Interactions of Amino Terminal Domains of *Shaker* K Channels with a Pore Blocking Site Studied with Synthetic Peptides

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ABSTRACT   Synthetic peptides of the five alternative NH_2-terminal sequences of *Shaker* when applied to the cytoplasmic side of ShB channels that have an NH_2-terminal deletion (ShBΔ6-46) block the channel with potencies correlated with the rate of inactivation in the corresponding variant. These peptides share no sequence similarity and yet three out of the five have apparent dissociation constants between 2 and 15 μM, suggesting that the specificity requirements for binding are low. To identify the primary structural determinants required for effective block of ShBΔ6-46, we examined the effects of substitutions made to the 20 residue ShB peptide on association and dissociation rates. Nonpolar residues within the peptide appear to be important in stabilizing the binding through hydrophobic interactions. Substitutions to leucine-7 showed there was a clear correlation between hydrophobicity and the dissociation rate constant (k_{off}) with little effect on the association rate constant (k_{on}). Substituting charged residues for hydrophobic residues within the region 4–8 disrupted binding. Within the COOH-terminal half of the peptide, substitutions that increased the net positive charge increased k_{on} with relatively small changes in k_{off}, suggesting the involvement of long-range electrostatic interactions in increasing the effective concentration of the peptide. Neutralizing charged residues produced small changes in k_{off}. Charges within the region 12–20 act equivalently; alterations which conserved net charge produced little effect on either k_{on} or k_{off}. The results are consistent with this region of the peptide having an extended conformation and suggest that when bound this region makes few contacts with the channel protein and remains relatively unconstrained. Analogous mutations within the NH_2-terminal domain of the intact ShB channel produced qualitatively similar effects on blocking and unblocking rates.

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INTRODUCTION

The ShB potassium channel produces a transient outward current in response to depolarization of the membrane potential. Functional analysis of mutant ShB channels has revealed that 20 amino acids at the NH_2 terminus are necessary for the rapid inactivation process that these channels undergo after opening (Hoshi, Zagotta, and Aldrich, 1990; Zagotta, Hoshi and Aldrich, 1990). The time constant of inactivation of macroscopic currents at 20°C and at +50 mV is 1–2 ms. Deleting the NH_2-terminal region produces a channel which inactivates with a time constant of 1–2 s. Application of a synthetic peptide of the 20 residue NH_2-terminal sequence to the cytoplasmic side of channels which have the deletion (ShBΔ6-46) will effectively restore the fast inactivation process (Zagotta et al., 1990). It has been proposed that the 20 residue NH_2-terminal domain acts like a tethered particle and binds to the internal mouth of the open channel pore thereby blocking ion flow (Choi, Aldrich, and Yellen, 1991; Demo and Yellen, 1991; Hoshi et al., 1990). This is similar to the "ball and chain" model for inactivation originally proposed by Bezanilla and Armstrong (1977) as the mechanism of inactivation in voltage-gated Na channels. Recent studies have suggested that similar inactivation mechanisms occur in some cloned mammalian rapidly inactivating K channels (Ruppersberg, Stocker, Pongs, Heinemann, Frank, and Koenenn, 1991) and also in Ca-activated K channels in adrenal chromaffin cells (Solaro and Lingle, 1992).

The functional ShB channel is a tetramer of identical subunits (Liman, Tytgat, and Hess, 1992; MacKinnon, 1991) and inactivation has been shown to involve only one of the four NH_2-terminal domains per channel blocking the pore (MacKinnon, Aldrich, and Lee, 1993). There are two pieces of evidence in support of the binding site for the NH_2-terminal region being in the vicinity of the pore. Firstly, internal tetraethylammonium (TEA), which is a pore blocker, competes with the rapid inactivation process (Choi et al., 1991), and secondly, the stability of the inactivated state is sensitive to the concentration of K^+ on the external side of the membrane (Demo and Yellen, 1991). There is some evidence to suggest that the ShB peptide binds to a similar site as the tethered NH_2-terminal domain. It appears to bind preferentially to open channels (Zagotta et al., 1990) and a point mutation at the NH_2 terminus of the intact ShB channel that effectively removes fast inactivation, (leucine-7 to glutamic acid), when made in the peptide, disrupts the ability of the peptide to block ShBΔ6-46 channels (Hoshi et al., 1990; Zagotta et al., 1990). The ShB peptide has been shown to block other K channels; for example, the Ca-activated K channel (Foster, Chung, Zagotta, Aldrich, and Levitan, 1992; Solaro and Lingle, 1992; Toro, Stefani and Latorre, 1992), the RCK1 channel (Zagotta et al., 1990), an RCK1-4 chimera (Ruppersberg et al., 1991), and a K channel in epithelial cells (Dubinsky, Mayorga, and Schultz, 1992). Although the blocking potency of the peptide varies among the channels this suggests that the region of the channel protein that forms the internal mouth of the pore may be very similar for these different K channels. However it raises the question of how specific is the binding of the peptide to the pore blocking site. The observation that substituting a single residue produced a dramatic reduction in the blocking potency of the peptide suggested that binding was fairly specific (Zagotta et al., 1990).

The ShB channel is one of five alternatively spliced NH_2-terminal variants of *Shaker* (Schwarz, Tempel, Papazian, Jan, and Jan, 1988; Pongs, Kecskemethy, Muller, Krah-Hentgens, Baumann, Kiltz, Canal, Llamazares, and Ferrus, 1988; Kamb, Tseng-Crank, and Tanouye, 1988). The five alternative sequences are of different lengths, between 9 and 61 residues, and share no sequence similarity with each other. However, three of the variants (ShB, ShC, and ShD) inactivate rapidly (Aldrich, Hoshi, and Zagotta, 1990; Timpe, Jan, and Jan, 1988a; Timpe, Schwarz, Tempel, Papazian, Jan and Jan, 1988b; Zagotta, Hoshi, and Aldrich, 1989) whereas the other two (ShD$_2$ and ShH37) inactivate much more slowly with time constants of hundreds of milliseconds (Iverson, Tanouye, Lester, Davidson, and Rudy, 1988; Stocker, Stühmer, Wittka, Wang, Müller, Ferrus, and Pongs, 1990).

The aims of this study were firstly to determine whether the differences in the inactivation kinetics among the *Shaker* NH_2-terminal variants are determined solely by the blocking activity of the NH_2-terminal region of the polypeptide chain. The blocking potencies of peptides corresponding to different lengths of the NH_2-terminal sequences of all five variants were compared to determine whether they correlated with the rates of inactivation in the intact *Shaker* variants. Secondly we aimed to determine the primary structural features of the ShB peptide required for channel blockade and to compare this with the features of the NH_2-terminal domain of the intact ShB channel that are important in determining the rates of inactivation. The approach used was to examine the effects of amino acid substitutions on the rates of association and dissociation of the ShB peptides to the ShBΔ6-46 channel.

MATERIAL AND METHODS

Generation of Mutant cDNAs

The ShBΔ6-46 deletion was generated as described previously (Hoshi et al., 1990). The mutations in the NH_2-terminal region of *Shaker* B were generated by a method involving the polymerase chain reaction as described in Hoshi, Zagotta, and Aldrich, 1991.

Expression of Channels in Xenopus Oocytes

Shaker channel RNA was transcribed and injected into *Xenopus laevis* oocytes as previously described (Zagotta et al., 1989). Recordings were made 1–10 d after the injection.

Peptide Synthesis and Purification

Peptides were obtained from the Protein and Nucleic Acid Facility at Stanford University. All peptides were amidated. They were purified to a single peak by reversed phase HPLC on a C18 column (Waters Chromatography Division, Milford, MA). Purity was assayed on a Hi-pore reversed phase analytical column (Bio-Rad Laboratories, Richmond, CA).

Electrophysiology and Data Analysis

Macroscopic currents from expressed channels were made using the inside-out configuration of the patch-clamp method as described previously (Hoshi et al., 1990). Data was acquired and analyzed using a Digital Equipment Corp. (Marlboro, MA). LSI 11/73-based minicomputer system. The output of the patch-clamp amplifier was low-pass filtered through an eight pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized at sampling intervals between 50

and 100 μs unless otherwise indicated. The pipettes had initial resistances < 1MΩ, and no series resistance compensation was made. Recordings were made at 20°C. The internal solution contained 140 mM KCl, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 11 mM EGTA and 10 mM HEPES (pH 7.2). The external solution contained 140 mM NaCl, 6 mM MgCl$_2$, 2 mM KCl, and 5 mM HEPES (pH 7.1). Peptides were dissolved in internal solution and applied to the cytoplasmic side of membrane patches.

Measuring Peptide Blocking and Unblocking Rates

To measure the rate of block of ShBΔ6-46 channels by the different peptides we took advantage of a previous finding that inactivation is largely coupled to activation (Zagotta et al., 1989) and likewise that the ShB peptide appears to bind preferentially to channels in their open conformation (Zagotta et al., 1990). Peptides were dissolved in bath solution and applied to the cytoplasmic side of inside-out membrane patches while holding the potential across the patch at −100 mV. At this potential, ShBΔ6-46 channels are in the resting closed state. A depolarizing step was then given to +50 mV to activate the channels rapidly (< 1 ms). The patches contained many channels (between 100 and 5,000) so the rate of block was measured from the time course of the decay phase of the macroscopic current. This was fitted with the sum of two exponential components. The slow component had a time constant of several hundred milliseconds and represented another distinct inactivation process (C-type) that remains even after deleting the NH$_2$ terminus (Hoshi et al., 1991). The time constant of the fast component (τ_{fast}) was dependent upon peptide concentration and with the concentrations that were used was generally easily separable from the slower component. The amplitude of the slow component was taken to be the steady-state level of current remaining after peptide block and was divided by the sum of the amplitudes of the fast and slow components to give an estimate of the steady-state current as a fraction of the peak current in the absence of peptide (I_s). This value together with τ_{fast} was used to calculate the blocking (k_b) and unblocking rates (k_{ub}) from the expressions:

$$k_b = \frac{1 - I_s}{\tau_{fast}} \tag{1}$$

and

$$k_{ub} = \frac{I_s}{\tau_{fast}}. \tag{2}$$

If we equate blocking rates with binding rates, as discussed later, the association rate constant (k_{on}) is obtained by dividing k_b by the peptide concentration whereas the dissociation rate constant (k_{off}) is simply k_{ub}.

