

Interaction Between Ion Channel-Inactivating Peptides and Anionic Phospholipid Vesicles as Model Targets

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ABSTRACT Studies of rapid (N-type) inactivation induced by different synthetic inactivating peptides in several voltage-dependent cation channels have concluded that the channel inactivation "entrance" (or "receptor" site for the inactivating peptide) consists of a hydrophobic vestibule within the internal mouth of the channel, separated from the cytoplasm by a region with a negative surface potential. These protein domains are conformed from alternative sequences in the different channels and thus are relatively unrestricted in terms of primary structure. We are reporting here on the interaction between the inactivating peptide of the *Shaker* B K⁺ channel (ShB peptide) or the noninactivating ShB-L7E mutant with anionic phospholipid vesicles, a model target that, as the channel's inactivation "entrance," contains a hydrophobic domain (the vesicle bilayer) separated from the aqueous media by a negatively charged vesicle surface. When challenged by the anionic phospholipid vesicles, the inactivating ShB peptide 1) binds to the vesicle surface with a relatively high affinity, 2) readily adopts a strongly hydrogen-bonded β -structure, likely an intramolecular β "hairpin," and 3) becomes inserted into the hydrophobic bilayer by its folded N-terminal portion, leaving its positively charged C-terminal end exposed to the extravesicular aqueous medium. Similar experiments carried out with the noninactivating, L7E-ShB mutant peptide show that this peptide 1) binds also to the anionic vesicles, although with a lower affinity than does the ShB peptide, 2) adopts only occasionally the characteristic β -structure, and 3) has completely lost the ability to traverse the anionic interphase at the vesicle surface and to insert into the hydrophobic vesicle bilayer. Because the negatively charged surface and the hydrophobic domains in the model target may partly imitate those conformed at the inactivation "entrance" of the channel proteins, we propose that channel inactivation likely includes molecular events similar to those observed in the interaction of the ShB peptide with the phospholipid vesicles, i.e., binding of the peptide to the region of negative surface potential, folding of the bound peptide as a β -structure, and its insertion into the channel's hydrophobic vestibule. Likewise, we relate the lack of channel inactivation seen with the mutant ShB-L7E peptide to the lack of ability shown by this peptide to cross through the anionic interphase and insert into the hydrophobic domains of the model vesicle target.

INTRODUCTION

Fast (or N-type) inactivation exhibited by many voltage-dependent ion channels specific for Na⁺ or K⁺ implies the physical occlusion of the channel's internal mouth by a flexible cytoplasmic domain of the channel protein itself, which acts as an open channel blocker (the "ball and chain" hypothesis of channel inactivation; Armstrong and Bezanilla, 1977). Studies in the *Shaker* B (ShB) potassium channel (Hoshi et al., 1990; Zagotta et al., 1990) have demonstrated that the inactivating "ball" peptide (ShB peptide) corresponds to the first 20 amino acids in the N-terminal region of each ShB channel subunit (H₂N-MAAVAG-LYGLGEDRQHRKKQ). Indeed, addition into the intracellular media of free, synthetic ShB peptide restores fast inactivation in mutant ShB channels that do not inactivate because of deletions in their N-terminal region, suggesting that the synthetic peptide contains all of the necessary

molecular determinants to recognize the channel protein and carry out its inactivating function (Zagotta et al., 1990). Moreover, the ability of the ShB peptide to induce inactivation has been found to depend on certain features of its primary structure (Murrell-Lagnado and Aldrich, 1993). For instance, substitution of a single nonpolar residue in the hydrophobic stretch in the N-terminal region of the ShB peptide by either positively or negatively charged amino acids, such as that in the ShB-L7E peptide (mutant of the ShB peptide in which the leucine 7 in the wild peptide is substituted by glutamate), results in the loss of the ability of the peptide to induce channel inactivation.

Additional studies using synthetic peptides and different voltage-dependent channels have suggested that the sites on the channels for binding of the inactivating peptides consist of a hydrophobic protein pocket, which becomes accessible only upon channel opening, separated from the cytoplasm by a region with a negative surface potential (Jan and Jan, 1992; Catterall, 1995; Kukuljan et al., 1995). In the ShB K⁺ channel in particular, it has been proposed that the region of negative surface potential is made up of a ring of glutamate residues plus the negatively charged ends of the S₄₋₅ helix dipoles contributed by each channel subunit (Jan and Jan, 1992; Durell and Guy, 1992). Similarly, the formation of the

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internal hydrophobic protein vestibule has been attributed to the rotation of the amphipathic S_{4-5} helices induced by channel activation (Durell and Guy, 1992). Regardless of whether such components of the channel inactivation "entrance" are correctly assigned, it is remarkable to observe that the ShB-inactivating peptide induces inactivation not only in the ShB channel, but also in a variety of other voltage-dependent K^+ channels (Zagotta et al., 1990; Isacoff et al., 1991; Dubinsky et al., 1992), high conductance Ca^{2+} -activated K^+ channels (Toro et al., 1992; Foster et al., 1992), or cyclic nucleotide-gated channels (Kramer et al., 1994), some of which do not normally inactivate. Furthermore, the presumed inactivating peptide from the voltage-dependent Na^+ channel serves also as an inactivation peptide for the ShB K^+ channel (Patton et al., 1993). From these observations of "crossed inactivation" and from the diversity in primary structure of the molecules involved, it follows that rather than highly specific amino acid sequences, the interaction between the inactivating peptide and the channel "entrance" is likely to imply the mutual recognition of domains conformed from alternative sequences in each of the channel proteins or the inactivating peptides showing crossed inactivation with foreign partners.

In this manuscript we have studied the interaction between the K^+ channel-inactivating ShB peptide or its non-inactivating ShB-L7E mutant with anionic phospholipid vesicles, a model target that, as the channel's inactivation "entrance," contains a region with a negative surface potential (the negatively charged vesicle surface) and a hydrophobic domain (the lipid bilayer). Our expectations are that by exploring in detail the interaction of the synthetic peptides with such a model target, we might be able to infer similar molecular events involved in the inactivation of the real channel and understand the molecular basis of how a single amino acid substitution in the sequence of the inactivating peptide, such as that in the L7E mutant, determines a loss of function. A previous report using this approach (Fernandez-Ballester et al., 1995) showed that the anionic phospholipid vesicles induce the adoption of distinct conformations by the synthetic ShB and ShB-L7E peptides. Whereas the mutant peptide remained in a nonordered conformation under most of the experimental conditions explored, the inactivating ShB peptide readily adopted a highly stable structure. Although the conformation adopted by the ShB peptide when it binds to the channel protein remains unknown, the proposal of a β -structure as the "active" conformation seems consistent with inferences made from electrophysiological recordings (Murrell-Lagnado and Aldrich, 1993), which predict an "extended" conformation for the channel-bound ShB peptide during inactivation. Additionally, it has been found that certain conditions known to make the ShB peptide unable to produce channel inactivation, such as trypsin hydrolysis, also prevent the peptide from interacting with the model anionic vesicle target (Fernandez-Ballester et al., 1995), thus providing further support for the hypothesis that the negatively charged and the hydrophobic domains in the anionic vesicle

model target could partly imitate some of the features of the inactivation "entrance" in the channel protein.

