Adoption of β Structure by the Inactivating "Ball" Peptide of the *Shaker* B Potassium Channel

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ABSTRACT The conformation of the inactivating peptide of the *Shaker* B K^+ channel (ShB peptide) and that of a noninactivating mutant (ShBL7E peptide) have been studied. Under all experimental conditions explored, the mutant peptide remains in a predominantly nonordered conformation. On the contrary, the inactivating ShB peptide has a great tendency to adopt a highly stable β structure, particularly when challenged "in vitro" by anionic phospholipid vesicles. Because the putative peptide binding elements at the inner mouth of the channel comprise a ring of anionic residues and a hydrophobic pocket, we hypothesize that the conformational restrictions imposed on the ShB peptide by its interaction with the anionic lipid vesicles could partly imitate those imposed by the above ion channel elements. Thus, we propose that adoption of β structure by the inactivating peptide may also occur during channel inactivation. Moreover, the difficulties encountered by the noninactivating ShBL7E peptide mutant to adopt β structure and the observation that trypsin hydrolysis of the ShB peptide prevents both structure formation and channel inactivation lend further support to the hypothesis that adoption of β structure by the inactivating peptide in a hydrophobic environment is important in determining channel blockade.

INTRODUCTION

Voltage-activated potassium channels are a diverse and ubiquitous class of ion channels involved in the regulation of many different cellular processes in both excitable and nonexcitable tissues (Jan and Jan, 1992; Pongs, 1993). Much of the current knowledge on these physiologically important protein molecules derives from the earlier characterization of the voltage-gated K^+ channels coded in the *Shaker* locus of *Drosophila* (Papazian et al., 1987; Kamb et al., 1988; Pongs et al., 1988). At negative membrane potentials (corresponding to the resting membrane potential), *Shaker* potassium channels are usually closed, whereas at membrane potentials sufficiently positive the channels open, or activate, and allow an efflux of potassium ions down thermodynamic gradients established across the cell membrane. Nevertheless, the potassium current produced is transient because shortly after activation, the channels enter into a nonconducting state, the inactivated state. This fast (N-type) inactivation of *Shaker* potassium channels is probably the best understood step in ion channel gating. Early work by Armstrong and Bezanilla (1977) to explain fast inactivation in Na^+ channels proposed that a flexible cytoplasmic domain of the protein itself could occlude the internal mouth of the channel to produce inactivation (the "ball and chain" model). More recent studies in *Shaker* B (ShB) potassium channel (Hoshi et al., 1990; Zagotta et al., 1990) in which the inactivated state is long lived have elegantly demonstrated that, indeed, the inactivating "ball" peptide (ShB peptide) corresponds to the first 20 amino acids in the N-terminal region of each ShB channel subunit (H_2N-MAAVAGLYGLGEDRQHRKKQ). An important piece of evidence from these studies was the finding that the addition of a synthetic ShB peptide, even when it is not covalently attached to the rest of the channel protein, was able to restore fast inactivation in mutant channels that did not inactivate because of deletions in their NH_2-terminal region (Zagotta et al., 1990). Also, addition of the same ShB inactivating free peptide produces fast inactivation in a variety of other K^+ channels that do not normally inactivate, such as the drk1 delayed rectifier-type K^+ channel (Isacoff et al., 1991), the *Shaker* homolog RBK1 from rat brain (Zagotta et al., 1990), a large conductance Ca^{2+}-activated K^+ channel from coronary artery smooth muscle (Toro et al., 1992) and others, suggesting that there are similarities in the interaction of the peptide with conserved, or equivalent, sites at the different channels that are important in forming the ion-conducting pore. Moreover, the ability of the ShB peptide to induce inactivation is exquisitely dependent on certain features of its primary structure (Murrell-Lagnado and Aldrich, 1993). For instance, amino acid changes in the hydrophobic stretch of the ShB peptide sequence, in which a single nonpolar residue is substituted by either positively or negatively-charged amino acids, such as that in the ShB-L7E peptide, result in the loss of the ability of the peptides to induce K^+ channel inactivation.

In this paper, we have used synthetic inactivating (ShB) and noninactivating (ShB-L7E) peptides to investigate whether the single amino acid change in the peptide sequence determines alterations in the conformation of the "ball" peptide that might explain the loss of function. To this end, we have made use of the conformational sensitivity of the amide

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I band in the infrared spectra of the peptides. Fourier-transform infrared spectroscopic (FT-IR) methods have shown great potential to detect structural differences between the various possible conformers of either polypeptides or complex proteins (Mendelsohn and Mantsch, 1986; Surewicz and Mantsch, 1988; Jackson et al., 1989; Rothschild, 1992; Arrondo and Goñi, 1993; Fernandez-Ballester et al., 1994). The strong amide I band, comprising the 1600–1700 cm^{-1} spectral region results primarily from stretching vibrations of C=O groups in peptide bonds, the exact frequencies of which depend on the nature of the hydrogen bonding involving the C=O groups which, in turn, is determined by the particular secondary structure adopted by the protein (Bandekar, 1992). Thus, the amide I band contour in a protein spectrum represents a complex composite of spectral components of characteristic frequencies, which can be correlated with different protein secondary structural motifs (Surewicz et al., 1993).