RESULTS

Time Course of Block by Shaker Variant Peptides

The alternatively spliced NH$_2$-terminal sequence of ShB is 61 residues in length. Only the first 20 residues appear to form the domain that blocks the channel pore. Deleting residues 23 to 37 in ShB speeded up inactivation suggesting that this region might act like a tether, keeping the pore blocking domain extended away from the rest of the channel (Hoshi et al., 1990). The ShB alternative sequence is the longest while the shortest is the ShC sequence which consists of only nine residues. However, there are 135 residues from the splicing point to the start of the first proposed

membrane spanning region of *Shaker* channels, some of which could contribute to the inactivation domain of the different variants. We made different length synthetic peptides based on the NH_2-terminal sequences of the five variants. Two of the ShC peptides included residues beyond the splicing point.

Intact ShB channels inactivate fastest with ShC and ShD channels 2–3 times slower (Aldrich et al., 1990). Fig. 1 shows the effects of peptides derived from ShB, ShC and
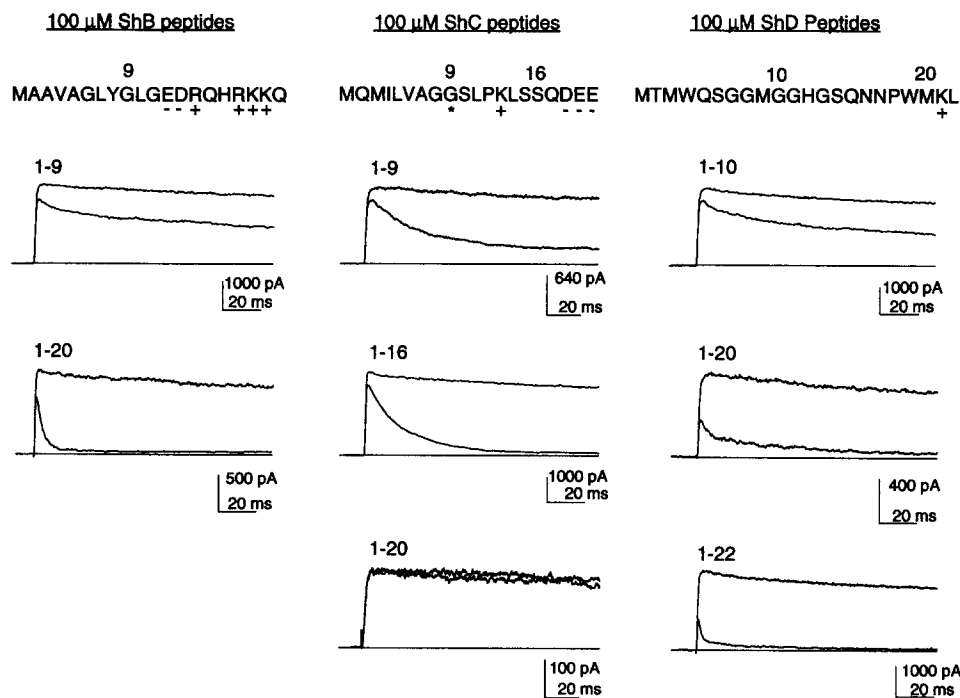

FIGURE 1. Effects of intracellular peptides derived from the NH_2-terminal regions of the ShB, ShC, and ShD variants on macroscopic currents from ShBΔ6-46 expressed in *Xenopus* oocytes. Currents were recorded with voltage steps to +50 mV from a holding voltage of −100 mV from inside out membrane patches before and after the application of peptides to the bath solution at a concentration of 100 μM. All peptides were amidated. The sequences shown at the top for ShB and ShD include only part of the alternatively spliced sequence, whereas the ShC alternative sequence consists of only nine amino acids so residues beyond the splicing point (*) which are common to all the variants are also shown. The numbers above the traces correspond to the residues at the NH_2- and COOH-terminal ends of the peptide from the above sequence. The currents were low pass filtered at 1.2 kHz and digitized at 100 μs per point.

ShD sequences applied to the cytoplasmic side of ShBΔ6-46 channels. Currents elicited by stepping the voltage from a holding voltage of −100 to +50 mV were recorded before and after addition of the peptides. In the presence of 100 μM of the 20 residue ShB peptide the current decayed with a time constant of 2.6 ± 0.15 ms and the mean steady-state level remaining was 3.5 ± 0.27% (SEM, n = 8). The nine residue ShB peptide, consisting of only uncharged residues, blocked much more

slowly, with a time constant of ~ 20 ms. Similarly the ShC and ShD peptides consisting of only the first 9–10 uncharged residues, blocked the current relatively slowly at this concentration. The 16 residue ShC peptide includes the positively charged lysine at position 13. In the presence of 100 μM of this peptide currents decayed with a time constant of 12.1 ± 1.5 ms (SEM, n = 4). In contrast the 20 residue peptide which includes three acidic residues produced no detectable block at 100 μM. This suggests that in the intact ShC channel these three negatively charged residues at positions 18, 19, and 20 are somehow shielded so that they do not disrupt the inactivation process. Of the ShD peptides the one consisting of the first 22 residues blocked most rapidly with a time constant of 1.6 ± 0.09 ms (SEM, n = 4). Again, this peptide had a positively charged lysine towards the COOH-terminal end. With the 20 residue ShD peptide there appeared to be significant block of channels in the closed state. The reduction in peak current amplitude was much greater than would be predicted given the time constant of decay, if the peptide was only blocking open channels.

The time course of inactivation in the intact NH_2-terminal *Shaker* variants has been described in several papers (Aldrich et al., 1990; Iverson et al., 1988; Pongs, et al., 1988; Timpe et al., 1988a, b; Zagotta et al., 1989). Inactivation in the intact ShB channel is the most rapid amongst the *Shaker* NH_2-terminal variants and has a time constant of 1–2 ms at +50 mV and at 20°C, whereas ShD and the ShC NH_2-terminal variants inactivate ~ twofold and threefold slower respectively. For these three variants there is very little steady-state current (<5% of peak current). By contrast, ShD_2 and ShH37 inactivate much more slowly, with time constants in the range of hundreds of milliseconds. Fig. 2 shows families of currents recorded from ShBΔ6-46 by stepping from −100 to between −25 to +50 mV in the absence of peptide and in the presence of peptides derived from all five *Shaker* variants plus a peptide derived from the mammalian rapidly inactivating K channel, Raw3 (Ruppersberg et al., 1991). In general the time course of block by 100 μM peptide was similar to the time course of inactivation in the corresponding variant. The blocking potencies of the ShD_2 and ShH37 peptides were considerably less than for the other three *Shaker* variant peptides. ShD_2 has only 16 residues from the splicing point to the NH_2-terminus. In the presence of the 16 residue peptide there was a small fast component of decay at +50 mV, but the steady-state level of current remaining was high, which is very similar to the time course of macroscopic currents recorded from the intact ShD_2 channel (Pongs et al., 1989). The 20 residue ShH37 peptide produced very little block and shown is just a single trace at +50 mV. The Raw3 peptide was shown by Ruppersberg et al. (1991) to block the RCK1-4 dimer with significantly higher affinity than the ShB peptide. However, it blocked the ShBΔ6-46 channel with similar potency to that of the ShB peptide. These results suggest that for all of the NH_2-terminal variants of *Shaker* the time course of inactivation is determined by the blocking activity of the region of the polypeptide chain at the NH_2-terminus.

For the rates of block and unblock of ShBΔ6-46 channel currents by the peptides to be a measure of the association and dissociation rate constants for binding, blockade of the channel has to involve the binding of a single peptide molecule to the channel and require no further rate limiting conformational change in the channel subsequent to binding. This type of simple bimolecular reaction predicts that the rate

of block should be linearly dependent on peptide concentration and the unblocking rate should be independent of concentration. In Fig. 3, blocking and unblocking rates are plotted versus peptide concentration for the ShB, ShC, and Raw3 peptides. Within the concentration range 5–200 µM, blocking rates increased linearly with peptide concentration whereas unblocking rates remained fairly constant. From the
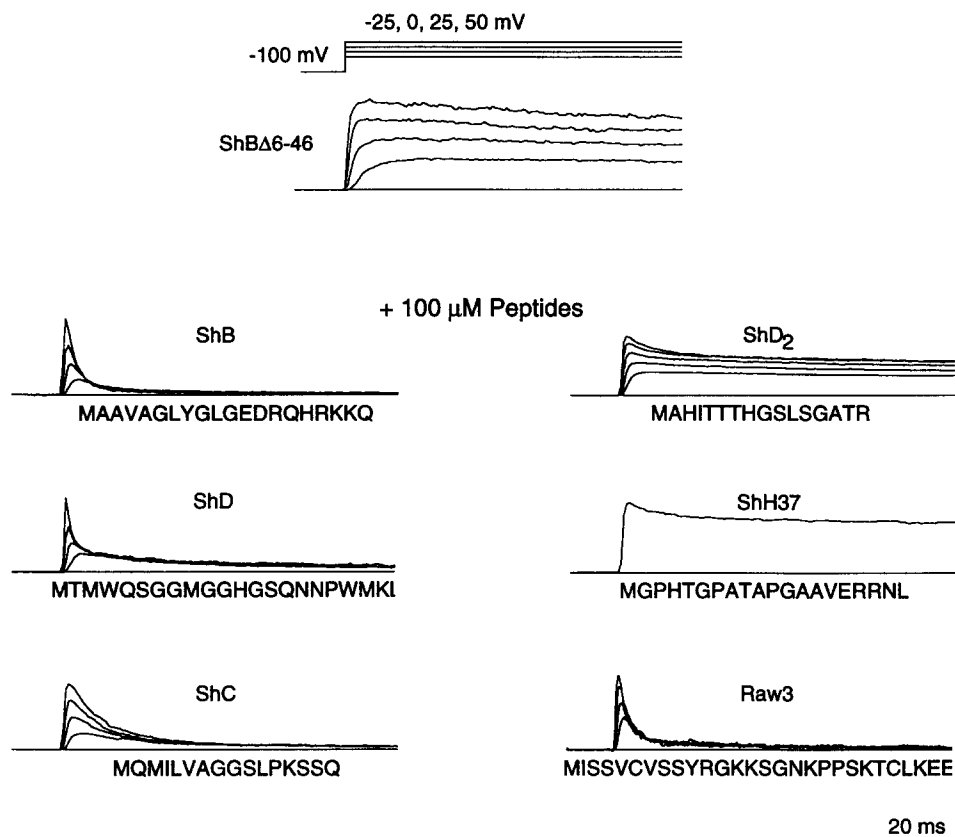