MATERIALS AND METHODS

Deuterium oxide (D_2O , 99.9% by atom) was purchased from Sigma. The phospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidic acid (PA) (Avanti Polar Lipids) were all derivatives of egg yolk PC and therefore had the same fatty acid composition. The synthetic 1,2-*sn*-dimyristoyl phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG), and 1,2-dimyristoyl-*sn*-glycero-3-phosphate (DMPA) were also from Avanti Polar Lipids. All phospholipids were dissolved in chloroform and divided into aliquots, and the solvent was driven off under a stream of nitrogen and under vacuum.

Peptide synthesis and characterization

The peptides ShB (MAAVAGLYGLGEDRQHRKKQ) and ShB-L7E (MAAVAGEYGLGEDRQHRKKQ) were synthesized as C-terminal amides on an automatic multiple synthesizer (AMS 422; Abimed) using a solid-phase procedure and standard (9-fluorenyl methoxy carbonyl) chemistry (Fernandez-Ballester et al., 1995). The peptides were purified by reverse-phase high-performance liquid chromatography to better than 95% purity, and their composition and molecular weight were confirmed by amino acid analysis and mass spectrometry, respectively (Fernandez-Ballester et al., 1995). Residual trifluoroacetic acid used both in the peptide synthesis and in the high-performance liquid chromatography mobile phase (the trifluoroacetate ion has a strong infrared absorbance around 1670 cm^{-1} , which interferes with the characterization of the amide I band; Surewicz et al., 1993) was removed by submitting the peptides to several lyophilization-solubilization cycles in 10 mM HCl (Zhang et al., 1992). The lyophilized peptides were divided into aliquots and kept frozen until use.

Fluorescent labeling of cysteine-containing peptides

The peptides ShB-21C (MAAVAGLYGLGEDRQHRKKQC) and ShB-L7E-21C (MAAVAGEYGLGEDRQHRKKQC) were synthesized as C-terminal amides as described above for the ShB and ShB-L7E peptides. These cysteine-containing peptides at their C-terminal ends were fluorescently labeled by alkylation with NBD-iodoacetamide (*N,N'*-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine) (Molecular Probes, Eugene, OR). Briefly, lyophilized aliquots containing 1–3 mg of either cysteine-peptide were dissolved in 0.6 ml of 0.5 M Tris buffer, pH 8.5, reacted during 1 h and in the dark with a fourfold molar excess of the reducing agent dithiothreitol and alkylated by the addition of an excess of the NBD-iodoacetamide (2.5-fold molar excess over the dithiothreitol used in each experiment) previously dissolved in dimethyl sulfoxide. The reaction mixtures were incubated for 1 h in the dark and under an inert atmosphere, then subjected to chromatography in a Biogel P-2 (Fine) column ($1.5 \times 15\text{ cm}$), with 0.05 M acetic acid as the eluant. The resulting NBD-labeled peptides eluted in the void volume and were subsequently lyophilized, rechromatographed in the Biogel P-2 column, divided into aliquots, lyophilized again, and kept frozen until further use. Peptide concentration in these samples was determined by amino acid analysis. The extent of peptide derivatization estimated by the carboxymethylcysteine/peptide molar ratios was better than 97%. Either the cysteine-containing ShB-21C and ShB-L7E-21C peptides, or their NBD derivatives, exhibited conformations similar to those of the ShB and ShB-L7E peptides, respectively, as judged by the spectral shape of their infrared amide I bands.

Fluorescence assay of peptide binding

Small unilamellar vesicles were used in these studies to minimize light-scattering phenomena. The lyophilized lipids (PC, PG, or PA) were resuspended in the appropriate buffers to give final concentrations of 0.05, 0.5, and 5 mM (in terms of lipid phosphorus), kept at 50°C for 90 min, frequently vortexed, and then sonicated in a bath-type sonicator until the samples became completely transparent. Small aliquots from these stock vesicle solutions were added in triplicate to 1.5-ml aliquots from a 0.1 μ M solution of NBD-labeled peptides at 25°C. Fluorescence intensity was measured immediately afterward as a function of the lipid:peptide molar ratio in a SLM 8000 spectrofluorometer with the excitation and emission wavelengths set at 470 and 530 nm, respectively. To account for the background (scattering) signal contributed by the lipids to the emitted fluorescence, the readings observed when titrating identical concentrations of unlabeled peptides with the same lipid vesicles were subtracted from each recording of fluorescence intensity. The binding isotherms were analyzed as partition equilibria (Peled and Shai, 1993), according to the expression $X_b^* = K_p^* C_f$, where X_b^* is the molar ratio of bound peptide per 60% of the total lipid (i.e., assuming that the peptides were initially partitioned only over the outer leaflet of the membrane), K_p^* is the partition coefficient, and C_f represents the concentration of free (unbound) peptide at equilibrium. F_∞ , the fluorescence signal obtained when all of the peptide is lipid-bound, was extrapolated from a double reciprocal plot of F , the observed fluorescence intensity, versus the total concentration of lipid. Knowing F and F_∞ , as well as F_0 (the fluorescence exhibited by the free, unbound peptide), the fraction of membrane-bound peptide (f_b) can be calculated by using the expression $f_b = (F - F_0)/(F_\infty - F_0)$. Once the f_b values for each of the experimental points in the binding curves are known, C_f and X_b^* can be easily calculated. Finally, the partition coefficient K_p^* is determined by extrapolating the initial slope in a X_b^* versus C_f plot to zero C_f values.

Differential scanning calorimetry

To facilitate the comparison with reported calorimetric data on lipid phase transitions, large multilamellar vesicles made from synthetic DMPA, DMPG, or DMPC were used in these studies. Dried lipid films were suspended in the required buffer, depending on the desired pH and composition (see figure legends for details), to give a final concentration of 2 mM in terms of lipid phosphorus. The suspended lipids were kept for 90 min above their phase transition temperatures and vortexed. The resulting liposomes were stored overnight, at 4°C, to ensure a complete hydration of the sample before the differential scanning calorimetry (DSC) experiments.

Thermograms were recorded on a high-resolution Microcal MC-2 differential scanning microcalorimeter, equipped with the DA-2 digital interface and data acquisition utility for automatic data collection, as described earlier (Villar et al., 1988). The lipid dispersions containing added ShB or ShB-L7E peptides at different molar ratios, and the corresponding buffer in the reference cell, were thermally equilibrated in the microcalorimeter, at $\sim 10^\circ\text{C}$ for 45 min, before heat was applied. Differences in the heat capacity between the sample and the reference cell were obtained by raising the temperature at a constant rate of 45°C/h. Transition temperatures and enthalpies were calculated by fitting the observed transitions to a single van't Hoff component. Reported transition temperatures correspond to those at which there is a maximum differential heat capacity, as observed in the thermograms.

Infrared measurements

For amide I band recordings, lyophilized aliquots of the synthetic peptides and the desired lipids were separately hydrated in 25 μ l of D₂O buffers (see figure legends for details on the composition of the buffers) to avoid the interference of H₂O infrared absorbance (1645 cm⁻¹; Mendelsohn and Mantsch, 1986). In the hydration of the phospholipid samples, either sonication of the samples in a bath-type sonicator or forcing the suspensions several times in and out of a micropipette tip was used, with identical

spectral results. The resulting solutions were mixed by placing them together in a liquid demountable cell (Harrick, Ossining, NY) equipped with CaF₂ windows and 50- μ m-thick mylar spacers, and maintained at room temperature for ~ 30 min to ensure that the isotopic H-D amide proton exchange reached equilibrium, as judged by a constant minimum absorbance at the residual amide II band in the peptide's infrared spectra. The peptide concentrations in the final mixtures usually ranged from 0.9 to 3.6 mM, whereas the phospholipids were mostly used at ~ 26 mM, thus resulting in phospholipid-to-peptide molar ratios ranging from 7 to 29.