MATERIALS AND METHODS

Deuterium oxide (D_2O, 99.9% by atom) was purchased from Sigma Chemical Co. (St. Louis, MO). The phospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidic acid (PA) (Avanti Polar Lipids, Alabaster AL)) were all derivatives of egg PC and, therefore, had the same fatty acid composition. All solvents used were of HPLC grade.

Peptide synthesis and characterization

The peptides ShB (MAAVAGLYGLGEDRQHRKKQ) and ShB-L7E (MAAVAGEYGLGEDRQHRKKQ) were synthesized as COOH-terminal amides on an automatic multiple synthesizer (AMS 422, Abimed) using a solid-phase procedure and standard Fmoc-chemistry in a base of 25 μmol. The synthesis was carried out on a N-β-Fmoc-DMP resin (4-(2′, 4′-dimethoxyphenyl-Fmoc-amino-methyl)-phenoxy resin (Novabiochem, La Jolla, CA)), with Fmoc-protected amino acids activated in situ with PyBOP (benzotriazole-1-*yl*-oxy-tris-pyrrolidinophosphonium hexafluorophosphate) in the presence of *N*-methyl morpholine and 20% piperidine/dimethylformamide for deprotection. The protecting groups were as follows: Gln (Trt), Glu (OtBu), Tyr (tBu), Asp (OtBu), Arg (pmc), His (Trt), and Lys (Boc). Peptides were cleaved from the resin with 82.5% trifluoroacetic acid and 5% phenol, 5% H_2O, 5% thioanisole, 2.5% ethane dithiol (King et al., 1990) as scavenger, precipitated, and washed with cold methyl *tert*-butyl ether, water-extracted, and lyophilized. The purity of the peptides was assessed to be more than 93% by reverse-phase HPLC analysis on an Ultrasphere-ODS C_{18} column (4.6 × 150 mm) with a linear gradient water/35% acetonitrile in 0.1% trifluoroacetic acid. Amino acid analysis indicated the expected composition in both peptides. Fast atom bombardment mass spectrometry yielded the following results: ShB, *m/z* 2227.36 (calculated 2227.62); ShB-L7E, *m/z* 2243.50 (calculated 2243.58).

Trypsin hydrolysis of both peptides was carried out with immobilized TPCK-trypsin (Pierce, Rockford, IL) following the manufacturer's protocol. Purification of the major tryptic fragments (residues 1–14) was accomplished by reverse-phase HPLC using an Ultrasphere-ODS C_{18} column (10 × 150 mm) and the mobile phase from above. The composition of these tryptic peptides was confirmed by amino acid analysis. To remove residual trifluoroacetic acid used both in the peptide synthesis and in the HPLC mobile phase (the trifluoroacetate ion has a strong infrared absorbance at 1670 cm^{-1}, which interferes with the characterization of the amide I band (Surewicz et al., 1993)), the peptides were submitted to several lyophilization-solubilization cycles in 10 mM HCl (Zhang et al., 1992).

Circular dichroism

CD spectra were taken in a Jovin Yvon Mark III dichrograph at a scanning speed of 0.2 nm/s, using 0.1-cm-path quartz cuvettes. CD results were the mean values of at least three determinations and were expressed as molar ellipticities (deg cm^2 $dmol^{-1}$).

Infrared measurements

Lyophilized aliquots of the synthetic peptides were dissolved at concentrations ranging 1–10 mg/ml in plain D_2O or in D_2O buffers (see figure legends for details on the composition of the buffers) to avoid the interference of H_2O infrared absorbance (1645 cm^{-1}; Mendelsohn and Mantsch, 1986) on the protein amide I band. Lyophilized lipids hydrated in the same D_2O buffers were either mixed with peptides immediately or submitted to sonication in a water bath for 20 min before or after the addition of the peptides. Use of such alternative procedures to prepare the peptide/lipid vesicles mixtures produces no differences in the observed spectral features of the samples. These solutions were placed into a liquid demountable cell (Harrick, Ossining, NY) equipped with CaF_2 windows and 50-μm-thick mylar spacers and maintained at room temperature for approximately 1 h to assure that the isotopic H-D amide proton exchange reached equilibrium, as judged by a constant minimal absorbance at the residual amide II band in the peptide's infrared spectra. FT-IR spectra were taken in a Nicolet 520 instrument equipped with a DTGS detector as described previously (Castresana et al., 1992). Fourier derivation and deconvolution of the spectra were carried out as reported by others (Moffatt and Mantsch, 1992). Derivation was performed using a power of 3, breakpoint of 0.3. Deconvolution was performed by using a lorenztian bandwidth of 12 cm^{-1}, a resolution enhancement factor ranging 1.8–2.0, and a Bessel apodization.