FIGURE 2. The time course of block by 100 µM of the NH_2-terminal peptides. Families were recorded using the voltage protocol shown at the top from ShBΔ6-46 channels in the absence of peptide and in the presence of the peptides indicated. The peptide sequences are given below the current families. The repetition interval between test pulses was 15 s. The current trace recorded from ShBΔ6-46 in the presence of the 20 residue ShH37 peptide was at +50 mV. The Raw3 peptide was the same as that used by Ruppersberg et al. (1991).

slope of the lines fitted to the data by the method of least squares we calculated the association rate constants (k_{on}) for ShB and Raw3 to be 4.8×10^6 M^{-1} s^{-1} and 5.0×10^6 M^{-1} s^{-1} respectively. This value for the ShB peptide agrees very closely with the value calculated from single channel records by Zagotta et al. (1990) which supports the method we have used to calculate rates from macroscopic currents as being valid.

The association rate constant for ShC peptide was lower, only 0.60×10^6 M^{-1} s^{-1}. However the dissociation rate (k_{off}) for ShC peptide was also lower than for the ShB peptide (7.7 ± 0.75 s^{-1} [SEM, $n = 13$]) compared with 13.8 ± 0.87 s^{-1} (SEM, $n = 20$), Table I). These results are similar to what was observed in single channel recordings from the corresponding channel variants (Aldrich et al., 1990). ShC inactivates more slowly than ShB but recovers from the inactivated state more slowly indicating that the inactivated state is relatively stable. In contrast, k_{on} calculated for
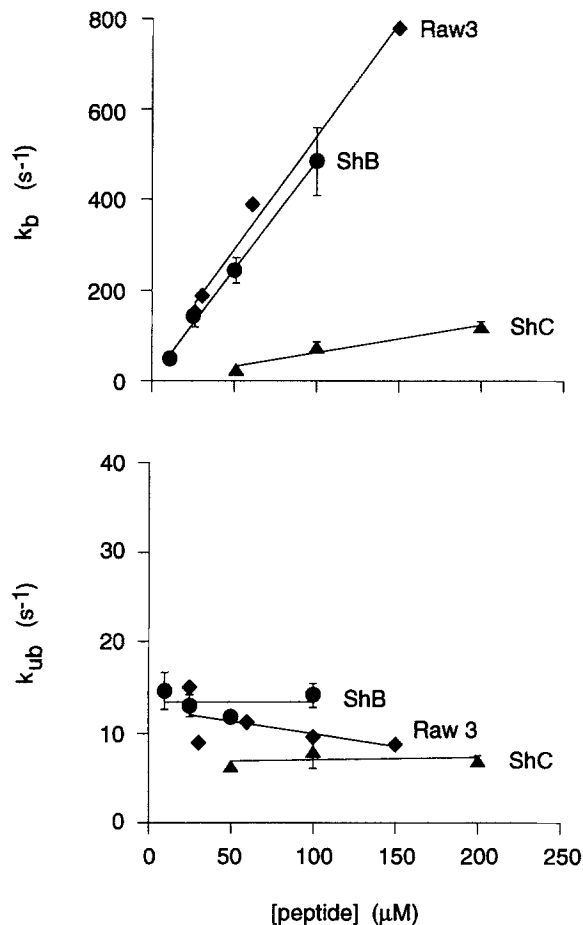

FIGURE 3. Blocking and unblocking rates as a function of the concentration of ShB, ShC, and Raw3 peptides. Error bars are ± S.E.M., $n = 2 - 5$. From a least square linear regression the slope of the lines fitted to the blocking rates were for the Raw3 peptide, 4.96×10^6 M^{-1} s^{-1}; for the ShB peptide, 4.7×10^6 M^{-1} s^{-1}; for the ShC peptide, 0.60×10^6 M^{-1} s^{-1}. Unblocking rates appeared to be independent of concentration of peptide.

the 22 residue ShD peptide was similar to that for the ShB peptide (5.5×10^6 M^{-1} s^{-1}) but k_{off} was fivefold faster (76 ± 4.3 s^{-1}).

The equilibrium constant for inactivation in the intact ShB channel is given by k_{ub}/k_b, where k_{ub} and k_b are from Eqs. 1 and 2. The mean value from two patches was 0.031. Dividing the equilibrium dissociation constant (K_d) for block of ShBΔ6-46 by the ShB peptide by this value for the intact channel, gives an estimate for the effective concentration of the tethered NH$_2$-terminal domain of ShB of 95 μM. This

TABLE I

Association and Dissociation Rate Constants for Binding of NH_2-Terminal Peptides and Altered ShB Peptides to ShBΔ6-46 Channels

NH_2-terminal peptides	k_{on}	k_{off}	K_d	n
	$\times 10^6\ M^{-1}\ s^{-1}$	s^{-1}	μM	
ShB	4.7 ± 0.38	13.8 ± 0.87	2.9 ± 0.030	20
ShD (22)	5.5 ± 0.35	76 ± 4.4	14 ± 1.2	4
ShC (16)	0.61 ± 0.06	7.7 ± 0.75	13 ± 1.7	13
ShD$_2$	1.0 ± 0.19	80 ± 9.2	80 ± 18	3
Raw3	5.8 ± 0.47	11 ± 1.1	1.8 ± 0.024	6
ShB peptides				
acetyl-ShB	1.6 ± 0.2	5.7 ± 2.0	3.6 ± 1.3	3
L10Q	2.9 ± 0.27	23 ± 3.3	8.0 ± 1.4	5
L7Q	3.8 ± 0.45	84 ± 6	22 ± 3.1	7
L7A	5.0 ± 0.7	43 ± 3.4	8.8 ± 1.4	5
L7I	4.7 ± 0.47	17 ± 1.5	3.7 ± 0.48	6
L7F	5.0 ± 0.62	11 ± 0.84	2.1 ± 0.32	7
Y8F	2.7 ± 0.47	6.9 ± 2.1	2.6 ± 0.90	4
L10K	4.7 ± 0.57	44 ± 1.2	9.6 ± 1.2	3
Y8K	4.9 ± 1.2	170 ± 4.3	34.7 ± 8.5	3
del A3A5	3.7 ± 0.37	15 ± 2.0	4.1 ± 0.67	5
ins A6, 7, 8	1.9 ± 0.088	11 ± 3.5	5.8 ± 1.9	4
L10P	2.8 ±	32 ±	11 ±	2
P ins 10	4.1 ± 0.52	20 ± 2.3	4.9 ± 0.84	3
P ins 6	2.9 ± 0.27	39 ± 4	13 ± 1.9	5
G11P	3 ±	18 ±	6.0 ±	2
R17QK18QK19Q	0.22 ± 0.026	30 ± 2.6	140 ± 20	7
H16QR17QK18QK19Q	0.29 ± 0.064	43 ± 6.3	140 ± 39	5
E12Q	11 ± 1.3	16 ± 2.6	1.5 ± 0.29	3
E12QD13Q	27 ± 4.5	14 ± 1.3	0.53 ± 0.099	4
E12QD13QR14QR17Q	2.7 ± 0.09	28 ± 2.8	10 ± 1.1	8
E12KD13K	140 ± 16	24 ± 1.5	0.17 ± 0.022	14
E12KD13KK18EK19D	3.9 ±	29 ±	7.4 ±	2

The rate constants were measured at +50 mV and at 20°C. K_d was calculated from k_{off}/k_{on}. The values are means ± S.E.M. and n is the number of determinations. The peptide, del A3A5 represents deletions of alanine residues at positions 3 and 5, and ins A6, 7, 8, represents insertions of alanines at positions 6, 7, and 8. P ins 10 and P ins 6 are proline insertions at positions 10 and 6 respectively. Sequences for the NH_2-terminal *Shaker* variants are given in Fig. 2.

is low for an intramolecular reaction. Where two groups within a protein are positioned nearby and in the correct orientation to bind the effective concentration can be in the range of 10 M (Creighton, 1992).