For the acquisition of the 950-1500 cm⁻¹ spectral region comprising the absorbance bands assigned to vibrations of the phosphoryl group of PA, ZnS windows were used in the spectrometer cell and the samples were prepared essentially as described above, but using an aqueous buffer (10 mM sodium acetate, 10 mM 3-(*N*-morpholino)propanesulfonic acid, 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 100 mM NaCl, 0.1 mM EDTA, and 0.01% NaN₃) adjusted to a pH ranging from 5.5 to 10.5 (Bhushan and McNamee, 1993). The usual peptide and PA concentrations used in these experiments were 14 and 58 mM, respectively.

Fourier-transform infrared spectroscopy (FTIR) spectra were taken in a Nicolet 520 instrument equipped with a DTGS detector, as previously described (Castresana et al., 1992), using 200 spectral scans to define the amide I band and 600 scans to define the absorbance bands arising from the phosphoryl group of PA. Given the good resolution of component bands observed in the original spectra, no resolution-enhancement techniques (Fourier derivation or deconvolution; Moffatt and Mantsch, 1992) were applied to the spectral data.

RESULTS

Binding of peptides to phospholipid vesicles

To study the binding of the synthesized peptides to the different phospholipid membranes, the peptides were selectively labeled by alkylation with NBD-iodoacetamide of the sulfhydryl group of a cysteine residue added at the C-terminal end of the ShB and ShB-L7E peptides. Labeling of the peptides with the environmentally sensitive NBD probe, the fluorescence of which increases greatly and shifts to shorter wavelengths when in a hydrophobic media, provides a highly reliable and sensitive method that has been used to study the binding of a variety of different peptides to phospholipid vesicles (see Peled and Shai, 1993, and references therein).

To evaluate peptide binding, aliquots of NBD-labeled peptides were mixed with increasing amounts of small unilamellar phospholipid vesicles, and the resulting increases in the fluorescence intensity were plotted versus the phospholipid:peptide molar ratio to generate binding curves (Fig. 1). The analysis of the binding data was carried out as described by Peled and Shai (1993) (see Materials and Methods) to determine the surface partition coefficient (K_p^*), assuming that the peptides initially partitioned only over the outer leaflet of the different phospholipid vesicles (Table 1). At pH 7.0, neither the ShB nor the ShB-L7E peptide binds significantly to PC vesicles. On the other hand, both peptides bind quite efficiently and in a saturable manner to PA and, to a lesser extent, to PG vesicles. The K_p^* values observed under our experimental conditions for binding of the ShB peptide to either PA or PG vesicles ($\sim 8 \times 10^5$ and $\sim 0.3 \times 10^5 \text{ M}^{-1}$, respectively) are higher or comparable to those reported for binding to zwitterionic

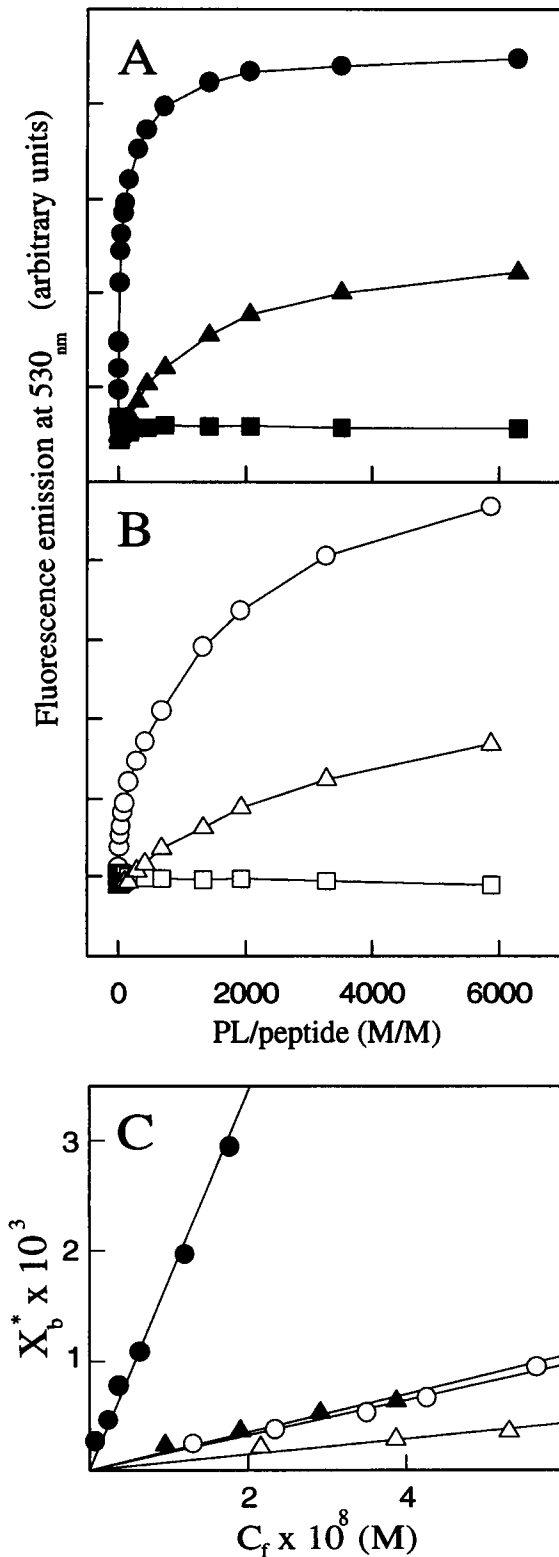


FIGURE 1 Representative changes in the fluorescence emitted by 0.1 μ M solutions of NBD-labeled ShB (A) or ShB-L7E (B) peptides, upon titration with PC (■, □), PG (▲, △), and PA (●, ○) vesicles at the indicated phospholipid/peptide molar ratios. (C) Examples of X_b^* versus C_f plots (see Materials and Methods) for several of the samples included in A and B, used to determine the partition coefficients (K_p^*) given in Table 1. The buffer used in these experiments was 10 mM HEPES, pH 7.0, containing 100 mM NaCl.

phospholipid vesicles of 1) peptides resembling transmembrane protein segments, 2) various signal peptides, or 3) membrane-permeating bioactive peptides known to form stable structures within membranes (see Peled and Shai, 1993, and references therein). K_p^* values obtained for the mutant ShB-L7E peptide and either PA or PG vesicles were always much lower than those corresponding to the ShB peptide under identical experimental conditions.

