structure [21,22]. The bands at 1620 and 1694 cm⁻¹ are consistent with an aggregated β -sheet structure. The amide I band of the spectra of P in 95% TFE shows a narrow band centred at 1654 cm⁻¹, corresponding to an α -helix structure.

In addition to the FTIR analysis of the P peptide in TFE, CD measurements were also performed in this medium. The hydrophobicity of the peptide prevented the solubilization of the

ROMK1 M1 Domain NH₂-VFITAFGLSWFLGGLWYVVAYV-COOH
 ROMK1 P Domain NH₂-SAFLFSLETQVTIGYGFRFVTEQCA-COOH
 ROMK1 M2 Domain NH₂-AIFLLIFQSILGVIINSFMCGAILA-COOH

Figure 1 Amino acid sequence of the three peptides synthesized corresponding to the M1, P and M2 domains of the inward rectifier channel ROMK1

The extents of the individual domains were predicted from a hydropathy plot of the ROMK1 channel.

Figure 2 FTIR and CD spectra of the P peptide in different concentrations of TFE

(A) FTIR absorbance spectra of the P peptide suspended in ²H₂O (solid line), 50% TFE/50% ²H₂O (broken line) and 95% TFE/5% ²H₂O (dotted line). (B) CD spectra of the P peptide suspended in 30% TFE/70% ¹H₂O (solid line), 50% TFE/50% ¹H₂O (broken line), 70% TFE/30% ¹H₂O (dotted line) and 90% TFE/10% ¹H₂O (dot-dashed line). All spectra were recorded at 20 °C.

peptide in concentrations of TFE in ¹H₂O below 30% (v/v). Consequently CD analysis was only possible at TFE concentrations of 30% and higher. CD spectra of the peptide in 30%, 50%, 70% and 90% TFE are presented in Figure 2(B). All the spectra have a minimum at 208 nm with a shoulder at 220 nm, characteristic of a predominantly α -helical conformation [23]. However, the $\Delta\epsilon$ of the minimum became more negative with increasing concentrations of TFE in ¹H₂O. Estimates of α -helical secondary structure content of the peptide in 30% and 70% TFE in ¹H₂O are 4% and 32% respectively, indicating that a greater proportion of the peptide participates in α -helical secondary structure at high concentrations of TFE in ¹H₂O.

P domain in DMPC and LPC

Figure 3(A) shows the absorbance spectrum of the P polypeptide in DMPC bilayers. The absorbance spectrum shows a narrow amide I band centred at 1654 cm⁻¹; the second-derivative spectrum did not show the presence of any other component in the amide I region (results not shown). This suggests that the P polypeptide adopts an α -helical structure in DMPC bilayers.

The spectrum of the P polypeptide reconstituted into LPC micelles is shown in Figure 3(B). The intense band at 1715 cm⁻¹ observed in the absorbance spectra arises from the vibrations of

Figure 3 Absorbance (left panels) and second-derivative (right panels) FTIR spectra of the P peptide reconstituted into DMPC vesicles (A) and LPC micelles (B) at 20 °C

phospholipid ester groups. The amide I band maximum is located at 1652 cm⁻¹. This indicates that the P polypeptide also adopts an α -helical structure in LPC micelles.

A high degree of ¹H-²H exchange in the P polypeptide was observed in all the TFE samples as well as in the LPC micelles and DMPC bilayers; this is evident from the weak intensity of the amide II band, which is normally observed near 1550 cm⁻¹.

Structural studies of the M1 domain: M1 domain in phospholipids

An FTIR analysis of M1 samples reconstituted into phospholipids with the use of the TFA film method indicated the presence of a significant proportion of intermolecular β -sheet structure, a structure that aggregated proteins have a propensity to form [20,24,25]. Samples were centrifuged on sucrose gradients in an attempt to separate aggregated structure from that of ordered secondary structure.

