Systematic Design and Validation of Ion Channel Stabilization of Amphipathic α-Helical Peptides Incorporating Tryptophan Residues

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ABSTRACT: Aromatic interactions such as π–π interaction and cation–π interaction are present in membrane proteins and play important roles in both structure and function. To systematically investigate the effect of aromatic residues on the structural stability and ion permeability of peptide-formed ion channels, we designed several peptides with one or two tryptophan (Trp) residues incorporated at different positions in amphipathic α-helical peptides. Circular dichroism (CD) studies revealed the preferable position of Trp residues for self-association in these designed peptides. Systematically designed di-substituted peptides with two Trps at each helix termini demonstrated intermolecular Trp–Trp interactions caused by aggregation. In the presence of liposomes, Trp on the hydrophilic face of the peptide enhanced interaction with the lipid membrane to increase the amphipathic α-helical contents. Appropriate incorporation and positioning of Trp enabled peptides to form more stable channels and had notable effects with Trp di-substituted peptides. The ion channel forming capability of a series of these peptides showed that the cation–π interactions between Trp and Lys residues in adjacent transmembrane helices contribute to remarkable stabilization of the channel structure.

INTRODUCTION

Ion channel proteins mediate the generation of electrical signals in the nervous system. Transmembrane domains of ion channel proteins are predominately α-helical and assemble to form multimeric structures via helix–helix interactions in lipid bilayers.1 Ion conducting pores can be also formed in lipid membranes by aggregation of peptides with simpler structures compared to proteins. Ion channel-forming peptides can adopt various helical structures (such as α-helix, 3_10-helix, and β-helix) in membranes.2–5 For example, alamethicin, a member of the peptaibol family isolated from soil fungi, is an ion channel-forming peptide with α-helical structures.6,7 Alamethicin forms helix-bundle (barrel-stave) channel structures through the self-association of 3 to 12 peptide monomers and has an antibiotic activity.6,8 Viroporins are a family of proteins encoded by pathogenic viruses. These proteins are essential to the life cycle of a diverse range of viruses and have ion channel activity due to the formation of oligomeric amphipathic α-helical units in the host cell membrane.9,10 Simplified synthetic peptides derived from protein transmembrane domains or de novo designed amphipathic peptides have also been shown to mimic the structure and function of native ion channel proteins and are, therefore, frequently used as models for understanding the function of ion channel proteins.3,11,12 Moreover, engineered model ion channel peptides may act as candidates for biologically functional molecules, such as antimicrobials,13,14 drug-delivery systems,15 or lytic peptides for cancer treatment.16

Control of the pore size and the stabilization of channel structures by appropriate modulation of the helix–helix interactions are important factors in developing functional molecules. The first step required to use an ion channel peptide as a functional module is to find a stabilizing factor for the helix–helix interaction. The helix–helix interactions in lipid bilayers are believed to be driven by van der Waals packing and electrostatic interactions.17–23 For example, the GxGxG motif has been reported to contribute to the stabilization of dimerized membrane proteins via van der Waals packing.24 Furthermore, interactions of aromatic residues (Phe, tryptophan (Trp), and Tyr), such as aromatic–aromatic interactions (π–π interaction and CH–π interaction) and cation–π interactions, can stabilize the associated forms of the membrane spanning domains. Dever et al. reported that the helix–helix interactions in model peptides supplemented with aromatic residues were facilitated through aromatic interactions, particularly cation–π interactions.25,26 Shai et al. suggested that an aromatic motif, WxxW, is involved in the transmembrane domain dimerization based on statistical analysis of a bacterial transmembrane...
interactions and stabilization effects on the concentration of these designed peptides and show that residues can promote peptide aggregation, that is, the formation of ion channels.

The effect of aromatic–aromatic interactions or cation–π interactions on the formation of ion channel has been highlighted in numerous studies. In site-specific mutation studies of natural ion channel proteins, substitution of aromatic residues in the transmembrane segments leads to loss of channel function, indicating that aromatic–aromatic interactions or cation–π interactions are crucial for the formation of the channel structure. In general, peptide–lipid interactions and the partitioning of peptides into membranes are critical in ion channel formation. The Trp residues of ion channel-forming peptides have been suggested to stabilize channel structures via Trp–Trp interactions among peptides and/or the high affinity of Trp residues for the lipid bilayer interface. Trp residues, with their amphipathic character and inherent dipole, can anchor peptides to the bilayer–water interface through interaction with the phospholipid head groups and stabilize the folded membrane proteins. Introduction of Trp residues and cationic residues (Lys, His, or Arg) into ion channel-forming peptides may promote both helix–helix and peptide–lipid membrane interactions and subsequent formation of higher-ordered structures.

