N-type Inactivation of the Potassium Channel KcsA by the Shaker B “Ball” Peptide

MAPPING THE INACTIVATING PEPTIDE-BINDING EPITOPE

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In vivo, the inactivating peptide from the eukaryotic Shaker B K^+ channel (the ShB peptide) on the prokaryotic KcsA channel have been studied using patch clamp methods. The data show that the peptide induces rapid, N-type inactivation in KcsA through a process that includes functional uncoupling of channel gating. We have also employed saturation transfer difference (STD) NMR methods to map the molecular interactions between the inactivating peptide and its channel target. The results indicate that the binding of the ShB peptide to KcsA involves the ortho and meta protons of Tyr^4, which exhibit the strongest STD effects; the C4H in the imidazole ring of His^16; the methyl protons of Val^4, Leu^2, and Leu^10 and the side chain amine protons of one, if not both, the Lys^18 and Lys^19 residues. When a noninactivating ShB-L7E mutant is used in the studies, binding to KcsA is still observed but involves different amino acids. Thus, the strongest STD effects are now seen on the methyl protons of Val^4 and Leu^10, whereas His^16 seems similarly affected as before. Conversely, STD effects on Tyr^8 are strongly diminished, and those on Lys^18 and/or Lys^19 are abolished. Additionally, Fourier transform infrared spectroscopy of KcsA in presence of ^13C-labeled peptide derivatives suggests that the ShB peptide, but not the ShB-L7E mutant, adopts a β-hairpin structure when bound to the KcsA channel. Indeed, docking such a β-hairpin structure into an open pore model for K^+ channels to simulate the inactivating peptide/channel complex predicts interactions well in agreement with the experimental observations.
phospholipid vesicles that, similar to the presumed inactivation site on the channel protein (3, 11), contain a hydrophobic domain (the acyl chains in the lipid bilayer), separated from the aqueous media by the negatively charged vesicle surface. These studies show that ball peptides are poorly structured in solution but adopt a β-structure when challenged by the channel-mimicking model target (19–21). Such β-structure has been identified as a β-hairpin that readily inserts into the hydrophobic domains provided by the lipid bilayer model target (20, 21, 23, 24).

Here we first demonstrate the occurrence of N-type inactivation mediated by the ShB peptide in KcsA, a prokaryotic potassium channel from *Streptomyces lividans* (25). Interestingly, KcsA inactivation is associated to functional uncoupling of channels that exhibited positively coupled gating prior to the addition of the ShB peptide (26). Also, saturation transfer difference (STD) NMR methods (27–29) have been used to identify interactions at atomic resolution in the KcsA-ShB complex. STD spectra are obtained after selective saturation of resonances of the KcsA protein, whereby the magnetization redistributes within the protein via intramolecular spin diffusion. Then, if a bound ligand is present, magnetization transfers from the protein to the ligand via 1H–1H dipolar contacts. An efficient intermolecular magnetization transfer from protons of the protein to the ligand requires very short distances, and therefore, ligand binding is strictly required for the transfer phenomenon. Our results demonstrate that indeed the wild-type ShB peptide binds to KcsA and also distinguish which amino acid residues are involved in binding. Finally, Fourier transform infrared spectroscopy using 13C-labeled ShB peptide supports the notion that the inactivating ShB peptide adopts a β-hairpin structure when channel-bound. Moreover, docking such a β-hairpin into the open channel conformation of KcsA modeled after MthK (14), a prokaryotic channel taken as a model for open K+ channels, predicts interactions that are well in agreement with those determined in the STD-NMR experiments.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Characterization**—The wild-type ShB and the mutant ShB-L7E peptides were synthesized as C-terminal amidated peptides in an automatic multiple synthesizer (AMS 422, Abimed, Lanfengeld, Germany) by using a solid phase procedure and standard Fmoc chemistry (19). The peptides were also acetylated at their N termini. The peptides were purified by reverse phase HPLC to better than 95% purity, and their composition and molecular mass were confirmed by matrix-assisted laser desorption ionization tryptic-peptide mass fingerprinting (30).