Internal TEA Competes with Peptide Block

Choi et al. (1991) showed that application of tetraethylammonium (TEA) to the internal side of ShB channels competed with the N-type inactivation process, thus providing evidence that the tethered domain binds in the vicinity of the channel

pore. We looked at the effects of internal TEA on block of ShBΔ6-46 channels by both the ShB and ShC (16 mer) peptides to test firstly whether the ShB peptide binds to a similar site as the tethered domain, and secondly, whether these two peptides which share no sequence similarity bind to a similar site on the channel. Fig. 4 shows macroscopic currents recorded in the presence of the ShB peptide and the ShC peptide before and after application of 1 mM TEA. TEA binds and unbinds very rapidly to ShBΔ6-46, producing a steady state level of block of macroscopic currents (Choi et al., 1991). The kinetics are too fast to resolve a relaxation phase after the activation of the channels. However, internal TEA slowed the decay phase of macroscopic currents in the presence of the peptides. In the presence of the ShC
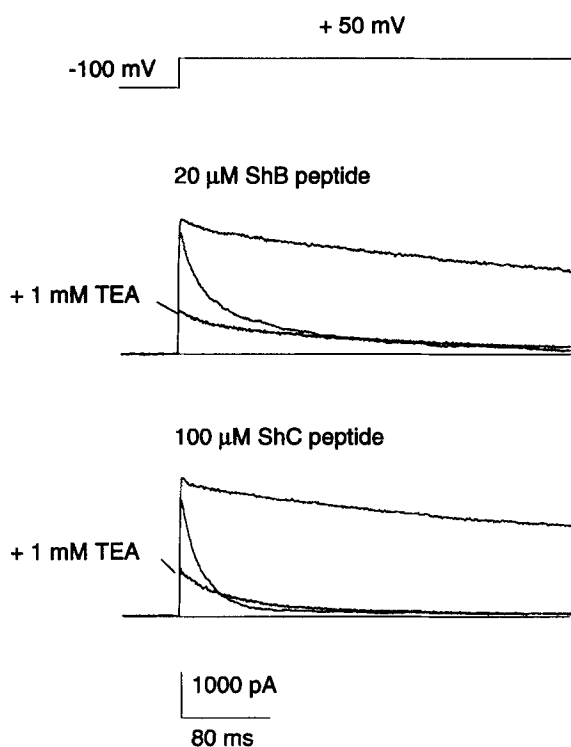

FIGURE 4. Effects of internal TEA on the apparent rate of block of ShBΔ6-46 by the ShB and ShC peptides. Macroscopic currents were recorded from an inside-out membrane patch with the voltage protocol shown above. Currents were recorded before and after application of 100 μM of the 16 residue ShC peptide. This was then washed out and applied for a second time in the presence of 1 mM TEA. Both were washed out and the experiment was repeated but this time applying 20 μM of the ShB peptide.

peptide alone the decay phase had a time constant of 16.6 ms, whereas with 1 mM TEA also present the time constant was 42 ms. Likewise, 1 mM TEA slowed the rate of block by the ShB peptide. On average 1 mM TEA produced a 2.1 ± 0.5 (SEM, n = 3)-fold increase in the time constant of decay in the presence of ShB peptide. The results indicate that TEA competes with the binding of both peptides, suggesting that they bind to nearby sites in the vicinity of the pore and physically occlude the pore. The free ShB peptide therefore appears to bind to a similar site on the channel as the tethered NH$_2$-terminal domain.

Comparing the sequences of the *Shaker* variant peptides that block effectively with those that produce little block few clear patterns emerge other than a suggestion that

a net positive charge is important. The 20 residue ShC peptide which has a net charge of -2 did not appear to have any blocking activity, although the ShH37 peptide which has a net charge of $+1$ was also ineffective.

To determine what are the features of the NH_2-terminal peptides that provide for their interaction with a region of the channel protein near the mouth of the pore we used the ShB peptide as the parent peptide with which to examine the effects of amino acid substitutions on the kinetics of binding to ShBΔ6-46. Site directed mutagenesis of the NH_2-terminal region of the intact ShB channel was also used to determine how its structure relates to its function as an inactivation domain. The ShB peptide has the sequence MAAVAGLYGLGEDRQHRKKQ and is amidated at the COOH-terminal end. The first half of the peptide (1-11) contains all uncharged
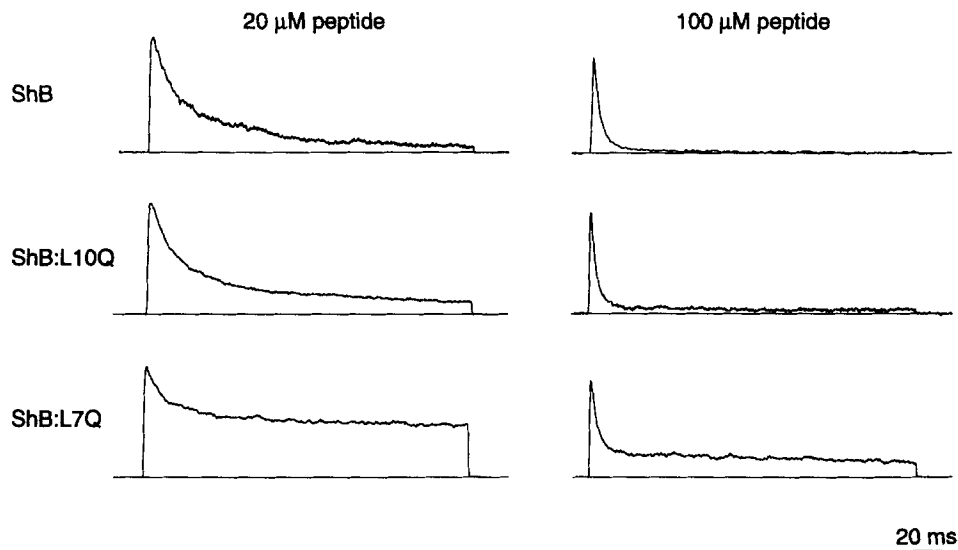

FIGURE 5. The effects of substituting glutamines for leucines at positions 7 and 10 in the ShB peptide on the kinetics of block of ShBΔ6-46 channels. Macroscopic currents were recorded from inside-out membrane patches in response to stepping the voltage to $+50$ mV from the holding voltage of -100 mV. Peptides were applied at the concentrations indicated to the bath solution. The current amplitudes ranged from 300 to 2,000 pA.

amino acids which are followed by two acidic residues in the middle of the peptide and then a series of basic residues. A peptide consisting of just the charged residues (12-20) was not effective at blocking the channel (Zagotta et al., 1990) and the peptide of just the first nine uncharged residues was also much less potent that the 20 residue ShB peptide. Substitutions were made to both the uncharged and charged region of the peptide to investigate the relative contributions of hydrophobic and electrostatic interactions to the binding association and dissociation rates.

k_{off} Is Related to Hydrophobicity

The only charge within the first 11 residues is the NH_2-terminal positive charge. Neutralizing this charge by acetylating the peptide produced a nearly threefold
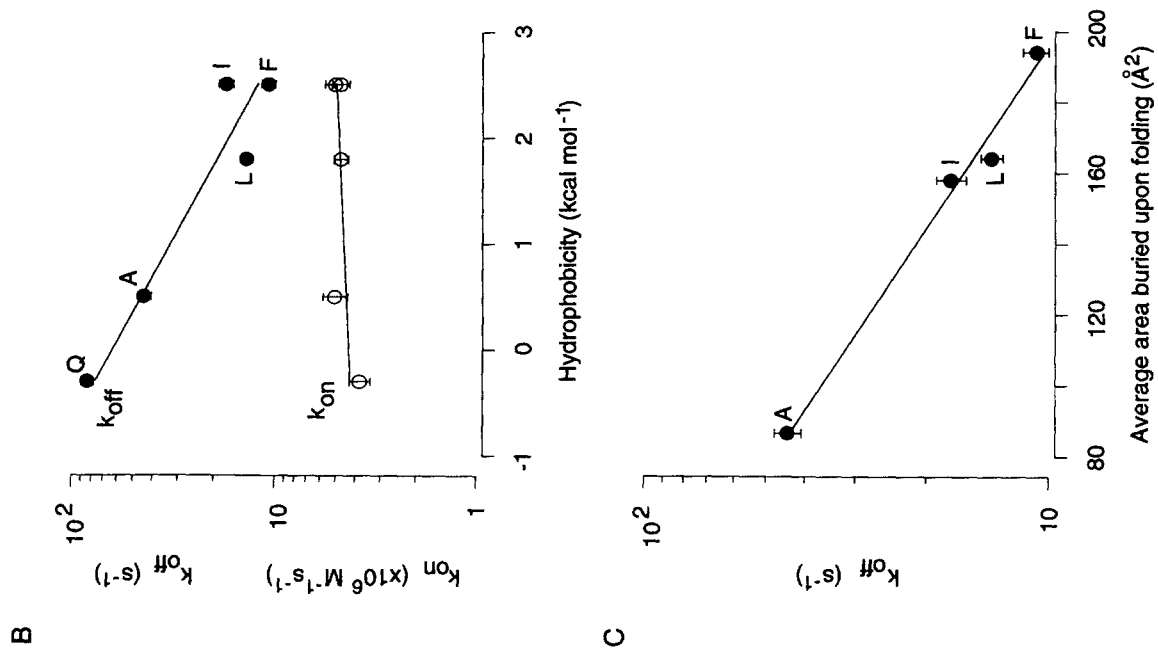
FIGURE 6.

decrease in k_{on} and a fourfold decrease in the k_{off} (Table I). The decrease in k_{off} suggests that the hydrophobicity of this region of the peptide might be important for binding. Hoshi et al. (1990) showed that substituting a charged residue for leucine-7 in the NH_2-terminal domain of ShB disrupted the rapid inactivation process. Similarly, substituting a glutamic acid for leucine-7 in the ShB peptide dramatically reduced the binding affinity of the peptide, so that a concentration of 100 μM produced no detectable block (Zagotta et al., 1990). There are two leucines, at positions 7 and 10 and we substituted glutamines for both these residues to test whether an uncharged but polar residue would be tolerated in these positions. Block of ShBΔ6-46 by 20 and 100 μM of the ShB:L7Q, ShB:L10Q and parent ShB peptides are shown in Fig. 5. The most noticeable effect was that the relative amplitude of the steady-state level of current remaining after block was greater for the altered peptides and in particular for the ShB:L7Q peptide, whereas the decay time constant was similar for each of the peptides. Whereas there was little change in k_{on}, the ShB:L10Q change increased k_{off} by a factor of 1.7 and the ShB:L7Q change increased k_{off} by a factor of 6.1 (Table I), suggesting that hydrophobic interactions in this region are important for binding stability and that in the bound complex the leucine-7 side chain is more effectively buried in a hydrophobic region than is the side chain of leucine-10. A series of more conservative substitutions were made at position 7 to test whether there was a correlation between the overall hydrophobicity of the side group and the stability of the bound complex and also whether the exact structure of the side group was important. Leucine was replaced with alanine, phenylalanine and isoleucine. The binding and unbinding rates of these three peptides were compared with the ShB and ShB:L7Q peptides (Fig. 6). Substituting isoleucine and phenylalanine produced only small changes in k_{off} and no changes in k_{on}, whereas substituting alanine increased k_{off} more than threefold (Table I). In Fig. 6 *B*, k_{on} and k_{off} are plotted against the hydrophobicity of the side group, as determined by Nozaki and Tanford (1971). There was a decrease in k_{off} with increasing hydrophobicity but little change in k_{on}. An alternative scale of hydrophobicity that is based on the accessibility to solvent for residues in proteins of known structure (Rose, Geselowitz, Lesser, Lee, and Zehfus, 1985) is used in the plot below (Fig. 6 *C*). This scale fits the data for the nonpolar residues slightly better than the Nozaki and Tanford scale in that it places leucine above isoleucine in terms of increasing hydrophobicity which is consistent with our findings that k_{off} for the parent ShB peptide is lower than for the ShB:L7I peptide.