The position of the fluorescence emission maxima exhibited by the NBD-labeled peptides upon binding to the phospholipid vesicles also provides information on the relative location within the vesicles of the peptides' C-termini, at which the environmentally sensitive NBD moiety is bound. Both the NBD-labeled ShB and the ShB-L7E peptides in solution exhibit emission maxima at 554–555 nm, which reflect a hydrophilic environment for the NBD moiety (Rajaraman et al., 1989). Similar emission maxima are also exhibited by the labeled peptides in the presence of PC vesicles (Table 1). However, binding of the peptides to either PA or PG vesicles determines a blue shift in the fluorescence emission maxima, which indicates a relocation of the NBD group to a more hydrophobic environment (Table 1). The extent of the observed blue shift for both peptides (531–533 nm and 531–534 nm in PA and PG vesicles, respectively) also suggests that penetration of the C-termini of the peptides into the hydrophobic bilayer provided by the anionic phospholipid is not as deep as that reported for pore-forming polypeptides (emission maxima 518–526 nm; Rapaport and Shai, 1992; Gazit and Shai, 1993), and it rather resembles that observed for an NBD group located at or near the membrane surface (emission maxima of 530–534 nm; Chattopadhyay and London, 1987; Rajaraman et al., 1989; Pouny et al., 1992).

The interaction of unlabeled ShB and ShB-L7E peptides with the anionic PA vesicles can also be assessed by monitoring the infrared absorbance bands at 1180 and 980 cm^{-1} arising from different stretching vibrations of the monoanionic and dianionic forms, respectively, of the PA phosphoryl group (Sanchez-Ruiz and Martinez-Carrion, 1986; Bhushan and McNamee, 1993). Monitoring of the pH dependence of the above infrared bands in plain PA vesicles, exactly as described by Bhushan and McNamee (1993), makes it possible to calculate a pK_a of ~ 8.0 corresponding to the deprotonation of the monoanionic phosphoryl group to produce the dianionic form (data not shown). The presence of either ShB or ShB-L7E peptides in these samples does not interfere with the spectral region of interest, which shows the same 1180 and 980 cm^{-1} bands observed in the plain PA samples, and therefore, these bands were also used to estimate the pK_a for the deprotonation of the peptide-bound phosphoryl group. For the ShB peptide/PA vesicle samples, the pK_a shift to ~ 7.4 at the peptide-to-lipid ratio used in these experiments. This indicates that the ShB peptide stabilizes the dianionic form of PA, thus suggesting that the phospholipid phosphoryl group is clearly involved in the binding of the ShB peptide. On the other hand, the shift in the pK_a observed in the presence of ShB-L7E is not

TABLE 1 Fluorescence emission maxima (λ_{\max}) and surface partitioning constants (k_p^*) exhibited by NBD-labeled peptides in the presence of phospholipid vesicles

| Peptide designation | pH | λ_{\max} (nm) | | | | $k_p^* \times 10^{-4}$ (M ⁻¹) | | |
|---------------------|-----|-----------------------|-----|-----|-----|---|----------------------|---------------------|
| | | Buffer | PC | PA | PG | PC | PA | PG |
| ShB-NBD | 7 | 555 | 551 | 531 | 531 | No binding | 77.1 (± 11.6) | 2.80 (± 1.10) |
| ShBL7E-NBD | 7 | 554 | 551 | 533 | 534 | No binding | 3.05 (± 1.45) | 0.72 (± 0.20) |
| ShB-NBD | 8.5 | 552 | — | 530 | 536 | — | 1.95 (± 0.65) | 0.64 (0.09) |
| ShBL7E-NBD | 8.5 | 553 | — | 536 | 552 | — | 0.62 (± 0.025) | ≥ 0.05 |

as large as that produced by the ShB peptide ($pK_a \sim 7.7$), which seems consistent with the experiments using fluorescently labeled peptides in which the surface partition coefficients estimated for the interaction of the ShB peptide with PA vesicles were always higher than those corresponding to the ShB-L7E peptide.

Differential scanning calorimetric studies of peptide insertion into lipid bilayers

The possible implication of the hydrophobic domains of the phospholipid bilayers in the association of the peptides with the vesicles was investigated by DSC using synthetic dimyristoyl derivatives of the different phospholipids, which have convenient phase transition temperatures at 23.5°C, 23.3°C, and 49.6°C for DMPC, DMPG, and DMPA, respectively. In these experiments, peptide insertion into the lipid bilayer and, therefore, its interaction with the phospholipid acyl chains are expected to prevent part of the phospholipid molecules from undergoing the phase transition characteristic of the “pure” phospholipid species and thus decrease the phase transition enthalpy in a peptide concentration-dependent manner (Papahadjopoulos et al., 1975). We observed that neither the ShB nor the ShB-L7E peptides cause any effects on the transition temperature or the transition enthalpy of DMPC vesicles (data not shown). On the other hand, confronting the ShB peptide to anionic phospholipid vesicles (Fig. 2, A and B) causes a large, concentration-dependent decrease in the transition enthalpy, which is slightly more pronounced in DMPG than in DMPA vesicles, without modifying the phase transition temperature. A plot of the observed changes in the transition enthalpies versus the peptide/phospholipid molar ratios used in these studies (Fig. 2 C) predicts that each ShB peptide molecule prevents an average of three to four anionic phospholipid molecules from undergoing the phase transition. As for the mutant ShB-L7E peptide, its effects on the DMPA or DMPG phase transition enthalpies are negligible compared to those of the ShB peptide (Fig. 2, A and B).

DMPG vesicles were also used to study whether the observed peptide insertion into the phospholipid bilayer is pH-dependent. DMPG was chosen for these studies because it lacks chemical groups whose titration within the pH region of interest could interfere with the peptide insertion phenomena determined by DSC. Fig. 3 shows that, indeed, the ability of the ShB peptide to insert into the hydrophobic

domains of the anionic bilayer is strongly pH-dependent. A maximum decrease in the DMPG phase transition enthalpy in the presence of the ShB peptide can be observed at pH 6.0, whereas increasing the pH to 8.5 or beyond renders the ShB peptide incapable of inserting into the phospholipid bilayer. In spite of the observed lack of peptide insertion under the latter conditions, peptide binding experiments, carried out at pH 8.5 using NBD-labeled peptides as described above, demonstrate that the ShB peptide still binds to anionic phospholipid vesicles, although with a much lower affinity (Table 1). As for the mutant ShB-L7E peptide, its effects on the DMPG phase transition enthalpies are negligible at all of the pHs explored (Fig. 3).

Fourier transform infrared spectroscopy studies of peptide conformation

Fig. 4 shows the infrared amide I region of the spectra corresponding to the ShB and ShB-L7E peptides in the presence of different phospholipid vesicles. Either peptide in the presence of PC vesicles (Fig. 4, A and B) (as well as in plain buffers in the absence of lipids) exhibits a bell-shaped amide I band with a maximum centered at 1645 cm⁻¹ characteristic of nonordered protein structures. The similarities observed between the spectra of the peptides in solution and in the presence of PC vesicles is consistent with the lack of binding of either peptide to the zwitterionic PC vesicles. On the other hand, the spectra of the ShB peptide in the presence of either PA or PG vesicles exhibit a very prominent amide I component at 1623 cm⁻¹ and a smaller one at 1689 cm⁻¹ (lower traces in Fig. 4, C and E), which have been related to the adoption of a strongly hydrogen-bonded structure (Demel et al., 1990). Such a 1623 cm⁻¹ component in the ShB spectra appears readily and has approximately the same relative importance regardless of the peptide concentration or the peptide-to-phospholipid molar ratio used in the FTIR experiments (ranging from 1.2 to 10 mg/ml and from 2 to 60 (by mole), respectively; Fernandez-Ballester et al., 1995). Even though they are similar, the ShB spectra taken in the presence of PG or PA vesicles differ in that the characteristic 1623 cm⁻¹ component is more heat-stable in the former, because at temperatures as high as 70°C, the absorbance at 1623 cm⁻¹ seen in the ShB/PG samples is still maintained at no less than 65% of that observed at room temperature, whereas that seen in the presence of PA vesicles decreases more