RESULTS

Fig. 1 *A* shows the amide I band region in the infrared spectra of the ShB and ShB-L7E peptides in D_2O solutions of different ionic strength and ion composition. In plain D_2O, the IR spectra of both peptides are practically identical and have a maximum absorbance centered near 1645 cm^{-1}, which is characteristic of a nonordered conformation. The only noticeable difference between the spectra of the two peptides under these conditions comes from a minor band near 1711 cm^{-1} that is somewhat more prominent in the ShB-L7E peptide and has been assigned to vibration of protonated carboxyl groups of glutamic acid side chains (Muga et al., 1990). Use of resolution-enhancement, band-narrowing techniques (Mantsch et al., 1988; Moffatt and Mantsch, 1992) such as Fourier derivation or deconvolution shows that the amide I region of the spectra in D_2O exhibits other maxima at 1627, 1638, 1660, and 1675 cm^{-1}, all of which have been assigned to vibration of the carbonyl group in peptide bonds within different secondary structural motifs: vibrations within the 1623–1636 cm^{-1} range are assigned to β structures, the 1660 cm^{-1} band to helices, and the 1675 cm^{-1} band includes contributions from turns as well as from the (0,β) β-structure vibration band (Fig. 2 *B*, *lower* trace) (Byler and Susi, 1986; Arrondo et al., 1987; Surewicz and Mantsch, 1988). Also, in addition to the 1711 cm^{-1} band referred to above, other bands at 1586, 1605, and 1613 cm^{-1} have been detected that correspond to different amino acid side-chain vibrations (Chirgadze et al., 1975; Muga et al., 1990).

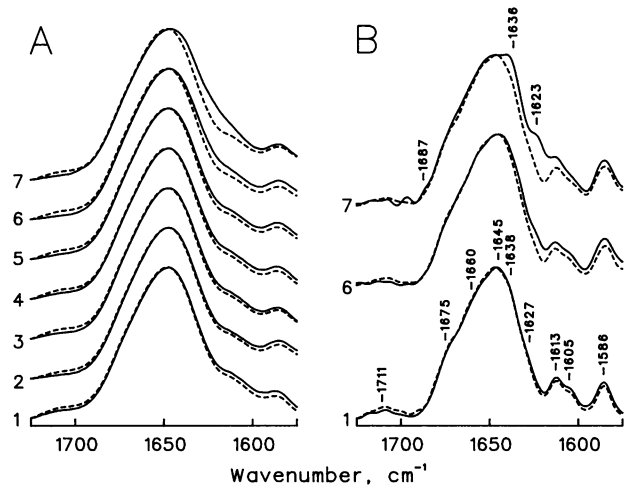

FIGURE 1 Infrared amide I band region of the original (*A*) and deconvolved (*B*) spectra of the ShB (——) and the ShB-L7E (- - - -) peptides in plain D_2O (traces 1) or in different D_2O media prepared from 10 mM HEPES, pH 7.4 (traces 2); 5 mM HEPES, pH 7.0, containing 130 mM KCl, 20 mM NaCl (traces 3); 5 mM HEPES, pH 7.0, containing 2 mM $CaCl_2$, 115 mM NaCl, 2 mM KCl (traces 4); or 10 mM HEPES, pH 7.4, containing 100 mM (traces 5), 200 mM (traces 6), or 300 mM $NaNO_3$ (traces 7). In this and in all other figures, the spectra of the different buffers alone were subtracted from those of the peptide-containing samples. Numbers on top of the spectra in *B* and in all other figures represent the wavenumbers of the most characteristics components detected upon deconvolution. Temperature was maintained at 20°C.

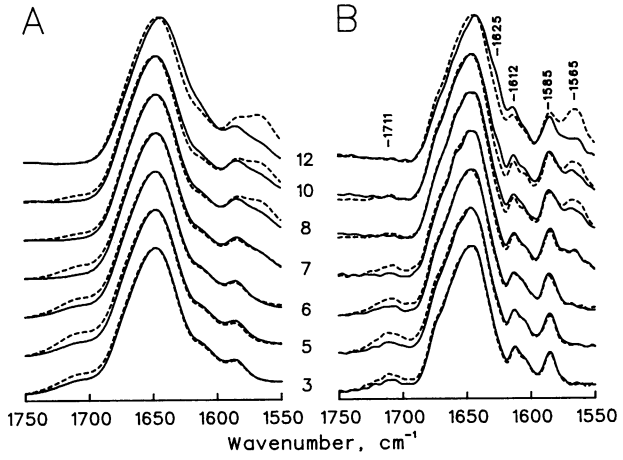

FIGURE 2 Infrared amide I band region of the original (*A*) and deconvolved (*B*) spectra of the ShB (——) and the ShB-L7E (- - - -) peptides in D_2O media prepared from 10 mM phosphate buffers at the indicated pH values (3–12), containing 130 mM KCl and 20 mM NaCl.