Figure 4 shows the results of Bradford assays performed on the fractions collected. Fractions that corresponded to the first two peaks were pooled (fractions 15–18). A second sample was collected that corresponded to the large narrow band that appeared towards the 10% sucrose concentration end of the gradient. These two samples were dialysed and concentrated for FTIR analysis. Figure 4 shows the FTIR second-derivative spectra corresponding to the two samples collected from the sucrose gradient. The spectra corresponding to the first two peaks display an intense band at 1725 cm⁻¹, corresponding to vibrations within the LPC molecule. The amide I band is located at 1652 cm⁻¹, which is characteristic of α -helical structure. In contrast, the second-derivative spectrum of the second sample is largely dominated by a band at 1626 cm⁻¹, which was due to peptide aggregation; a smaller amount of absorbance due to α -helical structure was also observed.

Figure 4 Graph showing typical results obtained from centrifuging the M1 peptide reconstituted in LPC on 2–10% (w/v) linear sucrose gradients and the corresponding second-derivative FTIR spectra obtained from pooled fractions

Sample fractions (70 μl) were collected from the top of the sucrose gradient; the absorbance (shown as OD_{595}) was calculated by the Bradford assay technique.

Structural studies of the M2 domain

M2 segment in $^2\text{H}_2\text{O}$

Owing to the hydrophobicity of the M2 peptide it was not possible to solubilize it in $^2\text{H}_2\text{O}$. However, an FTIR spectrum of the peptide suspension was recorded. Figure 5(A) shows the amide I band of the resulting spectrum. A broad band with maximum absorbance at 1627 cm^{-1} was seen. Significant absorbance was also observed at 1694 cm^{-1} . These peaks correspond to intermolecular β -sheet structure, which is associated with aggregation.

M2 segment in TFE

Figure 5(A) shows the effect of adding TFE to the peptide suspension. FTIR spectra of the peptide in 50% and 95% TFE are shown. At a ratio of 1:1 TFE to $^2\text{H}_2\text{O}$ the resulting spectrum indicated a shift of the absorbance maximum to 1654 cm^{-1} . Further absorbance occurred at 1620 cm^{-1} and to a smaller extent at 1694 cm^{-1} . The band present at 1654 cm^{-1} corresponds to α -helical structure. The other components are consistent with the aggregation previously mentioned. These latter bands were completely absent in 95% TFE; the only visible peak occurred at 1656 cm^{-1} , suggesting that M2 adopts a predominantly α -helical structure in 95% TFE.

CD spectra were recorded of the M2 peptide suspended in 30%, 50%, 70% and 90% (v/v) TFE. Recordings at TFE

Figure 5 FTIR and CD spectra of the M2 peptide in different concentrations of TFE

(A) FTIR absorbance spectra of the M2 peptide suspended in $^2\text{H}_2\text{O}$ (solid line), 50% TFE/50% $^2\text{H}_2\text{O}$ (broken line) and 95% TFE/5% $^2\text{H}_2\text{O}$ (dotted line). (B) CD spectra of the M2 peptide suspended in 30% TFE/70% $^1\text{H}_2\text{O}$ (solid line), 50% TFE/50% $^1\text{H}_2\text{O}$ (broken line), 70% TFE/30% $^1\text{H}_2\text{O}$ (dotted line) and 90% TFE/10% $^1\text{H}_2\text{O}$ (dot-dashed line). All spectra were recorded at 20°C .

concentrations below 30% were rendered impossible by the insolubility of the peptide. The spectra of the peptide in 50%, 70% and 90% were almost identical (Figure 5B). The minimum of the spectrum is located at 207 nm; a shoulder is observed at 223 nm. This pattern is characteristic of proteins with some degree of α -helical structure. Estimates of α -helical secondary structure content of this peptide in 30% TFE/70% $^2\text{H}_2\text{O}$ and 70% TFE/30% $^2\text{H}_2\text{O}$ were 6% and 63% respectively. The spectrum of the peptide in 30% TFE had a similar pattern to that described but the spectrum was less intense. This indicates that the peptide adopts some disordered structure.