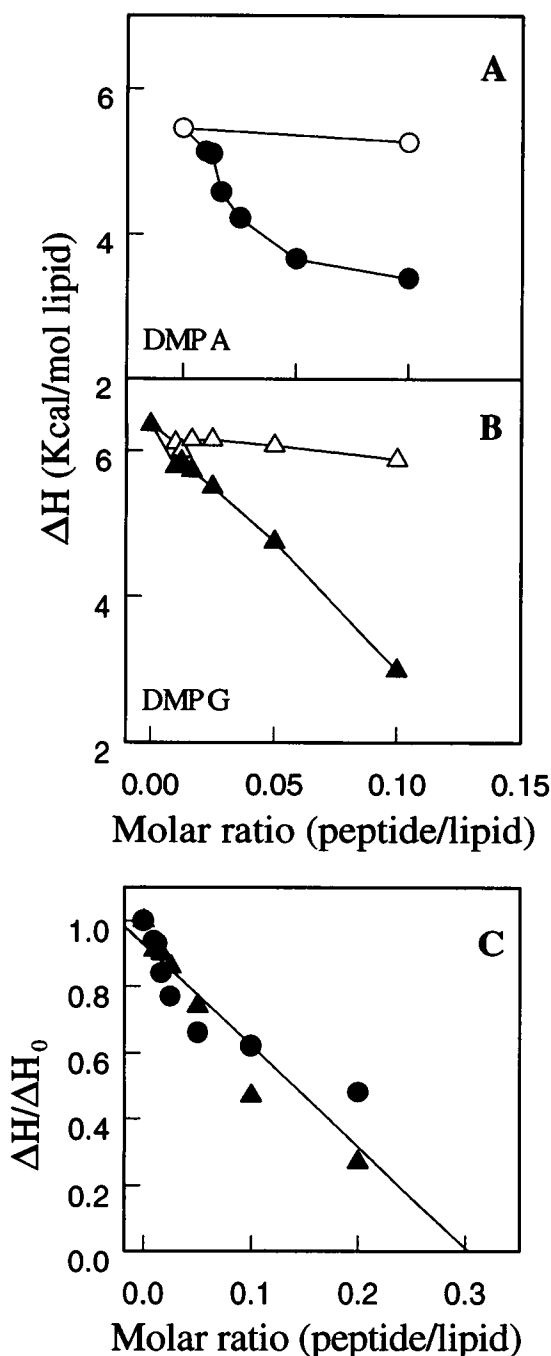


FIGURE 2 Changes in the gel to liquid crystal phase transition enthalpies of DMPA (A, ○, ●) or DMPG (B, △, ▲) large multilamellar vesicles in the presence of increasing concentrations of either ShB (●, ▲) or ShB-L7E (○, △) peptides, as determined by differential scanning calorimetry of the indicated phospholipid/peptide mixtures in 10 mM HEPES, pH 7.0, 100 mM NaCl. Error bars are within the size of the symbols used to plot the experimental results. In the absence of added peptides, the estimated phase transition enthalpies in vesicles made from pure DMPA or DMPG were 5.4 and 6.5 kcal/mol of phospholipid, respectively. (C) Plot of the observed $\Delta H/\Delta H_0$ from above (transition enthalpies of either DMPA (●) or DMPG (▲) determined in the presence (ΔH) and in the absence (ΔH_0) of added ShB peptide) versus the ShB peptide/phospholipid ratios. In spite of the heterogeneity arising from the use of data from DMPA and DMPG samples altogether, a linear extrapolation of such plot indicates that the addition of ShB peptide in this sample at a peptide/phospholipid ratio of ~ 0.3 will completely avoid the occurrence of the cooperative lipid phase transition.

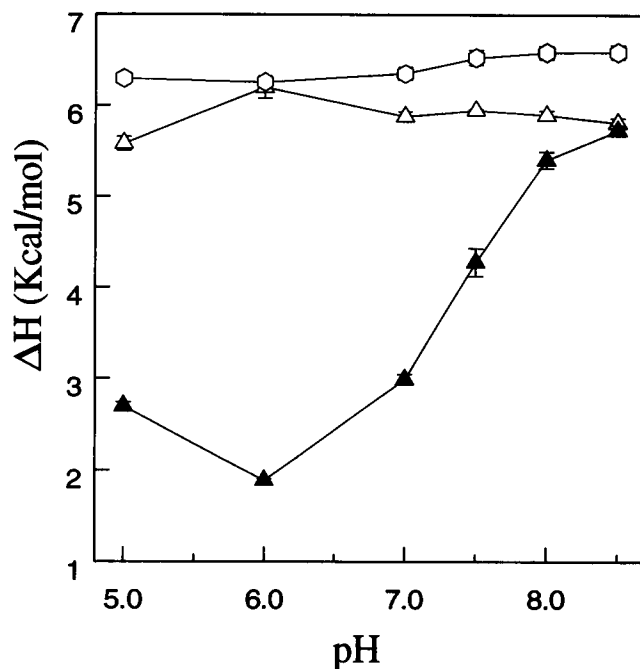


FIGURE 3 pH dependence of the observed gel to liquid crystal phase transition enthalpy of large multilamellar DMPG vesicles, as determined by differential scanning calorimetry. Symbols correspond to DMPG vesicles alone (○) and in the presence of either ShB (▲) or ShB-L7E (△) peptides, both at a peptide/DMPG molar ratio of 0.1. The buffers used at each of the indicated pHs were: pH 5.0, 25 mM citrate, 50 mM phosphate, 20 mM NaCl, 130 mM KCl; pH 6.0, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, 20 mM NaCl, 130 mM KCl; pH 7.0, 10 mM phosphate, 20 mM NaCl, 130 mM KCl; pH 7.5, 10 mM phosphate, 20 mM NaCl, 130 mM KCl; pH 8.0, 10 mM *N*-(2-hydroxyethyl) piperazine-*N'*-3-propanesulfonic acid Epps, 20 mM NaCl, 130 mM KCl; pH 8.5, 10 mM Epps, 20 mM NaCl, 130 mM KCl.

markedly with increasing temperature (Fig. 4, *C* and *E*, middle traces). Other properties attesting to a remarkable conformational stability exhibited by the ShB peptide in the presence of anionic phospholipid vesicles are that the absorbance at 1623 cm^{-1} 1) recovers quite efficiently upon cooling of previously heat-denatured samples (Fig. 4, *C* and *E*, upper traces); 2) is fairly insensitive to increasing the ionic strength of the aqueous media, unless the vesicles are preincubated, before the addition of the peptide, with NaCl at more than 1 M, which efficiently prevents the conformational event; 3) is insensitive to the presence of low concentrations of nonanionic detergents such as octyl-glucoside, unless the vesicles are completely disrupted by the detergents at concentrations above their critical micellar concentration; and 4) is insensitive to the presence of millimolar concentrations of EDTA (data not shown).