The lack of predominant ordered conformations exhibited by these peptides in solution has been confirmed also by CD using trifluoroethanol/water mixtures as the solvent (data not shown). In plain water, both peptides have practically identical CD spectra and clearly exhibit random coil (aperiodic) behavior, which is in agreement with the infrared spectroscopic results from above. Increasing the percent of trifluoroethanol in the trifluoroethanol/water mixtures results in the appearance of spectral features in both peptides characteristic of helical structure, such as the negative ellipticities at ~205 and 220 nm and a positive band near 190 nm (Dyson and Wright, 1991). This is similar to a previous report on the CD spectrum of the ShB peptide (Lee et al., 1993) but, also, it was expected from the known tendency of trifluoroethanol to induce helical structure on a variety of other peptides or proteins (Reddy and Nagaraj, 1989; Sönnichsen et al., 1992). Despite the limitations imposed by the use of trifluoroethanol, these CD experiments are interesting in that they suggest a potentially different conformational behavior of the two peptides because, as the percent of trifluoroethanol is increased, the native ShB peptide reaches a significantly higher content of the trifluoroethanol-induced helical structure than the mutant ShB-L7E peptide.

Fig. 1 *A* also shows that increasing the ionic strength in the D_2O solutions produces a shift in the infrared amide I band of the ShB peptide to lower frequencies that cannot be detected in those spectra corresponding to the mutant ShB-L7E peptide. Deconvolution of the spectra revealed that such spectral change corresponds mainly to the appearance of a larger β-structure component at 1636 cm^{-1} and a smaller one at 1623 cm^{-1} in the ShB peptide (Fig. 1 *B*), the intensities of which increase as the ionic strength is increased. Other experiments were made in buffers containing high concentrations of either K^+ or Na^+ in an attempt to imitate the "intracellular" or "extracellular" media, respectively (Fig. 1 *A*, traces 3 and 4). The spectra of the peptides obtained under such conditions were not significantly different from those taken from similar samples in the HEPES/$NaNO_3$ media at comparable ionic strength.

Fig. 2 shows the effects of pH on the infrared spectra of the ShB and ShB-L7E peptides. It is observed that the spectra of both peptides are practically identical within the pH 3–7 range. At pH 8 and above, there is a small shift in the amide I band of the ShB peptide to lower frequencies, which is similar to the observations made at moderately high ionic strength. On the other hand, the spectrum of the ShB-L7E peptide remains unperturbed at all pH values explored with the exception of the disappearance of the carboxyl side-chain vibration of glutamic acid (1711 cm^{-1}), which remains at an unusually high pH, and the appearance of a band near 1565 cm^{-1} above pH 7, which has been assigned to vibration of unprotonated carboxylate groups of glutamic acid side chains (Muga et al., 1990). Because this interconversion between protonated and unprotonated forms of glutamic acid at unusually high pH occurs only in the ShB-L7E peptide, we assume that it is due to titration of glutamic acid at position 7, which is the sequence substitution characteristic of the mutant peptide and has an unusually high pK_a, probably because of its involvement in some fairly stable structure that shields it from the aqueous solvent. In any case, the small pH dependence observed in the conformation of the two peptides suggests that the neutralization of either acidic or basic amino acid side chains in the peptide sequence has little relevance in determining the peptide structure in solution.

Fig. 3 *A* shows that the presence of the detergent sodium cholate at concentrations below the critical micellar concen-

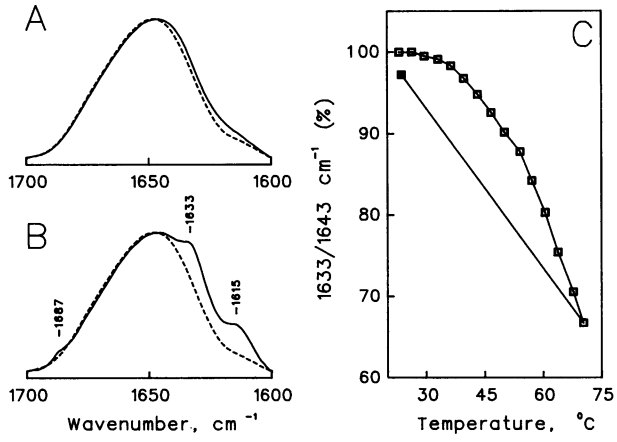

FIGURE 3 Effects of cholate at 5 mM (*A*) or 20 mM (*B*) on the original amide I infrared band of the ShB (——) and the ShB-L7E (- - - -) peptides in D_2O media prepared from 5 mM HEPES, pH 7.0, containing 130 mM KCl, 20 mM NaCl. (*C*) Temperature dependence of the absorbance ratio (1633/1643 cm^{-1}, *open symbols*) observed during thermal denaturation (spectra taken at temperatures ranging 23.1–70.3°C) and further recooling (*closed symbol*) of the ShB peptide in the presence of 20 mM cholate, as in *B*.