M2 segment in DMPC and LPC

The amide I band of the spectrum recorded of the M2 segment reconstituted in DMPC showed a narrow band at 1654 cm^{-1} (Figure 6A). This band is characteristic of α -helical secondary structure. Unlike the spectra of the P-domain peptide, the amide II band was observed in the spectra associated with the M2 peptide.

The absorbance and second-derivative spectra of the M2 peptide reconstituted into LPC micelles shows the amide I band to be centred at 1654 cm^{-1} (Figure 6B), which is characteristic of α -helical structure. This indicates that the M2 domain of ROMK1

Figure 6 Absorbance (left panels) and second-derivative (right panels) FTIR spectra of the M2 peptide reconstituted into DMPC vesicles (A) and LPC micelles (B) at 20 °C

adopts an α -helical structure when reconstituted into phospholipids.

DISCUSSION

Early structural models of the voltage-gated K⁺ channel pore were based solely on electrophysiological and mutagenesis experiments. These findings suggested that the H5 region was responsible for the conductance and ion-selectivity properties of the channel [3,4]. Furthermore the H5 entered and exited from the membrane from the extracellular fluid [26]. Durell and Guy [27] proposed that the four H5 segments of the channel formed an eight-stranded antiparallel β -barrel in which the entire pore was located. Other workers have suggested that four long β -strands formed the pore [28]. In more recent models it has been suggested that the H5 domain is arranged in a helix-loop-helix structure [29]. Molecular biological and electrophysiological studies also indicate that other domains of the channel have a fundamental role in pore formation, namely the S6 domain and S4-S5 linker, which form the lower part of the pore [10,11].

Until recently little structural evidence has been presented to support continually updated models of the structure/function relationship of the pore. An interesting approach towards obtaining information on the structure of ion channels has been to synthesize peptides corresponding to functionally important domains of ion channels and characterize them structurally by biophysical techniques. Spectroscopic studies of two peptides corresponding to the H5 domain of the *shaker* channel have been performed [12,30] that suggest that this region is largely helical.

The H5 domain of the voltage-gated K⁺ channel has a high degree of sequence identity with that of the P region of inward rectifier channels. A recent paper by Ben-Efraim and Shai [14] suggests that the P region of ROMK1 has a non-helical structure. According to these workers the CD signal of the P peptide was very low in 40 % TFE/60 % water and in SDS, so that it could

not be assigned to any particular structure. However, our studies conducted with both CD and FTIR spectroscopy clearly indicate a predominantly α -helical conformation for this peptide in TFE/water as well as in phospholipid membranes (DMPC bilayers and LPC micelles). Our results are also in agreement with previous FTIR and CD studies conducted on the pore-forming domains of the *shaker* voltage-gated K⁺ channel [12].

The discrepancy between our results and that of Ben-Efraim and Shai [14] can be explained by the different approaches used for reconstitution of peptides for spectroscopic analysis. We have performed a large number of experiments to determine the role of sample preparation and environment on the conformation adopted by the synthetic peptides. From these studies we found that under certain conditions (especially those favouring peptide aggregation) the P peptide adopted a predominantly β -sheet structure in an aqueous medium; transferring the same peptide to a hydrophobic medium resulted in a conformational change from intermolecular aggregated β -sheet to α -helix. It is therefore essential to perform spectroscopic studies on synthetic peptides under various conditions and reconstitution procedures before arriving at conclusions about their structure. The low CD signal observed by Ben-Efraim and Shai for the P peptide might have been due to insufficient solubility of the peptide, which we observed at low concentrations of TFE. To obtain accurate information on such systems it is important to identify conditions under which the peptides are soluble and give sufficiently strong CD and FTIR signals.