As for the mutant ShB-L7E peptide (Fig. 4, *D* and *F*), the 1623 cm^{-1} structural component can be observed only in PG vesicles (Fig. 4 *F*), at a moderately high peptide concentration and only in the virtual absence of EDTA (below $\sim 20\ \mu\text{M}$), and even then it is much less heat-stable than the component seen in the ShB peptide under similar conditions

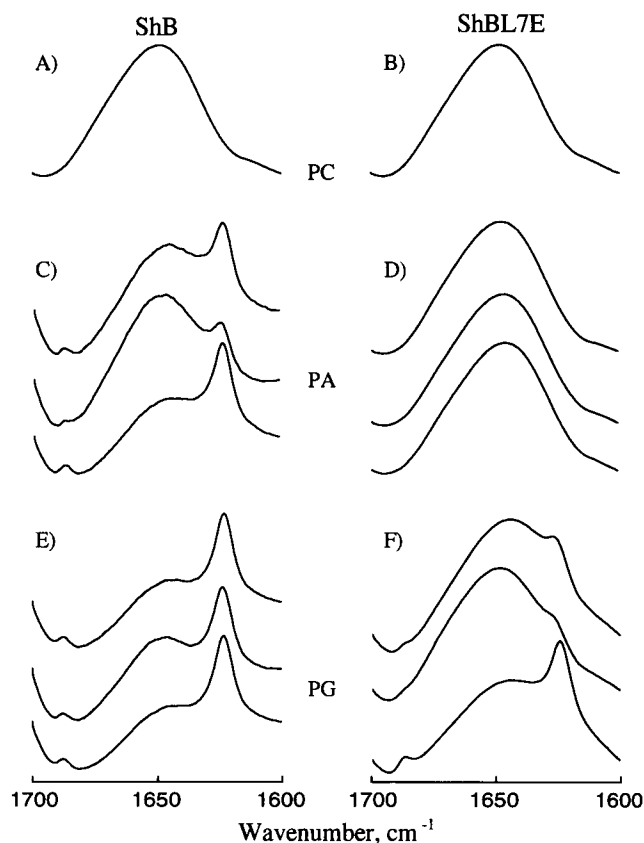


FIGURE 4 Infrared amide I band region of the original spectra of the ShB (A, C, and E) and the ShB-L7E (B, D, and F) peptides in the presence of PC (A and B), PA (C and D), and PG (E and F) vesicles. The samples were suspended in a D₂O medium prepared from 10 mM HEPES buffer, pH 7.4, containing 130 mM KCl and 20 mM NaCl. The final concentration of either peptide in all the samples was ~4 mM, and the phospholipid concentrations ranged from 32 to 35 mM, depending upon the different phospholipids. In this figure, as well as in Fig. 5, the spectra of the different buffers alone were subtracted from those of the peptide-containing samples. The triplet of traces shown in C, D, E, and F correspond to spectra of samples taken at 20°C (lower traces in each triplet), spectra taken in samples heated at 70°C (middle traces, "heat-denatured" samples), and spectra of the heated samples after recooling to 20°C (upper traces, "renatured" samples).

(Fig. 4 F, middle trace) and recovers very poorly from heat denaturation (Fig. 4 F, upper trace).

It should be noted that, based on the binding experiments using the fluorescence peptides described above, it is expected that at the peptide-to-lipid ratios used commonly in the FTIR measurements, only a portion of the added peptide is lipid-bound, and thus the observed peptide amide I bands shown in Fig. 4 should be the result of contributions from both lipid-bound and lipid-free peptide. In this respect, we found that the spectra taken from pellets obtained by ultracentrifugation of the peptide-lipid incubation mixtures, in which most or all of the peptide present is lipid-bound, have an even more conspicuous 1623 cm⁻¹ component (data not shown). These observations further emphasize that the appearance of the 1623 cm⁻¹ component in the infrared spec-

tra arises from peptide bound to the anionic phospholipid vesicles.

Because adoption of the β structure at 1623 cm⁻¹ by the ShB peptide occurs in the presence of anionic phospholipid vesicles but not in PC vesicles, additional experiments were done in the presence of vesicles made from mixtures of natural anionic phospholipids and PC to determine the minimum amounts of PA or PG required to induce such a structure. Fig. 5, A and C, shows that the appearance of the 1623 cm⁻¹ component in the ShB spectra requires a similarly high percentage (~50%) of either PA or PG in the

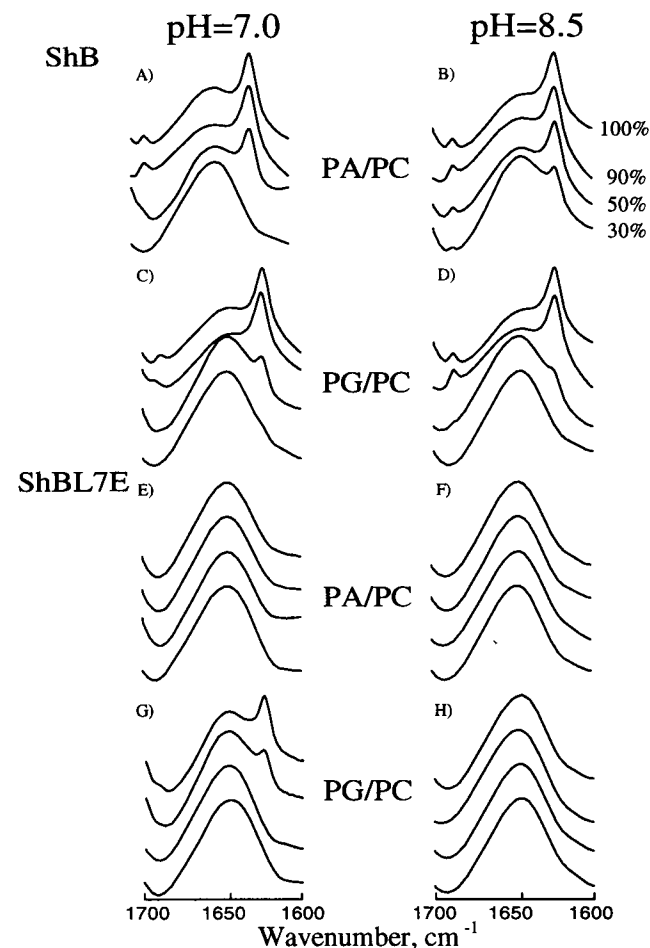


FIGURE 5 Infrared amide I band region of the original spectra of the ShB (A–D) and the ShB-L7E (E–H) peptides in the presence of phospholipid vesicles made from PA/PC (A, B, E, and F) or PG/PC (C, D, G, and H) mixtures. The spectra in the left column (A, C, E, and G) have been taken in a D₂O media prepared from 10 mM HEPES buffer, pH 7.4, containing 130 mM KCl and 20 mM NaCl, and in those in the right column (B, D, F, and H) the D₂O media was prepared from 10 mM Epps buffer, pH 8.5, containing 130 mM KCl and 20 mM NaCl. The stacks of spectra shown in each panel correspond to different samples in which the percentage of the acidic phospholipid (either PA or PG) in the acidic phospholipids/PC mixtures used to make the vesicles were 30%, 50%, 90%, or 100% (from the lower to the upper trace in each stack, respectively). To avoid an excessive complication of the figure, such percentages have been indicated only in B as an example. The peptide and phospholipid concentrations in these samples were approximately as in those in Fig. 4.

anionic phospholipids/PC mixed vesicles. Similar experiments were conducted at pH 8.5 (the pK_a for the deprotonation of the monoanionic PA is ~ 8) to investigate the possible role of an increased surface charge of the vesicles in the observed phenomena. At pH 8.5, most of the PA molecules should be in the dianionic form, thus providing a net negative charge at the surface of the PA/PC vesicles considerably higher than that found in the PG/PC vesicles. Under these conditions, the 1623 cm^{-1} component in the ShB spectra becomes noticeable at lower percentages of PA in the PA/PC mixed vesicles (Fig. 5 B), despite the fact that at this pH, at which some of the basic groups (most likely, the amino-terminal group and possibly the lysine side chains clustered near the C-terminus) in the peptide should be mostly deprotonated, the absorbance of the peptide at 1623 cm^{-1} in the PG/PC vesicles decreases slightly (comparison between Fig. 5, C and D).