**Expression of the Wild-type KcsA**—Large unilamellar vesicles of asolectin (soybean lipids, type II-S; Sigma) were prepared at 25 mg/ml in 10 mM Hepes, pH 7.5, 100 mM KCl (reconstitution buffer) and stored in liquid N2 (31). The purified, DDM-solubilized KcsA protein was mixed with the above asolectin vesicles previously resolubilized in 3 mM DDM at a lipid:protein subunit molar ratio of 500:1, for 2 h. Reconstituted liposomes were formed by removing the detergent by gel filtration on Sephadex G-50 (fine, 15–20–ml bed volume) previously swollen overnight in buffer without detergent. The detergent-solubilized lipid/protein mixture (2 ml) was loaded on top of the column, and the reconstituted liposomes were eluted in the void volume. The protein-containing reconstituted fractions were pooled and centrifuged for 30 min at 300,000 × g. The pellet was suspended into reconstitution buffer to a protein concentration of 1 mg/ml, divided into 50-μl aliquots, and stored in liquid N2 (30).

**Electrophysiological Recordings in Reconstituted Giant Liposomes**—Multilamellar giant liposomes (up to 50–100 μm in diameter) were prepared by submitting a mixture of the reconstituted vesicles from above (usually containing 50 μg of KcsA protein) and asolectin lipid vesicles (25 mg of total lipids) to a cycle of partial dehydration/rehydration (26, 30, 31). Standard inside-out patch clamp recordings (32) were carried out on excised patches from giant liposomes containing the wild-type KcsA, as reported previously (26). Recordings were obtained using either an Axopatch 200A (Axon Instruments) or an EPC-9 (Heka Electronic, Lambrecht/Pfalz, Germany) patch clamp amplifier, at a gain of 50 mV/pA. The holding potential was applied to the interior of the patch pipette, and the bath was maintained at virtual ground (V = Vbath − Vpipette). The recordings were filtered at 1 kHz, and the data were analyzed with the Clampfit-9 software (Axon Instruments). An Ag–AgCl wire was used as the reference electrode through an agar bridge. All of the measurements were made at room temperature. The pipette (extracellular) solution contained 10 mM Hepes buffer, pH 7, 100 mM KCl, and the bath (intracellular) solution contained 10 mM MES buffer, pH 4, 100 mM KCl.

**Nuclear Magnetic Resonance Spectroscopy**—One-dimensional 1H NMR experiments were recorded at 283 K on an Avance Bruker DRX-500 using a z axis gradient by using BBO or TXI probeheads. Two-dimensional NMR spectra were recorded in the phase sensitive mode by using the States time proportional phase incrementation method (33). All of the experiments were carried out using the WATERGATE pulse sequence for water suppression.
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(34). Two-dimensional $^1$H-$^1$H total correlation spectra were recorded by using a MLEV-17 spin-lock sequence (35) with a mixing time of 80 ms. NOE spectroscopy experiments were recorded with mixing times of 100 and 250 ms. Typically, spectra were acquired with 512 $t_1$ increments, 2048 data points, and a relaxation delay of 1 s. The spectral width was 6000 Hz in all cases. The spectra were processed using the BRUKER-XWIN-NMR software working on a PC work station. All of the spectra were zero-filled in the $F_1$ spectral dimension to 1024 data points, and a squared sine bell window function was applied in both dimensions prior to Fourier transformation. Sequential assignments of proton resonances of ShB and ShB-L7E peptides were based on characteristic sequential NOE connectivities between the $H_N$ of a particular residue, and the amide proton of the next residue in the NOE spectra was referred to internal 3-(trimethylsilyl) propionic acid-2,2,3,3-$^2$H$_3$-sodium salt.