FIGURE 6 (*opposite*). Effects of making substitutions for leucine-7 in ShB peptide on binding and unbinding rates. (*A*) Macroscopic currents from ShBΔ6-46 recorded in an inside out patch before and after application of 10 μM of the different peptides to the bath solution. Between the application of each peptide the bath solution was washed several times. (*B*) k_{on} and k_{off} for the L7 altered peptides plotted on a log scale against the hydrophobicity of the residue at position 7. The hydrophobicity index used is from Nozaki and Tanford (1971) and is based on the free energy of transfer of the side chain from water to organic solvent. The error bars are S.E.M. for $n = 3 - 6$. An exponential function is fitted by the method of least squares to the values for k_{on} and k_{off}. (*C*) k_{off} plotted against the hydrophobicity of residue 7 using a scale from Rose et al., (1985). The least square exponential regression is shown.

For nonpolar residues, the surface area of the side chain that is buried in a hydrophobic environment when two surfaces come together provides an estimate of its hydrophobic contribution to the stability of the complex. To quantify the interaction, every square angstrom that is buried in the bound state but not in the unbound state is assumed to contribute 25 cal mol^{-1} to the free energy (ΔG) (Creighton, 1992). Comparing a leucine with an alanine the difference in their surface area is about 75 Å^2 (Rose et al., 1985), so if the side groups went from being completely solvent accessible in the unbound state to completely buried, the difference in the values of ΔG ($\Delta\Delta$G) would be 1.9 kcal mol^{-1}. The free energy contribution to binding of the ShB peptide of a leucine at position 7 versus an alanine in this position was calculated from the expression:

$$\Delta\Delta G = RT\ln \frac{K_d^P}{K_d^{L7A}} \tag{3}$$

where K_d^P and K_d^{L7A} are the equilibrium dissociation constants for the parent ShB and ShB:L7A peptides respectively, R is the Gas constant and T is Absolute temperature. Comparing the parent ShB peptide with the ShB:L7A peptide, $\Delta\Delta G$ was 0.64 kcal mol^{-1}, which is considerably less than the value of 1.9 kcal/mol calculated above. This suggests that the leucine-7 side group does not go from being completely solvent accessible to completely buried upon binding. The change in the free energy difference between the bound state and the transition state ($\Delta\Delta G^\dagger$) is very similar to the value for $\Delta\Delta G$ because the differences in the binding affinities for the L7 substitutions reflected differences in the values for k_{off} and there was no apparent change in k_{on}. Therefore this suggests that in the bound ShB peptide-channel complex, leucine-7 is only partially buried. In Fig. 6 C, the point for phenylalanine lies above the line suggesting that the bulky aromatic side group is less effectively buried than the aliphatic side chain of leucine.

Apart from the leucines at positions 7 and 10 there are two other relatively hydrophobic residues in the peptide, valine-4 and tyrosine-8. Tyrosine has a hydroxyl group which also makes it fairly hydrophilic. Removing the hydroxyl group by substituting in a phenylalanine produced a small decrease in k_{off} (Table I) however not of sufficient magnitude to suggest that this group hydrogen bonds with residues at the binding site. We tested the effects of substituting a lysine for valine-4, tyrosine-8 and leucine-10 on the binding and unbinding rates (Fig. 7). Although the side chain of lysine is locally hydrophobic it has an amino group at the end and buries the smallest fractional side chain surface area on folding of any residue (Rose et al., 1985). A lysine at position-8 increased k_{off} 10-fold compared to the parent ShB peptide, whereas substituting lysine for leucine-10 increased k_{off} only threefold. Substituting lysine for valine-4 reduced both the relative amplitude and the rate of the fast component of the decay phase ($\tau_{fast} = 10$ ms and $a_{fast} = 0.39$ compared with $\tau_{fast} = 2.3$ ms and $a_{fast} = 0.96$ for the ShB peptide). The reduction in peak current, given the time course of decay, was greater than one would predict for a pure open channel blocker suggesting that this peptide blocked some of the channels before opening. This is unlikely to be cumulative inactivation because we measured the current elicited by the first test pulse after application of the peptide. The method

used to calculate k_{on} and k_{off} depends upon the peptide only binding to open channels and therefore these values were not calculated for the ShB:V4K peptide. However we estimate from the steady state level of block that the K_i was around 100 μM. A charged residue in the NH_2-terminal half of the peptide therefore reduces the binding energy, suggesting that in the bound peptide-channel complex, the region of the peptide between residues 4 and 10 is partially buried in a hydrophobic environment. There are other possible interpretations. Another way in which the binding energy could be affected is if the substitutions altered the conformational equilibria of the peptide in solution so affecting the proportion of time the peptide spent in the correct conformation for binding. For the ShB:Y8K and ShB:L10K
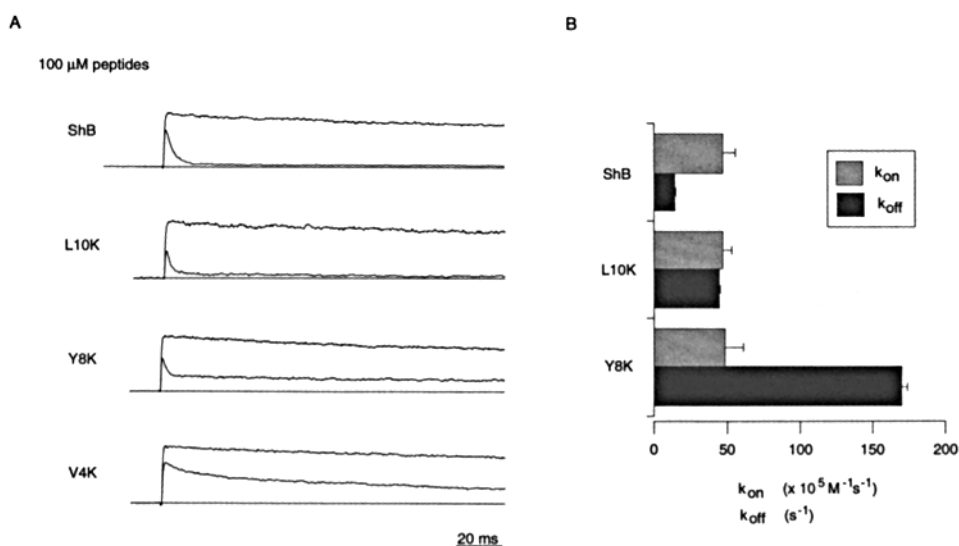

FIGURE 7. Effects of substituting lysines for hydrophobic residues in the NH_2-terminal half of the ShB peptide on binding and unbinding rates. (*A*) Macroscopic currents from ShBΔ6-46 recorded from inside-out patches before and after addition of 100 μM peptides to the bath solution. (*B*) k_{on} and k_{off} were calculated from the two exponential fits to the decay phase of the macroscopic currents as described in the text. The error bars are S.E.M. for $n = 3 - 20$. Values for ShB:V4K have not been included because this peptide appears to block channels before they open.

peptides this is unlikely because there was little change in k_{on}. Alternatively a lysine at positions 4 and 8 may alter the structure of the bound complex and thus prevent favorable interactions involving other residues on the peptide that normally occur, as opposed to reflecting a positive contribution of these residues.

The conformation that the peptide adopts upon binding is unknown. However, the solution structures of some of the peptides have been determined using the techniques of circular dichroism and 2D-NMR (Lee, Aldrich, and Gierasch, 1992). Although the ShB peptide in aqueous solution does not adopt a stable folded conformation there is a region between residues 3–15 which has a propensity to form a helix. This might be important for binding, particularly if the interaction between

the peptide and its binding site stabilizes a helical conformation. In this case increasing the stability of a helical conformation in the free peptide should increase the binding affinity. The three glycines at positions 6, 9, and 11 will have a destabilizing effect because they confer a large degree of freedom of movement to the peptide. Replacing all three glycines with valines increased the stability of a helix within this region of the peptide (Lee et al., 1992), however it also caused the peptide to aggregate into micelles, so it was not possible to determine accurately what the effective concentration of free peptide molecules was and hence calculate k_{on}. Addition of three alanines at positions 6, 7, and 8 also increased the propensity for helix formation, although the effect was less than with the glycine to valine substitutions (Lee, Murrell-Lagnado, Aldrich, and Gierasch, in preparation) and was only apparent in the presence of trifluoroethanol. This alteration produced little change in the binding affinity (Table I). A negative result is difficult to interpret; either there was not a significant increase in the helical character of the peptide under the conditions of the electrophysiological recordings or the peptide does not adopt a helical conformation when it binds. Similarly, removal of two alanines at positions 3 and 5 had little effect on the binding affinity (Table I).