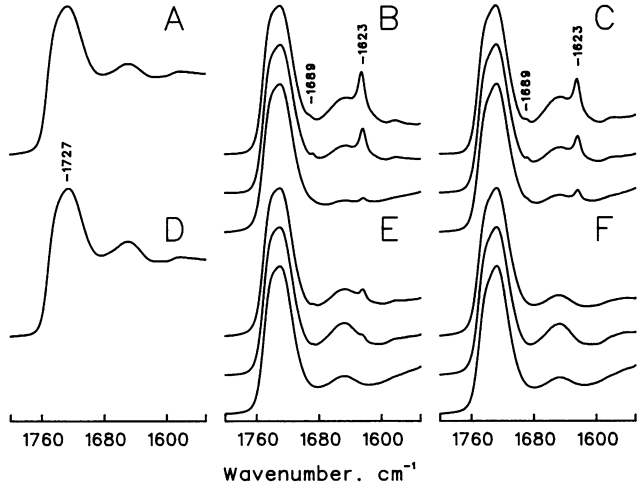

FIGURE 4 Original 1550–1800 cm^{-1} region of the spectra of the ShB (*A–C*) and ShB-L7E (*D–F*) peptides in the presence of phosphatidylcholine (*A* and *D*), phosphatidylglycerol (*B* and *E*), or phosphatidic acid (*C* and *F*) vesicles in D_2O media prepared from 5 mM HEPES, pH 7.0, containing 130 mM KCl, 20 mM NaCl. In all of the experiments shown in this figure, the phospholipid concentration was 25 mg/ml. The concentrations of either peptide were 5 mg/ml in *A* and *D*, whereas for the triple traces shown in *B*, *C*, *E*, and *F* were 5 mg/ml (*upper* traces), 2.5 mg/ml (*middle* traces), and 1.25 mg/ml (*lower* traces). None of the spectra shown here have been normalized for concentration of the peptide in the sample.

tration produces only minor changes in the infrared amide I band of either peptide. However, presence of detergent micelles (at detergent concentrations above 14 mM), determines that the ShB peptide becomes structured, showing a negative shoulder at approximately 225 nm in the CD spectra (data not shown) and a strong β-structure component at 1633 cm^{-1} in the infrared amide I band, accompanied by a characteristic, high wavenumber, minor β component near 1687 cm^{-1} and a second component at 1615 cm^{-1} (Fig. 3 *B*) that has been related to the presence of hydrogen-bonded, intermolecular β sheets (Muga et al., 1990). On the other hand, the ShB-L7E peptide remains in a predominantly nonordered conformation (Fig. 3 *B*) under identical experimental conditions. The stability of the β-structure components observed in the ShB peptide in the presence of cholate micelles was examined by submitting the samples to thermal denaturation. In these experiments, infrared spectra are taken at progressively higher temperatures to produce denaturation of the peptide samples, which can be monitored by measuring the alteration induced on the amide I band (Fernandez-Ballester et al., 1992). Fig. 3 *C* shows that there is a progressive loss of the β components with increasing temperature. Nevertheless, most of the initial β structure can be regained upon cooling of the heat-denatured sample, indicating that the observed thermal denaturation is a reversible process of thermal unfolding.

The infrared spectra of the ShB and ShB-L7E peptides taken in the presence of PC vesicles (Fig. 4, *A* and *D*) reveal that the two peptides have a similar, featureless amide I band contour in which, similarly to those seen in aqueous solution, a nonordered structural component near 1645 cm^{-1} is predominant (in these and in all spectra taken in the presence of phospholipids, the strong absorbance centered near 1727 cm^{-1} corresponds to vibration of the phospholipid carbonyl ester groups). On the contrary, the presence of vesicles formed by negatively charged PG or PA (Fig. 4, *A* and *C*, respectively) induces a remarkable alteration in the amide I band of the ShB peptide that shows a very prominent component at 1623 cm^{-1} accompanied by a smaller one at 1689 cm^{-1}, which in other peptides has been related to the adoption of strongly hydrogen-bonded β structure in hydrophobic environments (Demel et al., 1990). Such spectral components in the ShB peptide are always conspicuously present and exhibit a similar relative importance (the 1623 to 1645 cm^{-1} absorbance ratio remains constant), regardless of the concentration of peptide (ranging 1.2–10 mg/ml) or the phospholipid to peptide ratio (ranging 2–60, by mole) used in these studies. Spectral components of identical frequencies can also be detected in the ShB-L7E peptide/acidic phospholipids samples, but they are always much less prominent and their appearance is markedly dependent on the concentration of peptide and the type of phospholipid used (it is produced only in PG vesicles) (Fig. 4, *E* and *F*). Experiments carried out at pH 8, at which the band assigned to vibration of the protonated carboxylic group of glutamic acid at position 7 disappears (see text above and Fig. 2 *B*), cause a complete disappearance of the β-structure components from the spectra of the ShB-L7E peptide in PG vesicles under those conditions, whereas they have no effects on that of the ShB peptide (not shown). Therefore, we conclude that the difficulties encountered by the ShB-L7E peptide to adopt such β structure at pH 8 are related to the presence of a net negative charge at the hydrophobic stretch of the mutant peptide where the glutamic acid in position 7 is located.