In the STD experiments, 2 mg of freeze-dried peptide were dissolved in 500 μl of buffer (50 mM d$_1$-acetic acid, 100 mM KCl, 10% D$_2$O), containing KcsA either reconstituted in asolectin vesicles or solubilized in 5 mM of the detergent DDM. Deuterated acetic acid and its sodium salt were purchased from Sigma. Peptide and KcsA final concentrations were 2 mM and 45 μM, respectively. The one-dimensional $^1$H STD NMR (27–29) spectra of ShB or ShB-L7E peptides-KcsA complexes were recorded with usually 4000 scans. Selective saturation of protein resonances was set at −3 ppm (on resonance). Control experiments at this frequency showed that the entire protein (either in reconstituted lipid vesicles or solubilized in DDM micelles) can be saturated uniformly and then efficiently used for the STD NMR technique. In this region, the methyl resonances of the protein appear, and irradiation at protein methyl groups has previously been found to yield the most effective saturation (36); furthermore, this frequency is far away from the most up-field shifted methyl protons of the peptides (see Ref. 22 and our own observations), such that no perturbation of this signal can be induced by the selectively shaped pulse. Investigation of the time dependence of the saturation transfer with saturation times ranging from 0.2 to 2.5 s showed that 1.5 s were needed for efficient transfer saturation from the protein to the ligand protons. STD NMR spectra were acquired using a series of equally spaced 50-ms Gaussian-shaped pulses for selective saturation. The radio frequency field strength of the Gaussian-shaped pulses was ~100 Hz, with a 1-ms delay between the pulses. Residual water signal was eliminated by using excitation sculpting procedures (37). A spin-lock sequence was applied before the excitation sculpting sequence to remove the signals of KcsA (27, 38). The reference STD spectra were recorded with the off resonance set at 30 ppm. The saturated spectra were subtracted from the reference spectra via phase cycling. Experiments with ShB-L7E peptide were only carried out with KcsA reconstituted in asolectin vesicles.

To ensure that the observed signals arose from the STD effect, the following controls were carried out. STD-NMR experiments with the on and off resonance frequencies set either at −3 and 30 ppm, respectively, were acquired with samples containing the ShB peptide in the absence of KcsA, either in plain aqueous buffer or in the presence of DDM; the saturation time was 2 s. No signals were present in the difference spectra, indicating that the effects observed in the presence of KcsA were due to true saturation transfer. In addition, we recorded control experiments in a sample of DDM micelles with on and off saturation frequencies set to identical values (either at −3 or 30 ppm). The difference spectra did not contain any signals, proving that good subtraction was achieved.

To allow for comparison among the different signals in the STD experiments, the signal intensities of the different protons were analyzed by the use of the fractional STD amplification factor, which expresses the signal intensity in the STD spectrum, $I_0 - I_{sat}$, as a fraction of an unsaturated reference spectrum, $I_0$, and the excess of added ligand (28).

$$\text{STD amplification factor} = \frac{I_0 - I_{sat}}{I_0} \times \text{[ligand excess]} \quad (\text{Eq. 1})$$

The errors in the STD amplification factor were estimated from the determination of the STD effects measured in two identical samples at 1.5 s and processed simultaneously. The differences obtained were lower than 10% for all the measured resonances.

Fourier Transform Infrared Spectroscopy—Lyophilized aliquots of synthetic peptides, with or without the desired lipid vesicles, were processed in D$_2$O buffer as reported earlier (20). For the samples containing detergent-solubilized KcsA, both in the presence and in the absence of added ShB or ShB-L7E peptides, aliquots of KcsA in 10 mM Hepes buffer, pH 7.0, 100 mM KCl, and 1 mM DDM were processes as described earlier (39), except that 10 mM acetate buffer, pH 4.0, 100 mM KCl, 1 mM DDM was used for the washings. Routinely, amide I bands in these experiments were 0.4 absorbance units or larger.

For spectral acquisition, the aliquots of 20 μl of each sample were placed between a pair of CaF$_2$ windows separated by a 50-μm-thick mylar spacers and mounted in a Harrick (Ossining, NY) demountable cell. Temperature (~22 °C) was maintained constant with a circulating water bath. The spectra were recorded on a Bruker IF66s instrument equipped with a DTGS detector, and the sample chamber was continuously purged with dry air. A minimum of 600 scans/spectrum were taken, averaged, apodized with a Happl-Genzel function, and Fourier-transformed to give a nominal resolution of 2 cm$^{-1}$ (40). Three spectra were recorded for each sample, and the buffer contribution was subtracted. Derivation was performed using a power of 3 and a breakpoint of 0.3. Self-deconvolution was performed using a Lorentzian bandwidth of 18 cm$^{-1}$ and a resolution enhancement factor of 2.0 (39).