As for the behavior of the ShB-L7E mutant peptide (Fig. 5, E-H), it is observed that the 1623 cm^{-1} component appears only in the PG/PC vesicles and at a very high percentage of PG (Fig. 5 G). Furthermore, increasing the pH to 8.5 causes a complete disappearance of such spectral components (Fig. 5 H).

Lipid phase separation studies

Additional DSC experiments were performed using vesicles made from mixtures of DMPA and DMPC to explore whether the presence of the ShB peptide in such vesicles induces lipid phase separation. DMPC and the monoanionic form of DMPA exhibit an ideal mixing at pH 6.0 (Fig. 6), which, depending on the proportion of the two phospholipids used in the mixtures, produces a wide endotherm with a transition temperature intermediate to those corresponding to the pure phospholipid components. The addition of ShB peptide to such mixtures, at a fairly high peptide-to-phospholipid ratio (see legend to Fig. 6), reduces the enthalpy of the wide transition to $\sim 70\%$ of the initial value but does not promote the appearance of additional endotherms such as those characteristic of the pure phospholipid components, as would have been expected if segregation of lipid domains had occurred.

DISCUSSION

Interaction of the ShB and ShB-L7E peptides with the model anionic vesicle target

Binding studies using NBD-labeled peptides show that peptide binding to zwitterionic phospholipid vesicles does not occur, but there is peptide binding to vesicles made from anionic phospholipids. The electrostatic component of this binding process is evidenced by 1) the effects observed by FTIR on the stabilization of the dianionic phosphoryl group of PA by the presence of either peptide, and 2) the decreased binding observed upon increasing the pH to ~ 8.5 , which is likely to cause partial deprotonation of both the amino

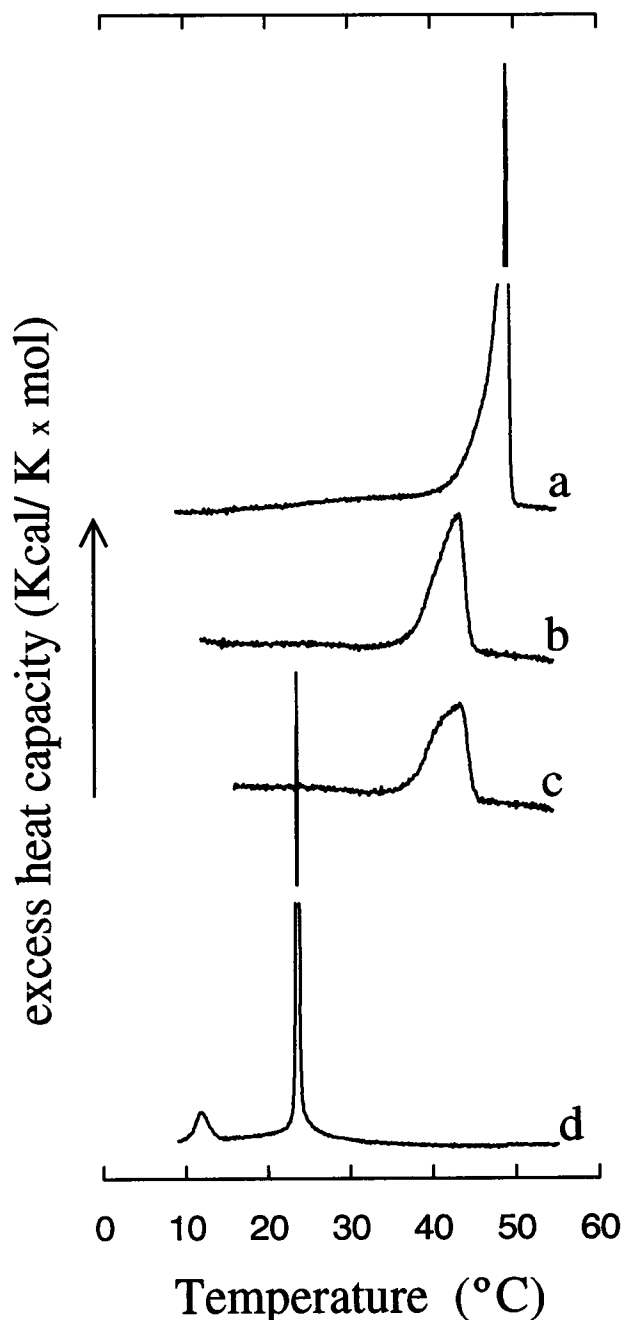


FIGURE 6 Effects of the addition of ShB peptide on the ideal lipid mixing exhibited by DMPA and DMPC at pH 6.0. For the experiment shown in the figure, large multilamellar vesicles were made from a DMPA:DMPC mixture (60:40, by mole) in 50 mM 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 6.0, containing 100 mM NaCl, 1 mM EDTA. Differential scanning calorimetry thermograms are shown for such mixed vesicles at a concentration of 2 mM in terms of total phospholipids, in the absence (scan b) and in the presence (scan c) of added ShB peptide at a 0.1 peptide/phospholipid molar ratio. Traces a and d represent thermograms corresponding to pure DMPA and DMPC vesicles, respectively.

terminal group and the Lys side chains at the peptide C-terminal ends and thus reduce electrostatic interaction with the negatively charged vesicle surface. Quantitative differences observed in the binding of either ShB or ShB-L7E

peptide to vesicles made from either PA or PG indicate that 1) the binding of the ShB peptide derivative is much tighter than that seen with the ShB-L7E labeled peptide, and 2) the binding of either fluorescent peptide to PA vesicles occurs with higher affinity than to PG vesicles. On a purely electrostatic basis, the former could be explained by the presence of the additional net negative charge consequence of the L7E substitution in the mutant peptide, whereas the latter should be expected to arise from the above-mentioned peptide-induced stabilization of dianionic lipid species, which can occur only in the PA vesicles.