Prolines are thought of as helix disrupters, although they are found within helices where they introduce a bend in the helix axis (Creighton, 1992). Four different peptides containing prolines in the NH_2-terminal half were synthesized. Two had insertions, a proline into position 6 and into position 10, and two had substitutions, a proline for leucine-10 and glycine-11. All four peptides were effective in blocking ShBΔ6-46 channels (Table I). Insertion of a proline into position 6 would be expected to distort a helix between valine-4 and leucine-7. It produced a 2.8-fold increase in k_{off} with little effect on k_{on}. (Table I). Similarly the insertion of a proline into position 10 and the L10P substitution produced relatively little change in k_{on} and k_{off}. Proline, in contrast with glycine, has a high additional constraint on the backbone relative to any other amino acid. The lack of an apparent effect of the G11P substitution on k_{on} and k_{off} indicates also that restricting rotational freedom at this position is not important in determining how well the peptide binds to the channel.

Long-range Electrostatic Interactions Increase k_{on}

Hoshi et al. (1990) showed that deleting charged residues within the NH_2-terminal domain of the intact ShB channel slowed the rate of inactivation as did mutating the arginines at positions 14 and 17, to glutamines. This suggested an important role of positively charged residues within this region in the inactivation process. We made substitutions to both the acidic and basic residues in the COOH-terminal half of the peptide to determine the contributions of the individual charged groups and the overall net charge on the peptide towards the binding energy. The net charge on residues 12–20 (EDRQHRKKQ) is +2 plus any charge on histidine-16. Neutralization of the three basic residues at positions 17–19 by substituting glutamines (ShB:R17Q,K18Q,K19Q peptide) slowed the rate of block of macroscopic currents so that 100 μM of this peptide had a similar effect as only 10 μM of the wild type peptide (Fig. 8). Discounting the histidine and the single positive charge at the NH_2-terminus this peptide has a net charge of −1. The subsequent substitution of glutamine for histidine-16 produced no additional effect suggesting that the side
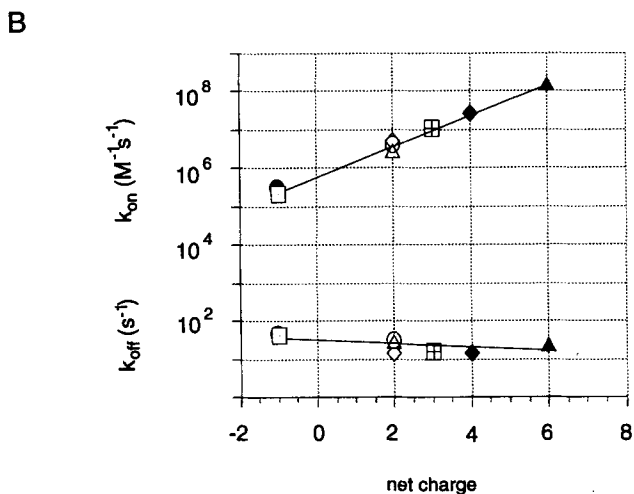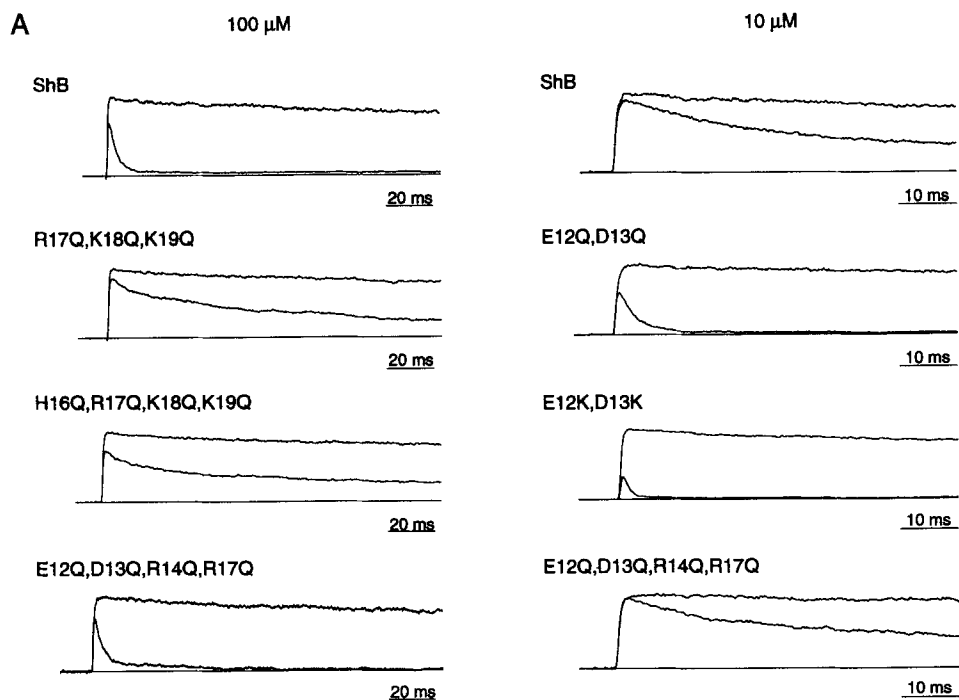
A

100 μM   10 μM

ShB   ShB

20 ms   10 ms

R17Q,K18Q,K19Q   E12Q,D13Q

20 ms   10 ms

H16Q,R17Q,K18Q,K19Q   E12K,D13K

20 ms   10 ms

E12Q,D13Q,R14Q,R17Q   E12Q,D13Q,R14Q,R17Q

20 ms   10 ms

B

k_{on} (M^{-1}s^{-1})

10^8

10^6

10^4

k_{off} (s^{-1})

10^2

-2   0   2   4   6   8

net charge

FIGURE 8. Effects of charged residues in the COOH-terminal half of the peptide on binding and unbinding rates. (*A*) Macroscopic currents from ShBΔ6-46 recorded in inside out patches before and after addition of peptides to the bath. Peak current amplitudes ranged from 300 to 2,000 pA. (*B*) k_{on} and k_{off} are plotted on a log scale against the net charge on the COOH-terminal half of the peptide. The charge at the NH_2 terminus has not been included and neither has any charge on histidine-16. The individual symbols correspond to the rates for the following peptides; ◇ ShB: ■ E12Q: ◆ E12Q,D13Q: ▲ E12K,D13K: △ E12Q,D13Q,R14Q, R17Q: ○ E12K,D13K,K18E,K19D: ● H16Q,R17Q,K18Q,K19Q: □ R17Q,K18Q,K19Q. Each data point represents the mean value of between 3–20 experiments. The standard errors are given in Table I. The data for k_{on} and k_{off} have been fitted with the equation, $k = k(0)$ $\exp(-zF\psi/RT)$, where $k(0)$ is the rate constant with zero local potential, z is the valence of the species, and ψ is the local potential at the binding site. From the least square regression the value for ψ for the association rate data was −23 mV.

chain of histidine was not charged. The pK_a of the imidazole ring can vary between 4.5 and 8 depending upon its environment. We saw no pH dependence of block by ShB:R17Q,K18Q,K19Q between pH 6.2 and 8 (results not shown) which suggests that this pH range was not large enough to change the charge on the histidine. We did not use lower pH's because there was significant proton block of outward currents. As shown in Fig. 8, substituting glutamines for the two acidic residues (ShB:E12Q,D13Q) increased the rate of block and substituting lysines (ShB: E12K,D13K) increased the rate of block still further ($\tau_{fast} = 0.8$ ms). Conserving the net charge by neutralizing two basic residues together with the two acidic residues (ShB:E12Q,D13Q,R14Q,R17Q), produced a peptide with similar blocking potency to the parent ShB peptide. We plotted the values for k_{on} and k_{off} for each of these peptides on a log scale versus the net charge on the peptide (Fig. 8 B). There was a clear relationship between the overall net charge on the peptide and k_{on}, with k_{on} increasing with increasing positive charge, suggesting that long-range electrostatic interactions might be enhancing diffusion of the peptide towards its binding site. There was no clear relationship however between net charge and the dissociation rate. A number of the substitutions appeared to slightly destabilize the peptide-channel complex (Table I). The dissociation rate constants for ShB: R17Q,K18Q,K19Q and ShB:E12Q,D13Q,R14QR17Q were approximately twofold faster than for the wild type peptide. However, the effects on k_{off} were much smaller than one would expect if the charged side groups on the peptide were forming salt bridges with oppositely charged residues on the channel.

For a simple long-range electrostatic interaction the effect of the local potential at the binding site on the association rate constant of a charged species is given by the expression:

$$k_{on} = k_{on}(0)e^{-zF\psi/RT} \tag{4}$$

where $k_{on}(0)$ is the association rate constant with zero local potential, z is the valence of the species, and ψ is the local potential at the binding site. Plotted on a log scale the least squares fit is shown as the straight line through the symbols and gives a value for the surface potential of -23 mV. Eq. 4 assumes that the blocking species is a point charge and that the local potential is uniformly distributed rather than being localized at discrete sites. In the case of the peptides the charge is distributed over several residues, which encompasses a distance several fold larger than the Debye length (~ 9 Å). One would predict therefore that not all the charged residues would be equivalent and that charges on residues at positions 12 and 13 might, for example, contribute more to the association rate than charges on residues 18 and 19, depending upon how the side groups were oriented with respect to the binding site. To test this we switched the positions of the acidic residues (E12 and D13) with the two lysines (K18 and K19). Values for k_{on} and k_{off} for the peptide ShB: E12K,D13K,K18E,K19D were very similar to those of the parent ShB peptide and have been included in the plot (Fig. 8 B). The spatial distribution of charges within this region does not appear to be critical. This suggests that on average the probability of any of the charged residues within the COOH-terminal half of the peptide being oriented towards the binding site at the transition state is similar.