The problem associated with peptide aggregation and subsequent problems with spectroscopic interpretation was further highlighted by the M1 peptide. The preparation of samples by the same method as that successfully applied to both the M2 and P peptides led to spectra dominated by absorbance arising from β -sheet structure. Furthermore this β -sheet structure was intermolecular in nature, indicating that the peptide had aggregated. We have successfully used sucrose gradients to separate the aggregated peptide from populations of phospholipid micelles containing successfully incorporated peptide. FTIR spectra of these populations show that the M1 peptide adopts a predominantly α -helical structure. This is in agreement with the finding that CD spectra of the M1 peptide in TFE were characteristic of α -helical structure [14].

It has been shown that the binding of Mg²⁺ ions to the intracellular side of the pore in response to membrane depolarization is essential for inward rectification. Mutations of residues within the M2 domain have been shown to affect the affinity of Mg²⁺ blockade, suggesting that the M2 region is either located in close proximity to the pore or that it lines part of the pore. Our study has shown that the M2 peptide adopts an α -helical conformation when reconstituted into both DMPC bilayers and LPC micelles and when dissolved in TFE. This is in agreement with the structure previously proposed from CD spectroscopy [14].

Recently a major breakthrough was made in our understanding of K⁺ channel structure when Doyle et al. [16] elucidated the X-ray structure of a K⁺ channel from *Streptomyces lividans* (KcsA). Although this channel has only two transmembrane domains it is more closely related to the six transmembrane K⁺ channels than to the inward rectifiers. However, their finding that the two membrane-spanning domains are helical (the second helix lining the pore) is in good agreement with the structures presented here for M1 and M2. Furthermore the ion-selective filter of this channel was found to be largely helical, as is the P domain of ROMK1.

In conclusion we have determined the secondary structure of the membranous domains of ROMK1. Furthermore these struc-

Figure 7 Schematic model of the ROMK1 channel in the membrane bilayer

Abbreviations: e.c., extracellular; i.c., intracellular.

tures have been elucidated in a wide range of environments, including phospholipids that more closely resemble the channel's physiological environment than organic solvents. We have used this structural information, alongside current understanding of K^+ channels, to predict a model of the ROMK1 channel. Our model (Figure 7) suggests that the P domain forms the ion-selective filter of the channel; the finding that the P peptide was predominantly α -helical is in good agreement with recent modelling studies of Durell and Guy [31] that suggest a helix-turn-helix structure for the similar H5 domain of the voltage-gated K^+ channels. Furthermore the P domain is modelled as being almost perpendicular to the membrane, as suggested by mapping of toxin binding to the H5 domain [32] and from the X-ray structure of the KcsA K^+ channel [16]. Both the M1 and M2 membrane-spanning domains have been modelled as α -helices, the lower section of the M2 domain probably lining the lower vestibule of the channel. Mutagenesis studies also indicate that the proximal C-terminal region is also involved in determining conductance and rectification properties of the channel, and could therefore also be involved in pore formation. Mutagenesis studies on the M1 domain suggest that this domain has no specific role in channel activity but it is likely that this region acts to shield the pore from the hydrophobic nature of the membrane.

The high level of structural similarity between the membrane-associated domains of ROMK1 and the comparable domains of the KcsA K^+ channel suggests that the structure of the ion-conducting pathway might have been preserved throughout the evolution of the K^+ channel family.

We thank Dr. Giuliano Siligardi (National Chiroptical Spectroscopy Facility, EPSRC, King's College, London, U.K.) for the CD measurements. S.P.B. is supported by a studentship from BBSRC and SmithKline Beecham.