Modeling the ShB-KcsA Complex—The modeling of KcsA in the open conformation was done by homology, using the crystal structure at 3.3 Å resolution of the calcium-gated potassium channel MthK as a template (Protein Data Bank code 1LNQ). The multiple sequence alignment was made with CLUSTALW at the European Bioinformatics Institute site and manually supervised (final 24% identity and 40% similarity). The homology modeling was performed in the Swiss-Model Protein Modeling Server at the ExPASy Molecular Biology site. For model refinements, the orientation and optimization of the side chains was carried out in two steps. First, those residues making van
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The Inactivating ShB Peptide Uncouples and Inactivates KcsA—The prokaryotic potassium channel KcsA behaves in many respects similarly to its structurally more complex eukaryotic counterparts (46), including the occurrence of slow, C-type inactivation (47). Perhaps for these reasons and also because of the promiscuity of the ShB ball peptide in inducing N-type, rapid inactivation in many different potassium channels (4–10), it has been assumed that the ShB peptide should also induce N-type inactivation in KcsA (13). Nonetheless, no experimental evidence on this matter has been reported to date. Here, we have used patch clamp techniques to study the effects of synthetic ShB peptide on KcsA reconstituted into asolectin giant liposomes. Fig. 1 illustrates the effects of the addition of the ShB peptide to the bath solution (cytoplasmic side of the channel) in an excised, inside-out membrane patch exhibiting a low opening probability pattern, the most frequent functional pattern exhibited by the reconstituted KcsA channel (27). No effects on the ion currents were observed when the ShB peptide was added into the pipette solution (extracellular side of the channel).

Prior to the addition of the peptide (Fig. 1A), KcsA exhibits “single channel-like” currents of different sizes (mainly 8 pA, but also a few 12 pA events at +150 mV), which are integer multiples of the smaller, truly KcsA single-channel current of 4 pA, and result from the simultaneous (coupled) gating of two or three KcsA channels (see Ref. 27 for more details on the positive coupling of KcsA). Also, as in the original report on the inactivation of Shaker B Δ6–46 channel by the synthetic ShB peptide (4), inactivation of KcsA occurs in a peptide concentration-dependent manner and most noticeably at positive (depolarizing) voltages, where the peptide (Fig. 1B) causes only a few, uncoupled 4 pA currents to remain that eventually disappear as the peptide concentration is increased, leaving little or no activity in the recording. An apparently similar uncoupling of channel gating seemed also present in the original report on the effects of the ShB peptide on the Shaker B Δ6–46 channel (see Fig. 3 in Ref. 4), although it was not discussed by the authors. On the other hand and also similar to the report on the Shaker B channel, only minor effects of the inactivating peptide were observed at negative (up to −200 mV) voltages (not shown). Moreover, Fig. 1C shows that washing the ShB peptide off the membrane patch by replacing the bath solution with fresh peptide-free buffer results in the recovery of channel activity and in the reappearance of coupled gating. Finally, no significant effects on the currents are seen at any voltage when, instead of the wild-type ShB peptide, the noninactivating ShB-L7E peptide mutant is used in the experiments (Fig. 1D), which is also reminiscent of the original report on the inactivation of the Shaker B Δ6–46 channel by ShB peptide derivatives (4).

NMR Assignments and Peptide Structure—The above demonstration that the ShB peptide induces N-type inactivation of the KcsA channel, along with the fact that both the channel and the synthetic peptide can be prepared easily and in sufficiently large amounts, makes this an unique experimental system to learn on the molecular interactions behind the rapid inactivation phenomena through the application of spectroscopic techniques. Here, we have used STD-NMR methods in an attempt to explore such interactions between the inactivating peptide and KcsA as the ion channel target. To this end, we first assigned the resonances seen in the proton NMR spectra of the wild-type ShB peptide in aqueous solution at pH 5.0. Such acidic conditions (pH 4–5.5) have been reported as optimal for opening the KcsA channel (27, 46, 48–51), and indeed, the inactivation patch clamp experiments from above were conducted at pH 4 in the solution bathing the membrane patches. Proton assignment was carried out by using homonuclear standard two-dimensional total correlation spectroscopy and NOE spectroscopy experiments (52). Chemical shifts essentially agreed with those reported previously by Kalbitzer and co-workers at pH 7.2 (see Table 1 in Ref. 22), except for small changes in the side chains of the amino acid His16 and its neighboring residues, probably because of protonation of the imid-
ordered conformation, as concluded from: (i) the absence of medium or long range NOEs (Fig. 2A); (ii) the small H-NOEs conformational shifts that, with the exception of residues D13 to Gln, are within the usually accepted limits for random coil conformational shifts (Fig. 2B) (53, 54); and (iii) the absence of chemical shift dispersion in both the amide and the methyl group regions of the spectra (data not shown).