Adoption of β structure by the ShB peptide was also studied in lipid vesicles made from synthetic dimyristoyl PA. Fig. 5 shows that the appearance of the characteristic 1623 cm^{-1} component occurs at temperatures above the gel-to-fluid phase transition temperature ($T_m \approx 50°C$) of the dimyristoyl PA bilayer, suggesting that the ShB peptide requires access to the hydrophobic bilayer to form the observed β structure.

The stability of the 1623 cm^{-1} β-structure component observed in the presence of negatively charged PG vesicles was examined by submitting the samples to thermal denaturation. Fig. 6 *A* shows that the ShB peptide was extremely resistant to heat denaturation and that at temperatures as high as 70°C, the β component at 1623 cm^{-1} is still maintained at no less than 65% of that seen at room temperature (Fig. 6 *C*). Furthermore, the starting absorbance at that frequency can be almost fully recovered upon cooling of the heated sample (Fig. 6 *A*, *upper* trace). On the contrary, the β-structure component at 1623 cm^{-1} in the ShB-L7E peptide, which is seen only at high peptide concentration, seems much more labile, disappears almost completely from the spectra taken at high temperature, and is only partly recovered upon recooling (Fig. 6, *B* and *C*).

Treatment with proteases such as trypsin has been reported to result in the loss of the ability of the ShB peptide to inactivate the ShB K^+ channel (Zagotta et al., 1990). For this reason, we wanted to test the effects of trypsin on the infrared spectra of the ShB peptide. There are four potential trypsin cleavage sites on either ShB or ShB-L7E peptides at positions 14, 17, 18, and 19 in the amino acid sequence. Under our experimental conditions, trypsinization of either peptide is complete after 2 h of incubation with the protease, as monitored by reverse-phase HPLC of the reaction mixture. Infrared spectra of the proteolytic peptide mixtures from either peptide are very similar and reveal that the ability of the ShB peptide to organize as a β structure in presence of negatively charged phospholipids is completely lost upon trypsin hydrolysis. Moreover, the infrared spectra of the purified major tryptic fragments (including amino acid residues 1–14) from either peptide are essentially identical and show no signs of the β-structure component at 1623 cm^{-1} (Fig. 7).

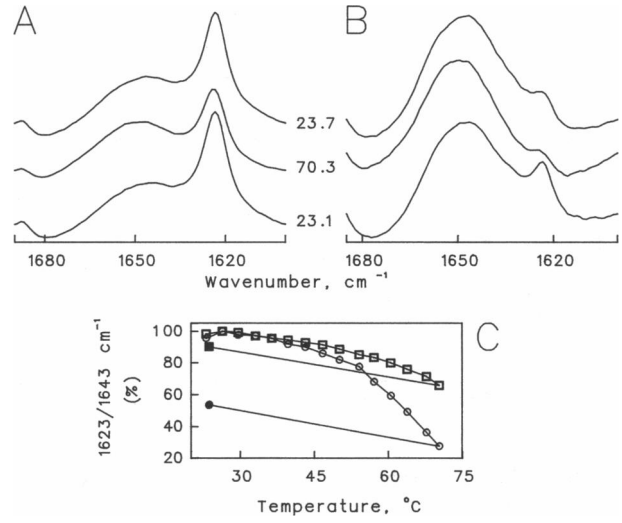

FIGURE 6 Temperature dependence of the infrared amide I band of the ShB (*A*) and ShB-L7E peptide (*B*) at 2.5 mg/ml in D_2O media prepared from 5 mM HEPES, pH 7.0, containing 130 mM KCl, 20 mM NaCl, and in the presence of phosphatidylglycerol vesicles (25 mg of phospholipid/ml). Spectra were taken at temperatures ranging 23.1–70°C during a heating cycle lasting approximately 2 h, after which the samples were brought near room temperature (*upper* spectrum in *A* and *B*) to assess the reversibility of the heat denaturation process. (*C*) Temperature dependence of the absorbance ratio 1623/1643 cm^{-1} in the ShB (*squares*) and ShBL7E (*circles*) samples. Closed symbols correspond to the recooling process.

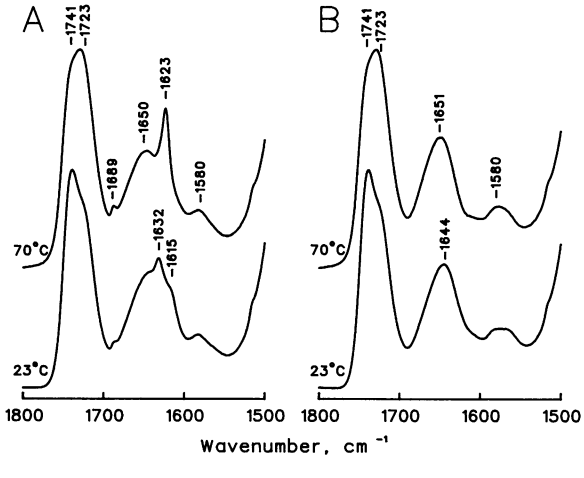

FIGURE 5 Spectral changes observed in the ShB (*A*) and ShBL7E (*B*) peptides in the presence of dimyristoyl PA at temperatures above (*upper* traces) and below (*lower* traces) the gel-to-fluid phase transition temperature ($T_m \approx 50°C$). Samples were prepared at room temperature at a 5 mg/ml peptide concentration in D_2O media containing 5 mM HEPES, pH 7, 130 mM KCl, and 20 mM NaCl, and mixed with previously formed dimyristoyl PA vesicles (25 mg of phospholipid/ml) prepared in the same buffer.