Decrease in Blocking Potency of Peptides Reflects Decrease in Binding Affinity

Our analysis of the results is dependent upon the binding of the altered peptides completely blocking ion conduction through the pore. If one of the altered peptides bound to a similar site as the parent ShB peptide and only partially blocked the channel then it will appear to have reduced affinity. In this case one would predict that the peptide would act similar to a partial agonist and under certain conditions reduce channel blockade by one of the more potent blocking peptides. Fig. 9 shows an experiment where 10 μM of the ShB:E12KD13K peptide was applied to the patch alone and then together with 100 μM of the ShB:V4K peptide, which is relatively ineffective as a blocker as shown in the bottom trace where it was added alone to the

10 µM E12KD13K

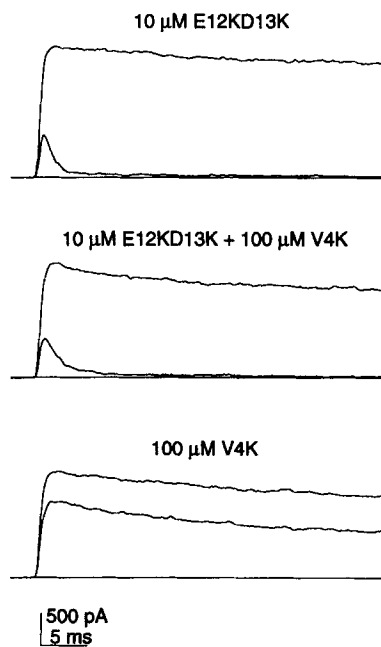**10 µM E12KD13K + 100 µM V4K**

100 µM V4K

500 pA
5 ms

FIGURE 9. Effects of peptide with low blocking potency on the rate of block by a relatively potent peptide. Macroscopic currents from ShBΔ6-46 were recorded from an inside-out patch with a voltage step to +50 mV from a holding potential of -110 mV. Currents were recorded directly before and after application of peptides to the bath. 10 μM ShB:E12KD13K peptide was applied first alone and then washed out and applied together with 100 μM ShB:V4K peptide. Both peptides were then washed out and 100 μM ShB:V4K peptide was applied alone. Two exponential components were fitted to the decay phase of currents in the presence of peptides. The fits were as follows: in the presence of 10 μM E12KD13K, τ_{fast} = 1.0 ms, a_{fast} = 2,000 pA, τ_{slow} = 180 ms, a_{slow} = 50 pA: in the presence of both peptides, τ_{fast} = 1.5 ms, a_{fast} = 1,280 pA, τ_{slow} = 180 ms, a_{slow} = 56 pA, in the presence of 100 μM V4K peptide, τ_{fast} = 16.2 ms, a_{fast} = 460 pA, τ_{slow} = 180 ms, a_{slow} = 810 pA.

patch. With the ShB:E12KD13K peptide present greater than 95% of the current was blocked and there was no evidence for 100 μM of the ShB:V4K peptide reducing the effects of only 10 μM of this peptide. This suggests that the low blocking potency of the ShB:V4K peptide does reflect a low binding affinity. Similarly the V4K peptide did not compete with block by the parent ShB peptide (not shown).

Mutations at the NH$_2$ Terminus of ShB

To compare the binding of the tethered NH$_2$-terminal domain of ShB with that of the free ShB peptide, mutations were made within the NH$_2$-terminal region that corresponded to some of the substitutions made to the ShB peptide. Macroscopic

currents recorded from wild type ShB, ShB:R17Q,K18Q,K19Q, ShB:L7A and ShB: E12K,D13K channels are shown in Fig. 10 and alongside are currents recorded from ShBΔ6-46 in the presence of the corresponding peptides. Qualitatively there was good agreement between the time course of inactivation in the mutant channels and the time course of peptide block. Mutating charged residues appeared to affect the rate of inactivation whereas mutating leucine-7 affected the rate of returning from
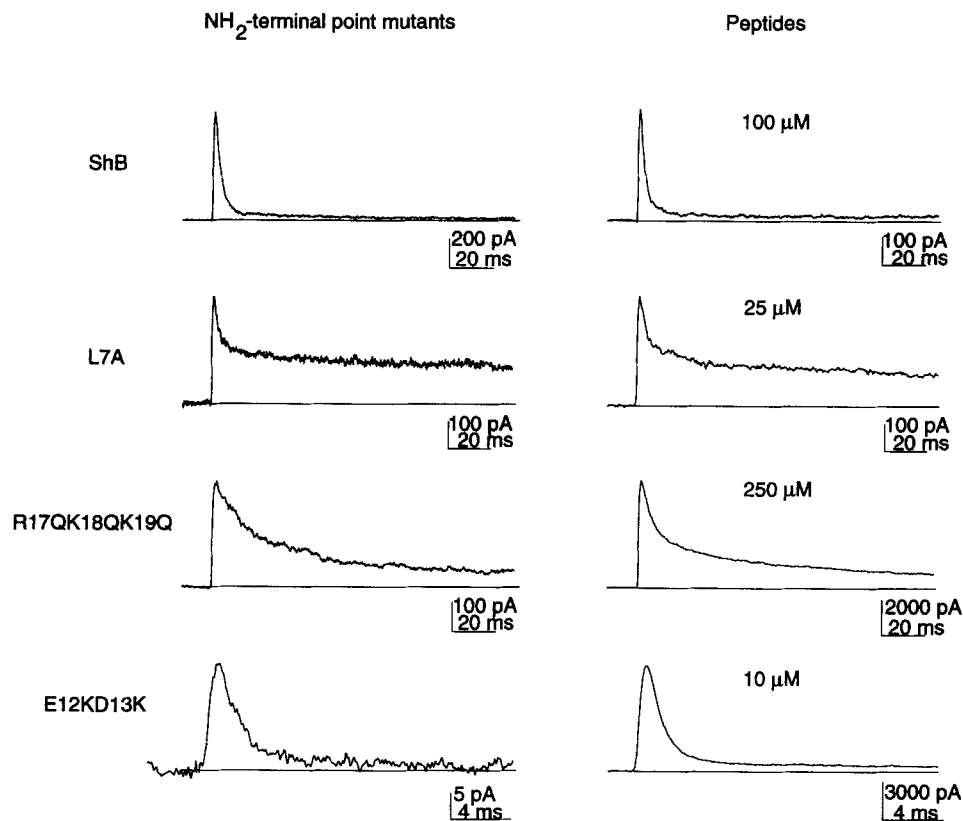

FIGURE 10. Comparing the effects of substitutions within the ShB peptide and mutations within the NH$_2$-terminal domain of ShB on inactivation kinetics. Currents were recorded from inside-out patches by stepping the voltage to +50 mV from a holding voltage of −100 mV. The current trace top left is from ShB and those below are from ShB channels which have mutations within the NH$_2$-terminal domain. Currents on the right hand side are from ShBΔ6-46 in the presence of the corresponding peptides at the concentrations indicated.

the inactivated state back to the open state. However, calculation of k_{on} and k_{off} revealed some quantitative differences. Neutralizing the three basic residues in positions 17–19 of ShB reduced the rate of inactivation 6.6-fold, from 2.6 to 17 ms. This compares to a 21-fold decrease in the rate of block when the equivalent substitutions were made in the peptide. Similarly the E12K,D13K mutation increased the rate of inactivation twofold as compared to the 30-fold increase in k_{on} when these

substitutions were made in the ShB peptide. These results suggest that long-range electrostatic interactions make a smaller contribution to the binding of the tethered domain compared to the binding of the free peptide. However they are still important, given the differences in the time course of inactivation of ShB: R17Q,K18Q,K19Q compared with ShB:E12K,D13K. Another quantitative difference was the extent to which the leucine-7 to alanine mutation destabilized the inactivated state. We calculated that this mutation in the intact channel decreased the value of $\Delta G^\dagger$ for unbinding by 1.3 kcal mol^{-1}, which is nearly twice the $\Delta\Delta G^\dagger$ value for the equivalent substitution in the peptide. This suggests that there are some differences in the conformation of the bound peptide and the bound NH_2-terminal domain.

DISCUSSION

The different inactivation kinetics of the 5 NH_2-terminal variants of *Shaker* appear to be determined solely by the blocking activity of a region at the NH_2-terminus consisting of between 16 to 22 residues. For each of the variants we identified a synthetic peptide, corresponding to a continuous segment of the polypeptide chain at the NH_2-terminus, that at a concentration of ~ 100 μM mimicked the time course of inactivation in the intact channel. The linear increase in the rate of channel block with increasing peptide concentration together with the competition between internal TEA binding and peptide binding is consistent with a simple bimolecular scheme in which one peptide molecule binds and occludes the pore. This is similar to inactivation in the intact ShB channel, which has been shown to involve the binding of only one of the four NH_2-terminal domains per channel to a pore blocking site (MacKinnon et al., 1993), a process that is also competed by internal TEA (Choi et al., 1991).

The five NH_2-terminal peptides from *Shaker* plus the Raw3 peptide share no sequence similarity and yet four out of the six blocked ShBΔ6-46 channels with similar potencies, suggesting that the interaction of peptides with the channel is not very specific. However, peptides derived from the slowly inactivating variants, ShD$_2$ and ShH37, were much less potent blockers although they have a net positive charge similar to the other peptides. By making alterations to the structure of the ShB peptide and analyzing the effects on its function we determined some of the important structural features for binding to ShBΔ6-46. These same features appear to be important in the binding of the NH_2-terminal domain of ShB to the pore blocking site on the channel. The presence of hydrophobic residues in the NH_2-terminal half of the peptide are important for stabilizing the bound peptide-channel complex, suggesting that the partial burial of these nonpolar residues in the bound complex makes a hydrophobic contribution to the binding free energy. Charged residues within the COOH-terminal half of the peptide influence the association rate of the peptides but have relatively little effect on the dissociation rate suggesting that they are not involved in forming salt bridges with oppositely charged residues at the binding site. The increase in k_{on} with increasing net positive charge suggests that long-range electrostatic interactions increase the effective concentration of the peptide nearby the binding site. In addition to the *Shaker* variant peptides, those derived from the NH_2-terminal regions of other rapidly inactivating K channels have been shown to block K channels with affinities in the submicromolar to tens of

micromolar range (Ruppersberg et al., 1991). As we and others have shown they block some of the same channels, suggesting that the nature of the binding interactions are similar.