In the presence of KcsA reconstituted into asolectin vesicles, the spectrum of the ShB peptide experienced a significant line broadening with compared with that in solution, although the chemical shifts of the different protons were not modified (data not shown). We interpret such broadening as a clear indication of an interaction between ShB and KcsA, particularly because it occurs at a high ShB peptide to KcsA molar ratio (2 mM of peptide versus 45 mM of KcsA), where the spectral features are dominated by signals arising mostly from the free form of the peptide. Interestingly, either in aqueous solution or in the presence of KcsA reconstituted into asolectin vesicles, the methyl protons of Val appeared at the same chemical shift (0.98 ppm), but those of Leu (0.85 and 0.91 ppm) and Leu (0.93 and 1.00 ppm) appeared at different chemical shifts, suggesting some preferred local orientations, as reported in other random coil ensembles (53, 54).

Proton assignments were also obtained for the non-inactivating ShB-L7E mutant peptide both in aqueous solution and in the presence of KcsA reconstituted into lipid vesicles. In either condition, the ShB-L7E peptide showed similar chemical shifts to those exhibited by the wild-type ShB peptide, except for amino acids Gly and Tyr, which are adjacent to the mutation site. Despite the similarities, two observations were made in the ShB-L7E peptide that differed from those made in the wild-type ShB. First, the line broadening of peptide signals observed in the presence of KcsA was not as large as that observed for the wild-type ShB. Second, the resonances of the methyl groups of Leu were degenerated in the ShB-L7E, suggesting that the preferred local conformation observed in the wild-type ShB peptide around these methyl groups is disrupted in the non-inactivating mutant.

**Probing Wild-type ShB Peptide-KcsA Channel Interactions in STD-NMR Experiments**—Fig. 3 shows the amide region of a reference, nonsaturated $^3$H NMR spectrum of a sample containing the wild-type ShB peptide and KcsA reconstituted into asolectin vesicles (Fig. 3A), along with STD-NMR spectra from the same sample, taken at different times of saturation (1 s, panel C and 2.5 s, panel E). Several resonances can be observed within the one-dimensional $^3$H-STD NMR spectra, which (i) are sufficiently resolved from the rest of the peptide reso-
that Tyr effects. This seems consistent with previous findings indicating among the ShB peptide resonances showing the largest STD measurements. were lower than 10%, as concluded from two different experimental series of imidazole ring of His present in ShB-L7E), and Leu also experienced significant STD effects (Fig. 4), although their magnitude was not as large as those observed for the aromatic protons of Tyr. Taken together, the above results indicated that binding of the ShB peptide to KcsA reconstituted into lipid vesicles involved mainly the aromatic rings of both Tyr and His in the inactivating peptide.

When the experiments were performed using DDM-solubilized KcsA as the target for the ShB peptide, instead of the reconstituted KcsA vesicles, a similar line broadening of the ShB peptide signals was observed. Moreover, practically identical STD effects were seen in such samples, suggesting that KcsA in the detergent-solubilized form is as capable as the reconstituted KcsA in allowing binding of the ShB peptide. Unfortunately, however, the presence of signals in the aliphatic region arising from the detergent overlapped with some of the peptide signals, thus preventing an adequate integration. Therefore, we decided not to study any further the detergent-solubilized system.