When challenged by anionic lipid vesicles, the native ShB peptide undergoes a dramatic structural change, from the predominantly nonordered conformation exhibited in solution or in the presence of zwitterionic lipid vesicles, to a strongly hydrogen-bonded β -structure characterized by a prominent spectral component at 1623 cm⁻¹. Such an adopted conformation does not result from peptide aggregation, because the absorbance at 1623 cm⁻¹ is not dependent upon the peptide concentration or the lipid-to-peptide ratios used in these studies (Fernandez-Ballester et al., 1995). Furthermore, the 1623 cm⁻¹ β -structure component present in these samples exhibits a great stability to drastic changes in the experimental conditions, including heating at high temperatures, large changes in ionic strength or pH, and the addition of detergents or EDTA. On the other hand, cleavage of residues 15 to 20 by hydrolysis with trypsin was found to impede the adoption by the ShB 1–14 peptide of the characteristic β -structure seen in the whole ShB peptide (Fernandez-Ballester et al., 1995), suggesting that the anchoring of the cationic C-terminal portion of the peptide to the negatively charged vesicle surface plays an important role in determining the final conformation. The overall conclusion from the above observations is that the intact ShB peptide, when challenged by the anionic vesicle targets, has a remarkable tendency to rapidly adopt a highly stable β -structure. This, however, has certain requirements that must be satisfied. For instance, the experiments using vesicles made from anionic phospholipids/PC mixtures show that such an adoption of β -structure by the ShB peptide requires that the vesicle target has a relatively high density of negative charges on the vesicle surface (high percentage of anionic phospholipids in the mixed vesicles). In fact, the proportion of anionic phospholipids to PC in the mixed vesicles must be maintained above 50% to allow the adoption of β -structure by the peptide. Such a proportion could only be lowered when using PA/PC mixtures at alkaline pH, at which dianionic PA species are present. Second, based on the lack of peptide-induced lipid phase separation observed by DSC, it is concluded that such an adequate negatively charged vesicle surface has to preexist as an organized structure before peptide addition, because the ShB peptide appears unable to reorganize its own microenvironment upon interaction with the phospholipid membrane.

In contrast to the observations made with the native ShB peptide, the mutant ShB-L7E peptide bound to the anionic

vesicles adopts β -structure in a much more restrictive manner, i.e., at high peptide concentrations and only in the presence of PG vesicles, and at pH values below 8 and in the absence of EDTA, and even when such structure is finally adopted it is quite heat-unstable.

The ability of the peptides to insert into the hydrophobic vesicle bilayer is probably what distinguishes best between the ShB and ShB-L7E peptides in their interaction with the anionic vesicle targets. Thus, the mutant ShB-L7E peptide, even under conditions leading to the adoption of β -structure, does not insert into the vesicle bilayer. On the other hand, the native ShB peptide inserts quite efficiently in either DMPG or DMPA vesicles, causing a concentration-dependent decrease in the enthalpy of the lipid phase transition, while leaving the phase transition temperature unaltered. This behavior is entirely similar to that described by Papahadjopoulos et al. (1975) for the interaction of integral membrane proteins with lipid bilayers. The insertion into the bilayer, however, should not include the C-terminal region of the ShB peptide, as suggested 1) by the value of the emission maximum at 530–535 nm exhibited by the NBD-labeled peptide bound to either PA or PG vesicles, which indicates that rather than being embedded in the bilayer, the NBD group covalently attached to the C-terminus is located at or near the membrane surface, and 2) by the fact that most of the potential trypsin cleavage sites in the vesicle-inserted peptide remain accessible to polymer-immobilized trypsin added to the extravesicular aqueous medium (unpublished observation).

The study of the pH dependence of peptide insertion in DMPG vesicles shows a maximum decrease in the enthalpy of the lipid phase transition at pH 6, whereas increasing the pH to near 8.5, at which the ShB peptide is still perfectly able to adopt the characteristic β structure, prevents the peptide from inserting into the phospholipid bilayer. This demonstrates (along with the lack of insertion seen under all experimental conditions with the mutant peptide) that adoption of β -structure may occur separately and does not necessarily imply peptide insertion into the bilayer. In our interpretation, the folding of the peptide caused by the adoption of an intramolecular β -structure by the N-terminal half of the ShB peptide at neutral pH is likely to bring amino acid residues of different signs closer together, thus favoring the formation of salt bridges. For instance, this could be the case for the positively charged amino terminal and the negatively charged E12 or D13. This would certainly increase the stability contributed by hydrogen bonding to the overall structure, but perhaps more importantly, it would have an effect of “net charge cancellation,” which is expected to make the folded N-terminal half of the peptide capable of 1) traversing the anionic interphase formed by the phospholipid polar headgroups and 2) partitioning into the hydrophobic bilayer. According to this idea, the lack of insertion of the ShB peptide seen at pH 8.5 should be ascribed to an altered charge balance caused by deprotonation of basic groups. Furthermore, it follows from this interpretation that the mutant ShB-L7E peptide would not

be able to insert into the bilayer because the net negative charge from the E7 substitution can not be canceled out, regardless of whether the mutant peptide adopts β -structure. The latter receives further support from the observation that mutants of the ShB channel in which the L7 residue was substituted by either glutamate or lysine residues (in which either a negatively or a positively charged group, respectively, could not be canceled out) do not show inactivation (Hoshi et al., 1990).

Predicted behavior of the inactivating peptide during channel inactivation

The observation of "crossed inactivation" between channel proteins and inactivating peptides of different origins (see the Introduction) suggests that channel inactivation occurs as a consequence of the interaction between complementary domains, relatively unrestricted in terms of their primary structure, contained in the two interacting molecules. These observations make it conceivable that indeed the interaction of the inactivating peptide with the model vesicle target, which also contains an anionic surface and a hydrophobic region, could imitate somewhat the interaction occurring during the association of the peptide with the corresponding domains in the inactivation "entrance" of the real channel protein. This assumption does not mean that the relatively simple structural elements in the model system exactly mimic those more complex elements configuring the peptide receptor site in the real channel protein. However, we believe that based on the behavior exhibited by the ShB peptide in our simple model target, several predictions can be reasonably made as to the molecular events that might occur during channel inactivation. First, according to the model proposed by Durell and Guy (1992), it could be assumed that the organized protein region with a negative surface potential that becomes exposed to the intracellular medium upon channel opening indeed provides a site with a sufficiently high density of negatively charged groups for binding of the ShB peptide, as seen with the surface of the anionic vesicles. This, in turn, should immediately determine the folding of the peptide from the nonordered conformation exhibited in solution into an intramolecular β -sheet "hairpin" structure that, having an adequate charge balance, should readily insert into the hydrophobic vestibule within the channel protein. According to this proposal, the channel would remain inactivated as long as the peptide β -hairpin remains inserted and would reactivate upon peptide withdrawal, which seems consistent with the idea that channels reopen during recovery from inactivation (Demo and Yellen, 1991; Ruppertsberg et al., 1991). The above proposal also seems consistent with observations made from electrophysiological recordings, which indicate that electrostatic interaction of the peptide with the anionic site on the channel protein determines primarily the "on" rate (the rate of onset of

inactivation), whereas hydrophobic interactions determine the unblocking or "off" rate (Murrell-Lagnado and Aldrich, 1993; Toro et al., 1994).

It should be noted here that we considered it unlikely that the cytoplasmic leaflet of the plasma membrane would act as an alternative target for the peptide "in vivo," precisely because natural membranes do not contain sufficient anionic phospholipids (and the peptide seems unable to draw them together around it) to provide the high density of negative charges needed for the interaction with the peptide.

As for the mutant ShB-L7E peptide, which should also be expected to bind to the anionic site although with a lower affinity, its lack of capacity to induce channel inactivation could perhaps be explained by the practical impossibility that its N-terminal portion, carrying a net negative charge, would traverse the anionic interphase and insert into the hydrophobic domain of the channel protein. Finally, the known effect of trypsin of preventing channel inactivation by the ShB peptide could be tentatively explained by the loss of the positively charged groups present at the peptide's C-terminal end produced upon trypsin cleavage, which, by preventing the peptide from anchoring to the anionic site in the channel "entrance," leaves no chance that the peptide folding and insertion steps will occur.

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