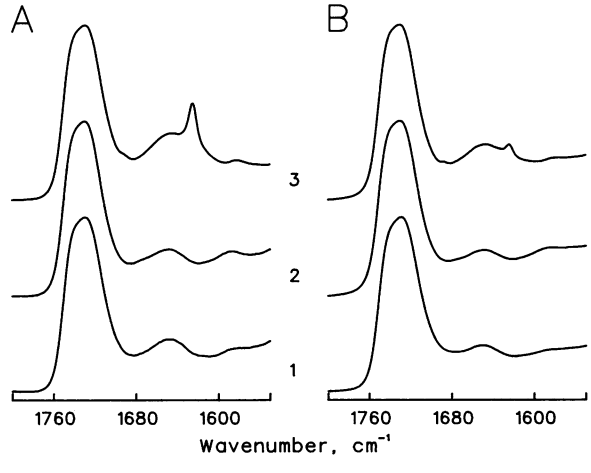

FIGURE 7 Effects of trypsin on the infrared amide I band of the ShB (*A*) and ShB-L7E peptide (*B*) at 2.5 mg/ml in D_2O media prepared from 5 mM HEPES, pH 7.0, containing 130 mM KCl, 20 mM NaCl, and in the presence of phosphatidylglycerol vesicles (25 mg of phospholipid/ml). The upper traces correspond to the spectra of untreated, control peptides. The middle traces are the spectra of the resulting tryptic peptide digests, whereas the lower traces correspond to the spectra of the purified major tryptic fragments (residues 1–14) from both peptides under identical experimental conditions.

DISCUSSION

The observation by Aldrich and co-workers that free, synthetic peptides having the sequence of the NH_2-terminal portion of the ShB K^+ channel retain the ability to induce channel inactivation (Zagotta et al., 1990) has opened the possibility to undertake structural studies on these important regulatory elements in ion channel gating. No experimental information on this subject is currently available other than a preliminary report on CD and nuclear magnetic resonance studies on the conformation of the ShB peptide in trifluoroethanol-water mixtures (Lee et al., 1993). These authors indicate that the peptide exhibited a random coil behavior in water whereas, as seen in other peptides or proteins (Reddy and Nagaraj, 1989; Sönnichsen et al., 1992), it had a tendency to form helical structures as the percent of trifluoroethanol was increased. Our own CD observations on both the ShB and ShB-L7E peptides confirm such report on the ShB peptide and more importantly, regardless of the fact that helical formation might just be a consequence of the use of trifluoroethanol, indicate that the two peptides have a different potential capacity to become structured into a given conformation.

Analysis of the FTIR spectra obtained in plain D_2O shows that the amide I band of both peptides consists of an ensemble of rapidly interconverting conformations, as should be generally expected for short linear peptides in solution (Dyson and Wright, 1991), with a predominance of nonordered structures. Altering the solution media such as changing the ionic strength, ion composition, or pH, produces little or no effect on the relative amounts of interconverting conformers in the noninactivating ShB-L7E peptide but induces detectable changes in the conformational preference of the K^+ channel-inactivating ShB peptide, which partly adopts a β structure seen at 1636 and 1623 cm^{-1} in the infrared amide I band. Such conformation in solution seems only slightly dependent on electrostatic interactions because neutralization of either acidic (low pH) or basic (high pH) amino acid residues, mostly located at the COOH-terminal end of the peptide, results in only minor alteration of the infrared spectra in solution. On the other hand, the presence of anionic detergent micelles seems a much more important factor in determining adoption of structure by the ShB peptide, while leaving the ShB-L7E peptide in a nonordered conformation similar to that seen in solution. The low propensity of the ShB-L7E peptide to adopt β structure could arise from the known strong β-breaker character of glutamic acid (Chou and Fasman, 1974) present at the NH_2-terminal half of the mutant peptide.