STD-NMR Monitoring of the Interaction of the Noninactivating ShB-L7E Peptide with KcsA Reconstituted into Lipid Vesicles—As concluded from the above patch clamp experiments, the ShB-L7E peptide does not inactivate the KcsA channel, which also resembles the original observation by Aldrich and co-workers (4) on the Shaker B Δ6–46 channel. Interestingly, despite such dramatic functional differences between the noninactivating ShB-L7E peptide and the wild-type ShB, both peptides are able to bind to the KcsA channel, as suggested by the line broadening observed in the NMR spectral bands (see above). However, the STD effects are quite different from those observed in the ShB peptide (Figs. 3 and 4). For instance, the STD effects on the aromatic protons of Tyr are clearly smaller in the mutant ShB-L7E peptide than in the wild-type ShB peptide. Conversely, STD effects on the methyl groups of Val and Leu are clearly more important in the mutant peptide than in its wild-type counterpart, despite the fact that in the latter, this contribution includes also that of Leu, which is obviously lacking in the mutant L7E peptide (Fig. 4). Furthermore, the side chains of Lys and/or Lys displayed STD effects in the wild-type ShB peptide but do not give rise to any STDs in the ShB-L7E peptide, indicating that the side chains of these residues do not participate in the binding of the mutant peptide to KcsA. Therefore, the main conclusion from these experiments is that binding of the noninactivating ShB-L7E peptide to KcsA results in less important STD effects on the key residue Tyr, as well as none at all on Lys and/or Lys, being the interaction between the KcsA protein with the aliphatic Val and Leu methyl groups clearly more intense. Finally, the involvement of the aromatic moiety of His in the binding to the channel protein remains similar, within experimental error, for both peptides.

We also observed a strong STD effect on the C4H of the imidazole ring of His (at 7.36 ppm), as well as a much weaker STD effect on the amine protons of the side chains of Lys and/or Lys (at ~7.70 ppm and close to one of the protons of the side chains of Glu and Gin). Finally, the amide proton of Ala (at 8.85 ppm) also shows STD effects, but they were smaller than any other described here and will no be further discussed. Moreover, the methyl protons of Val, Leu, and Leu also experienced significant STD effects (Fig. 4), although their magnitude was not as large as those observed for the aromatic protons of Tyr. Taken together, the above results indicated that binding of the ShB peptide to KcsA reconstituted into lipid vesicles involved mainly the aromatic rings of both Tyr and His in the inactivating peptide.

FIGURE 4. STD amplification factors for ShB and ShB-L7E. The STD amplification factors at different saturation times were calculated for the interaction of wild-type ShB (A) and ShB-L7E (B) to KcsA reconstituted into asolectin lipid vesicles. The different symbols correspond to the ortho (blank circles) and meta (filled circles) protons of Tyr; the methyl protons of Val, Leu (not present in ShB-L7E), and Leu (blank squares); and the C4H proton of the imidazole ring of His (filled squares). The errors at each experimental point were lower than 10%, as concluded from two different experimental series of STD measurements.
Does the ShB Peptide Adopt a Defined Conformation when Bound to the KcsA Channel?—The above NMR measurements suggested that the inactivating ShB peptide and the noninactivating ShB-L7E peptide mutant associate in different manners to the KcsA channel protein. Hypothetically, such a different association could be due to the adoption of different conformations by the peptides once they become channel-bound. Thus, we thought of characterizing such putative peptide structures by means of NOE transfer measurements (56). Unfortunately, however, no such transfer NOEs could be obtained, even when employing different channel to peptide ratios (ranging from 45:1 to 15:1, in molar terms) and mixing times (ranging from 100 to 400 ms). Detection of transfer NOEs requires a fast exchange between free and bound forms of the peptide ligand (57), and therefore, it is conceivable that they could not be detected here, because of an unfavorable slow exchange of the ligand from the ShB-KcsA complex. This could perhaps be the case, because the dissociation constant estimated from fluorescence titrations of the binding of ShB peptide to the KcsA channel is of \( \sim 4 \times 10^{-7} \) M.\(^6\) Whatever the reason might be, we turned out to conformation-sensitive Fourier transform infrared spectroscopy measurements as an alternative means to detect the possible adoption of a defined structure by the ShB peptide upon interaction with the KcsA channel. For technical simplicity, because the interaction of ShB with KcsA reconstituted into asolectin vesicles is similar to that seen in detergent solution (see above), the Fourier transform infrared spectroscopy measurements were carried out on detergent-solubilized channel/peptide mixtures. Also, the chymotryptic derivative 1–125 KcsA was used in these experiments instead of the wild-type channel, because it was found that cleavage of the cytoplasmic C terminus facilitated channel inactivation by the ShB peptide and allows the use of lower peptide concentrations.\(^7\) Finally, because the relevant infrared bands in both the peptide and the channel protein are fully overlapped, we performed these experiments using a heavy isotope-labeled ShB peptide, in which all the alanine (Ala\(^2\), Ala\(^3\), and Ala\(^5\)) and leucine (Leu\(^7\) and Leu\(^10\)) residues were substituted by their 1-\(^{13}\)C analogs. Under such conditions, an isotopic shift to lower vibrational frequencies in the infrared bands of the heavy isotope-labeled peptide is expected, which should permit its monitoring separately from the spectral bands arising from the nonlabeled channel protein or other sample components.