Peptide toxins which block K channels from the extracellular side have very much higher affinities, in the subnanomolar to nanomolar range. The best studied example is charybdotoxin (CTX) block of the calcium-activated K channel (Anderson, Mac-Kinnon, Smith, and Miller, 1988; Miller, Moczydlowski, Latorre, and Philips, 1985). The free energy of binding of CTX is $\sim$ -10.5 kcal mol^{-1} (Anderson et al., 1988) compared to -7.4 kcal mol^{-1} for the binding of the ShB peptide to ShBΔ6-46. The stability of the bound CTX is attributable to the formation of salt bridges between oppositely charged residues on the toxin and the channel (Park and Miller, 1992) and also to the complementarity between the structure of CTX and its binding site which determines the surface area of hydrophobic residues that are buried upon binding (Stampe, Kolmakova-Paartensky, and Miller, 1992). The greater the area buried the greater the hydrophobic contribution to the free energy. Clearly it is important for the NH$_2$-terminal domains to have relatively fast dissociation rates in order for these rapidly inactivating K channels to play a role in the high frequency response of cells. The relatively fast dissociation rate of the ShB peptide reflects a lack of complementarity between the surface of the peptides and the binding site. Hence peptides with no exact sequence similarity bind with similar affinities, and substitutions to the ShB peptide that conserve hydrophobicity but significantly alter the structure of the side group (e.g. L7F) are tolerated well. Isacoff, Jan, and Jan (1991) showed that mutating the leucine at position 385 in ShB to either an alanine or valine apparently increased the binding affinity of the NH$_2$-terminal domain, suggesting that this residue forms part of the binding site and that a smaller non-polar residue in this position improves the complementarity between the binding surface of the domain and this site. The change in the free energy of binding when leucine-7 in the ShB peptide was replaced with alanine was only $\sim$ 0.6 kcal mol^{-1}, whereas we would have predicted a value of 1.8 kcal mol^{-1} if this residue had gone from being completely solvent accessible when unbound, to completely buried in the bound complex. Making this substitution in the tethered inactivation domain of ShB produced a greater destabilization of the inactivated state. One possible explanation for this difference is that this residue in the bound peptide-channel complex is only partially buried, whereas the tethered domain adopts a slightly different bound conformation so that a greater surface area of the leucine-7 side chain is buried. An alternative explanation is that the peptide, which will have more conformational degrees of freedom than the tethered domain, binds in more than one conformation and thus partially compensates for the loss of a favorable interaction. Thus, the lowest energy bound conformation of the ShB:L7A peptide may be different from that of the parent ShB peptide.

Only at position 7 in the ShB peptide have we substituted in smaller nonpolar residues and demonstrated a relationship between the hydrophobicity of the side group and the stability of the peptide-channel complex. The simplest interpretation of the effects of substituting lysine for residues at positions 4 (V), 8 (Y) and 10 (L) is that these relatively hydrophobic residues are also partially buried in the bound complex and thus make a hydrophobic contribution to the binding free energy, with

leucine-10 being the least buried because polar residues in this position are tolerated fairly well. The ShB peptide in solution has a tendency to form a helix between residues 3–14 (Lee et al., 1992). Representing this region in the form of a helical wheel (Fig. 11), the three hydrophobic residues, valine-4, leucine-7 and tyrosine-8 are on one face of the helix and leucine-10 is slightly to one side. This hydrophobic face could come into close enough contact with a hydrophobic surface at the binding site to strip off some of the surrounding water molecules. An argument against this region forming a helix is that the proline substitutions and insertions produced only relatively small changes in binding affinity as did the addition and removal of alanine residues between valine-4 and leucine-7. These results are difficult to interpret given that a proline can be tolerated within a helix and also because a proline will tend to lower the entropy of the peptide. Another consideration is that these alterations might change the structure of the bound complex. For example, deleting two alanines will alter the spacing between the hydrophobic residues such that if this
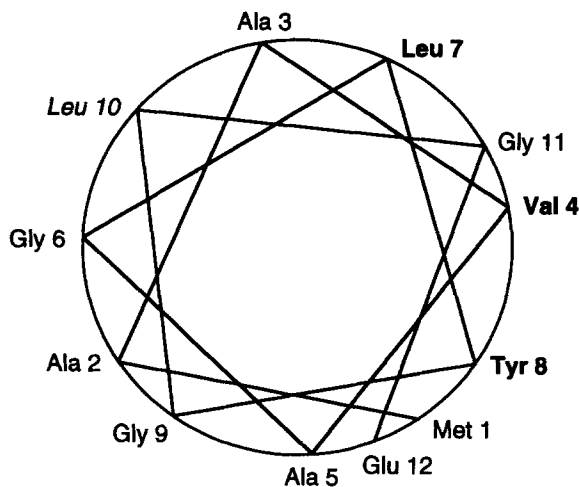

FIGURE 11. Residues 1–12 of the ShB peptide represented in the form of a helical wheel. The hydrophobic residues valine-4, leucine-7, and tyrosine-8 are all on one face of the helix.

region folds into a helix the two leucines are now on one face opposite to that of the valine-4. It may be more energetically favorable for this peptide to bind with the two leucines buried.

Inactivation in voltage-gated Na channels is proposed to involve an intracellular linker region between domains III and IV blocking the channel pore (Patton, West, Catterall, and Goldin, 1992). Hydrophobic residues within this region have been shown to be important in determining the macroscopic rate of inactivation (West, Patton, Scheuer, Wang, Goldin, and Catterall, 1992) suggesting that the interactions between a region within the III–IV loop and the internal mouth of the Na channel pore may be similar to those between the NH_2-terminal domain of *Shaker* and the pore blocking site.

Our finding that increasing the net positive charge within the COOH-terminal half of the peptides increased the association rate constant of the peptide is consistent with a mechanism whereby long-range electrostatic interactions between charges in

the vicinity of the binding site and on the peptide increase the frequency of encounters between the molecules. Electrostatic interactions might also be involved in orienting the peptide correctly for binding. Alternatively, altering the net charge on the peptide might affect its conformational equilibria in solution, so increasing the proportion of time that the peptide spends in the correct conformation for binding. The charged residues within the COOH-terminal half of the peptide (12–20) act fairly equivalently. Alterations which conserved the net charge within this region, for example switching the positions of basic and acidic residues or neutralizing both acidic and basic residues, produced relatively little change in either k_{on} or k_{off}. This is surprising because in an extended conformation the length of this region is several-fold greater than the Debye length (9 Å) at this ionic strength. It is in marked contrast to the results from structure-function studies of peptide toxin channel blockers (Becker, Prusak, Zamponi, Beck, Gordon, and French, 1992; Park and Miller, 1992). These toxins have a stable structure in solution (Bontems, Roume-stand, Boyot, Gilquin, Doljansky, Menez, and Toma, 1991a; Bontems, Roumestand, Gilquin, Menez, and Toma, 1991b) because of the presence of multiple disulfide bonds. The functional role of charged residues depends critically on their position within the peptide. The ShB peptide does not adopt a stable structure in solution and the COOH-terminal end of the peptide is proposed to have an extended chain conformation (Lee et al., manuscript in preparation) which would give this region considerable orientational and translational degrees of freedom. The small changes in k_{off} produced by substituting glutamines for charged residues suggests that this region does not closely contact residues at the binding site and in the bound peptide-channel complex probably remains fairly unconstrained.

There are many studies in which active peptide fragments have been used to study the binding interactions between two proteins or between two regions of the same protein. For example, Glass, Cheng, Mende, Reed, and Walsh (1989) used peptides corresponding to the active portion of the inhibitor protein of protein kinase A (PKI) to examine the primary structural determinants essential for its interaction with the catalytic subunits of protein kinase A (PKA). Peptides derived from the antibody hypervariable region sequences have been used to study the interaction with antigens (Williams, Moss, Kieber, Cohen, Myers, Weiner, and Greene, 1989). Sancho and Fersht (1992) have used protein fragments for studies of protein folding in Barnase. It is clear from these studies that very high affinity binding (in the nanomolar range) is only achieved when the peptides adopt a stable folded conformation in solution. PKI (5–22) amide adopts a stable folded structure in solution (Reed, De, Trewhella, Glass, Liddle, Bradbury, Kinzel, and Walsh, 1989; Reed, Kinzel, Cheng, and Walsh, 1987) and inhibits PKA with a potency of ~ 3 nM (Cheng, Kemp, Peaarson, Smith, Misconi, Van, and Walsh, 1986). Similarly peptide toxins that block ion channels with affinities in the nanomolar range or higher have stable structures (Anderson et al., 1988, Becker et al., 1992, Bontems et al., 1991a). Peptides like the ShB peptide which do not adopt a stable structure in solution tend to have affinities at least two orders of magnitude lower. Free in solution they have considerable entropy. Upon binding the contact surface area will either be large if the complementarity is good, in which case there will be a tremendous loss of conformational entropy, or if the complementarity is low the peptide will bind with only a few groups so maintaining a

greater number of degrees of freedom. Either way the Gibbs free energy of binding will not be very high. For the ShB peptide binding to ShBΔ6-46 channels it appears that the complementarity is not very high because the dissociation energies are fairly low and because specificity appears to be fairly low. We would predict therefore, that binding does not involve such a considerable loss of conformational entropy.

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