REFERENCES

- Ho, K., Colin, G., Nichols, W., Lederer, J., Lytton, J., Vassilev, P. M., Kanazirska, M. V. and Hebert, S. C. (1993) *Nature (London)* **362**, 31–37
- Yang, J., Jan, Y. N. and Jan, L. Y. (1995) *Neuron* **15**, 1441–1447
- Hartmann, H. A., Kirsch, G. E., Drewe, J. A., Tagliatela, M., Joho, R. H. and Brown, A. M. (1991) *Science* **251**, 942–944
- Yool, A. J. and Schwarz, T. L. (1991) *Nature (London)* **349**, 700–704
- Kirsch, G. E., Drewe, J. A., Hartmann, H. A., Tagliatela, M., De Baisi, M., Brown, A. M. and Joho, R. H. (1992) *Neuron* **8**, 499–505
- Heginbotham, L., Lu, Z., Abramson, T. and MacKinnon, R. (1994) *Biophys. J.* **66**, 1061–1067
- Tagliatela, M., Wible, B. A., Caporaso, R. and Brown, A. M. (1994) *Science* **264**, 844–847
- Wible, B. A., Tagliatela, M., Ficker, E. and Brown, A. M. (1994) *Nature (London)* **371**, 246–249
- Lu, Z. and MacKinnon, R. (1994) *Nature (London)* **371**, 243–246
- Lopez, G. A., Jan, Y. N. and Jan, L. Y. (1994) *Nature (London)* **367**, 179–182
- Slesinger, P., Jan, Y. N. and Jan, L. Y. (1993) *Neuron* **11**, 739–749
- Haris, P. I., Ramesh, B., Brazier, S. and Chapman, D. (1994) *FEBS Lett.* **349**, 371–374
- Haris, P. I., Ramesh, B., Sansom, S. P., Kerr, I. D., Srai, K. S. and Chapman, D. (1994) *Protein Eng.* **7**, 255–262
- Ben-Efraim, I. and Shai, Y. (1997) *Biophys. J.* **72**, 85–96
- Mercer, E. A. J., Abbott, G. W., Brazier, S. P., Ramesh, B., Haris, P. I. and Srai, K. S. (1997) *Biochem. J.* **325**, 475–479
- Doyle, D. A., Cabral, J. M., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. and MacKinnon, R. (1998) *Science* **280**, 69–77
- Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85**, 2149–2154
- Carpino, L. A. and Han, G. Y. (1972) *J. Org. Chem.* **37**, 3404–3409
- Hennessey, J. P. and Johnson, W. C. (1981) *Biochemistry* **20**, 1085–1094
- Jackson, M., Haris, P. I. and Chapman, D. (1991) *Biochemistry* **30**, 9681–9686
- Haris, P. I. and Chapman, D. (1992) *Trends Biochem. Sci.* **17**, 328–333
- Krimm, S. and Bandekar, J. (1986) *Adv. Protein Chem.* **38**, 181–364
- Woody, R. W. (1995) *Methods Enzymol.* **246**, 34–71
- Sanders, J. C., Haris, P. I., Chapman, D., Otto, C. and Hemminga, M. A. (1993) *Biochemistry* **32**, 12446–12454
- Jackson, M. and Mantsch, H. H. (1995) *Crit. Rev. Biochem. Mol. Biol.* **30**, 95–120
- Yellen, G., Jurman, M. E., Abramson, T. and MacKinnon, R. (1991) *Science* **251**, 939–942
- Durell, S. R. and Guy, H. R. (1992) *Biophys. J.* **62**, 238–250
- Bogusz, S., Boxer, A. and Busath, D. D. (1992) *Protein Eng.* **5**, 285–293
- Guy, H. R. and Durell, S. R. (1994) *Biophys. J.* **66**, 248
- Shinozaki, K., Anzai, K., Kirino, Y., Lee, L. and Aoyagi, H. (1994) *Biochem. Biophys. Res. Commun.* **198**, 445–450
- Durell, S. R. and Guy, H. R. (1996) *Neuropharmacology* **35**, 761–773
- Aiyar, J., Withka, J. M., Rizzi, J. P., Singleton, D. H., Andrews, G. C., Lin, W., Boyd, J., Hanson, D., Simon, M., Dethlefs, B. et al. (1995) *Neuron* **15**, 1169–1181