Fig. 5A shows that the amide I band of the nonlabeled ShB peptide in aqueous solution, where the peptide is known to adopt a random conformation (see above) (20), is indeed dominated by a 1644 cm\(^{-1}\) spectral component, assigned to nonordered secondary structure. In the \(^{13}\)C-labeled peptide, an additional shoulder at 1610 cm\(^{-1}\) was observed, thus allowing for the separate detection of \(^{13}\)C=O and \(^{13}\)C-O vibrations, which differed in \( \sim 33 \) cm\(^{-1}\). Such extent in the isotopic shift was further confirmed by spectra of the ShB peptide taken in the presence of anionic lipid (dimyristoyl phosphatidic acid) vesicles (Fig. 5B), which is known to induce the adoption of a \( \beta \)-hairpin structure in the ShB peptide with a characteristic band at 1624 cm\(^{-1}\) (20). In the \(^{13}\)C-labeled peptide, this spectral component was shifted to \( \sim 1593 \) cm\(^{-1}\) (Fig. 5B). Similar results were obtained with DMPG vesicles (data not shown). Interestingly, the spectra of the labeled ShB peptide in the presence of anionic lipid vesicles showed little or none of the nonlabeled \( \beta \)-hairpin band at 1624 cm\(^{-1}\), suggesting that the amino acid residues substituted by their \(^{13}\)C-labeled counterparts are precisely those contributing the most to the observed \( \beta \)-hairpin peptide structure.

Fig. 5C shows the results obtained when using the \(^{13}\)C-labeled ShB peptide in presence of the KcsA channel. Despite the extent of the isotopic shift from above, the amide I band arising from the detergent-solubilized KcsA is too large and still overlaps considerably with the spectral region where the amide I band from the \(^{13}\)C-labeled ShB peptide is expected to appear (Fig. 5, A and B). Nevertheless, despite overlapping, use of res-

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\(^{6}\) F. N. Barrera, unpublished observation.

\(^{7}\) M. L. Molina, unpublished observation.
Despite the difficulties arising from spectral overlap, we interpret such spectral component as truly derived from the acquisition of a β-hairpin or similar structure by a presumably small population of 13C-labeled ShB peptide bound to the KcsA channel during the course of the experiment. A potential candidate for such a structure could be the β-turn resulting from Asp13, Arg14, and Gln15, as suggested by the slight deviation from the accepted random coil conformational shifts. This, however, seems unlikely because the only 13C-labeled residue near such putative turn is Leu10, which should not be able to produce the infrared component seen at 1596 cm$^{-1}$. A more likely possibility is that such structure corresponds to a β-turn similar to that causing the β-hairpin seen when the ShB peptide is confronted to anionic lipid vesicles, a much simpler experimental model system mimicking certain physicochemical features of the cytoplasmic channel mouth (21). This seems supported by the similarity observed in the wave number of the spectral components seen in the 13C-ShB when bound to the KcsA channel (1596 cm$^{-1}$) and in the 13C-ShB peptide in presence of anionic lipid vesicles (1593 cm$^{-1}$). Furthermore, the β-hairpin structures resulting from a β-turn either at VAGL (residues 4–7) or at AGLY (residues 5–8) (21) include hydrogen bonding of all the 13C-labeled amino acid residues in ShB, namely, Ala3, Ala5, Ala3, Leu7, and Leu10, thus making it possible to produce the observed 1596 cm$^{-1}$ component in the infrared spectrum. In either case, the putative β-hairpins would define a mostly hydrophobic domain comprising the N-terminal half of the ShB peptide that, as indicated in the Introduction section, should have a complementary domain into the cytoplasmic channel mouth, into which it should be able to enter and bind when the channel is in the open state. Size seems not to be a problem, because the width of a β-hairpin would not exceed that of the cytoplasmic mouth of KcsA (14).