Studies carried out in the presence of negatively charged liposomes provide further evidence to support a distinct capacity of the ShB and ShB-L7E peptides to adopt a β-type structure. In this case, the main β-spectral component in the ShB peptide occurs at 1623 cm^{-1}, appears readily at low peptide concentration, and it is much larger than any of the β components seen in solution. Such β structure cannot be formed by the ShB-L7E peptide in presence of negatively charged phospholipid vesicles (except at high peptide concentration and in PG vesicles); neither can it be formed by either ShB or ShB-L7E peptides when mixed with lipid vesicles made from zwitterionic phospholipids. These observations are remarkably similar to those reported for certain NH_2-terminal signal peptides required for translocation of membrane proteins (Reddy and Nagaraj, 1989; Demel et al., 1990). These signal peptides insert readily into bilayers containing anionic phospholipids, giving raise to the formation of β structures of low frequencies in those highly hydrophobic environments (Demel et al., 1990). Also, it is known that spectral components in the 1610–1629 cm^{-1} frequency range are characteristic of strongly hydrogen-bonded β structures, likely resulting from intermolecular aggregation (Muga et al., 1990; Fraser et al., 1991; Jackson et al., 1992). Based on the above considerations, our results could be interpreted as a consequence of the insertion of the predominantly hydrophobic NH_2-terminal half of the ShB peptide into the bilayer provided by the anionic vesicles, where the β structure would be formed through either intra- or intermolecular hydrogen bonding. This possibility of peptide insertion receives further support from experiments made in the presence of synthetic dimyristoyl PA vesicles in which the characteristic 1623 cm^{-1} band can only be detected at temperatures above that corresponding to the gel-to-liquid crystalline phase transition. Accordingly, the ShB-L7E peptide would not be able to adopt such β structure because the L7E change in the amino acid sequence makes difficult both, the insertion of the NH_2-terminal half of the peptide into the negatively charged bilayer and the adoption of the structure. The lack of β-structure formation in the presence of PC vesicles for either the ShB or the ShB-L7E peptides suggests that before insertion, the peptide should be held or positioned at the bilayer surface and this, for peptides having several net positive charges at their COOH-terminal half, should require electrostatic interaction with an anionic bilayer surface. A similar conclusion can also be reached from the lack of β-structure formation observed for the major tryptic fragment of the ShB peptide in the presence of anionic phospholipid vesicles.

However simple, the model proposed above for the interaction of the ShB and ShB-L7E peptide with anionic phospholipid vesicles could have certain similarities with current models proposed to explain how the peptides produce K^+ channel inactivation. According to those models (Jan and Jan, 1992; Durell and Guy, 1992), the positively-charged COOH-terminal end of the ShB peptide is presumed to interact with a negatively charged region at the internal mouth of the ShB K^+ channel, which becomes accessible only upon channel opening. Such negatively charged region is made up of a ring of highly conserved glutamic acid residues plus the negatively charged ends of the S_{4-5} helix dipoles and thus may be comparable with the negatively charged surface of our anionic liposomes. On the other hand, because mutations that make the ShB peptide more hydrophobic results in a tighter peptide binding to the channel, a hydrophobic pocket has been postulated at an internal vestibule of either the ShB

K^+ (Murrell-Lagnado and Aldrich, 1993) or the Ca^{2+}-activated K^+ (Toro et al., 1994) channels. The formation of such a hydrophobic pocket could perhaps be attributed to the rotation of the amphipathic S_{4-5} helix induced by channel activation (Durell and Guy, 1992) and may provide a convenient environment into which, similarly to the hydrophobic domains of the bilayer in the anionic liposomes, the NH_2-terminal half of the ShB peptide may partition. Based on studies of channel inactivation kinetics, Murrell-Lagnado and Aldrich (1993) have proposed an "extended" conformation for the inactivating peptide when bound to the channel protein, whereas the possibility of adoption of a helical structure was considered less likely. Therefore, assuming that the above channel components impose conformational restrictions on the inactivating peptide somewhat similar to those imposed by the anionic phospholipid vesicles, the observed β structure in our model system should correspond to the "extended" structures proposed by Aldrich's group in the interaction of the peptide with the channel protein. Whatever the case might be, it seems unquestionable that the ShB peptide has a remarkable propensity to adopt β structure when challenged by an appropriate hydrophobic environment, suggesting that such conformation is also adopted in channel inactivation. Moreover, the difficulties encountered by the noninactivating ShB-L7E peptide to form β structure further suggests that the ability to form β structures in hydrophobic environments by inactivating peptides is important for function.

In relation to this, it has long been known that treatment with certain proteases results in the loss of fast ion channel inactivation (Armstrong and Bezanilla, 1977). Furthermore, Aldrich and co-workers (Zagotta et al., 1990) reported that it was the proteolysis of the inactivating peptide that caused such phenomena. In this work, we have reported that trypsinization of the ShB peptide results also in the loss of its ability to form β structures. This apparent correlation does not necessarily mean that the two phenomena are related causally, but in our interpretation it seems consistent with the hypothesis that adoption of β structure by the inactivating peptide is required for channel inactivation. Additionally, the finding that the ShB peptide can be heated in boiling water without losing its channel inactivation activity (Zagotta et al., 1990) seems consistent with the observation that the β structure adopted by the ShB peptide is extremely heat-stable and that the small decrease in such structural motif lost during heating is almost fully recovered upon cooling of the sample.

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