In an attempt to integrate the above experimental information, we carried out docking of the ShB peptide modeled as a β-hairpin, into an open channel conformation of KcsA modeled after the x-ray structure of MthK, the open K$^+$ channel

![Figure 6](JBC-MC2-V283-P18074 Fig6.jpg)
model (Fig. 6). The amino acid residues indicated in the figure and their numbering correspond to the KcsA sequence after the homology alignment on the MthK sequence. Fig. 6A shows that the ShB hairpin predictably enters into the pore up to the hydrophobic pocket provided mainly by the Phe103 and Thr107 residues from all four KcsA subunits, ~8–9 Å below the N-terminal end of the selectivity filter. The docking model foresees interactions involving Val8, Leu7, Tyr8, and Leu10 in the ShB peptide and Phe103 and Thr107 in KcsA (Fig. 6B). Likewise, the model predicts that His16 and possibly Lys18 in the ShB peptide may be hydrogen-bonded to Glu118 and Glu120, respectively, in the KcsA channel (Fig. 6C). Interestingly, the energy estimates for the modeled ShB peptide/KcsA channel complex yield binding affinities in the micromolar range, similar to that determined experimentally by patch clamp measurements. Moreover, a previous report based on a double mutant cycle analysis (13) using the α subunit of rat K1, 1.4 channel, a mammalian homolog of Shaker, and the construct β 12 subunit as the inactivation gate, predicted that peptide binding to the channel should involve the residues equivalent to Ala5, Gly6, or Leu7 in the ShB peptide and Thr107 or Ala111 in the KcsA channel, which is in apparent agreement with our expectations from the docking model. Thus, the predictions from the docking model seem consistent with the experimental data and lend further support to both the identity of the amino acid residues comprising the ShB peptide-binding epitope and the adoption of the β-hairpin conformation when the inactivating peptide is channel-bound.

Conclusions and Final Remarks—Rapid inactivation of KcsA, a prokaryotic potassium channel, by a synthetic peptide (the ShB peptide) having the sequence of the inactivating ball peptide of the eukaryotic Shaker B K+ channel is reported. We found that channel inactivation is associated to functional uncoupling of positively coupled KcsA channels and exhibits similar features to those reported originally for the inactivation by the ShB peptide of Shaker and other K+ channels of eukaryotic origin. Such similarity emphasizes the conservation through evolution of important functional domains in these channels and further confirms KcsA as a bona fide model for studying potassium channels.

This report also introduces STD-NMR methods to study ion channel inactivation, drawing important information on molecular events that were largely unknown up to now. Thus, it is observed that regardless of whether the wild-type ShB peptide or the noninactivating ShB-L7E mutant peptide is used in the experiments, there is always peptide binding to the KcsA channel. However, clear differences in the binding of both peptides to KcsA were detected. For instance, Tyr8 in the ShB peptide displays a stronger interaction with KcsA than its homolog Tyr8 in the noninactivating ShB-L7E peptide. Furthermore, qualitative differences were observed for the side chains of Lys18 and/or Lys19, because the STDs observed for these residues in the ShB peptide were not observed in the ShB-L7E peptide. Finally, the STD effects obtained for the methyl groups of Val4 and Leu10 were clearly more intense in the mutant than in the wild-type peptide. These results indicate that the mutation at position 7 alters not only the binding of the peptide to the KcsA channel, but also the topology of the peptide atoms within the KcsA binding site. Unfortunately, transfer NOEs could not be obtained, and therefore, the adoption of preferred conformations by either peptide when bound to the KcsA channel could not be assessed by means of NMR. On the contrary, Fourier transform infrared spectroscopy measurements using 13C-labeled peptides could be applied successively, and although not fully conclusive because of spectral overlap, they suggest that the ShB peptide, but not the noninactivating ShB-L7E mutant, adopts a β-hairpin structure when bound to the KcsA channel. Indeed, such conclusions seem supported by the observations derived from docking the ShB peptide as a β-hairpin into a KcsA open channel model, which predicts the occurrence or interactions very similar to those observed experimentally.

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