Viroporins are highly abundant in aromatic residues as compared with cellular ion channel proteins; viroporin interactions are believed to have certain roles in their membrane perturbation and high fusogenic activity. The extraordinary multiple Trp residues in viroporins are present despite the risk of viral RNA mutation at the wobble base leading to a stop codon. The existence of aromatic residues may be indispensable for constructing helical assemblies of small viral proteins. In contrast, aromatic amino acids are rarely found in peptaibols and, when present, are exclusively located at either the N- or C-termini. Peptaibols are nonribosomally synthesized peptides containing large quantities of α-amino-isobutyric acid (Aib), which has been proposed to be a strong helix promoter. Thus, high Aib contents impose a predominantly helical structure, regardless of the sequence, and therefore may require less driving force for aromatic–aromatic interactions.

Considering the abundance of interactions of Trp residues in viroporins, we have been interested in understanding the extent of the stabilizing effect of Trp residues in peptaibols that typically possess much fewer Trp residues, in comparison. Previously, we developed Aib-containing model peptides based on an Aib–Xxx–Aib–Ala repeated structure and demonstrated that these membrane spanning peptides have well-defined ion channel activity and antimicrobial activity. To clarify the stabilizing factors provided by Trp residues in the Aib–Xxx–Aib–Ala-based ion channel-forming peptides, we incorporated Trp residues at different sites in amphipathic model peptides and validated their structure–function relationship using circular dichroism and single channel measurements. In this study, we demonstrate that directed positioning of Trp residues can affect the ion conductance, lifetime, and effective concentration of these designed peptides and show that stabilization effects can be mostly explained by cation–π interactions and π–π interactions contributed by the Trp side chains. These results on the role of aromatic side chains in stabilization and functional improvement of the designed ion channel peptides are of value in further development of these peptides for potential therapeutic functions.

**RESULTS**

**Peptide Design.** In a previous study, we reported the synthesis of an Aib-containing 20-meric α-helical peptide, Ac–(Aib–Lys–Aib–Ala)3–NH2 (termed BKBA20). BKBA20 is believed to form helical structures in aqueous solution and lipid vesicles owing to the presence of Aib. In Figure 1, the helical wheel projection of BKBA20 represents an amphipathic structure with separation of hydrophobic and hydrophilic groups on the opposite faces of the helix.

We have previously shown that BKBA20 could self-associate and form ion conducting pores in lipid bilayers. As a model peptide framework, these biophysical properties of BKBA20 can be employed to examine the effect of Trp residues on the channel function. Therefore, we designed a series of peptides that contained single Trp residues inserted in the middle of BKBA20 helical structure as a scaffold. Since the helix–helix interface of associated forms of BKBA20 in both aqueous and lipid environments is not clearly identified, we systematically substituted single Trp residues in place of an Ala or Aib residue on the hydrophobic (position 8, 12) and hydrophilic faces (position 10), as well as their boundaries (position 9, 11), as summarized in Table 1. Two Ala residues at the N- or C-termini were further replaced with Trp residues (positions 4 and 16) to assess whether Trp–Trp interhelical interaction could be detected in these analogues.

**Conformation and Helix–Helix Interaction in PB.** To obtain an insight into the influence of Trp residues on conformation and molecular interactions in different environments, we analyzed the far-ultraviolet (UV) CD spectra of Trp-containing peptides. Concentration-dependent CD spectra of peptides in 50 mM PB (pH 7.4) are shown in Figure 2.

All peptides exhibited a pronounced α-helical CD signature with characteristic double minima near 208 and 225 nm. The helix contents of the peptides in different environments are summarized in Table 1. The mean residue ellipticity (MRE) ratio \( \theta_{\pi}^{\alpha}/\theta_{\pi}^{\alpha} \) was 1.10 for BKBA20. The ellipticity ratios for [W11]BKBA decreased to 0.86, whereas the ellipticity ratios for [W10]BKBA20 and [W4W16]BKBA20 increased to 1.13 and 1.17, respectively. These results reflect a slight conformational difference as compared to BKBA20 helices and exclude the possibility of 3(10) helix conformations, with a characteristic more enhanced negative ellipticity at 208 nm (compared to 225 nm) and with \( \theta_{\pi}^{\alpha}/\theta_{\pi}^{\alpha} \) ratio...
values much less than 1.38,39 The MRE values of BKBA20, [W9]BKBA20, [W10]BKBA20, [W4]BKBA20, [W16]-BKBA20, and [W4W16]BKBA20 in PB solution were observed in a concentration-dependent manner, implying that helix–helix association is involved between these peptides. However, we did not observe concentration dependency, even over a 40-fold concentration range, for either [W8]BKBA20 and [W12]BKBA20, suggesting that the ability of these peptides to associate was diminished due to the introduction of a Trp residue. This implies that Ala 8 and Ala 12 at the helix–helix interface should be preserved to ensure helix–helix interaction in an aqueous environment; despite its hydrophobic nature, the local steric bulkiness of the indole ring apparently prevented helix–helix interactions. Compared to BKBA20, [W11]BKBA20 (which contained a Trp residue at the boundary of the hydrophobic face and the hydrophilic face) had less concentration dependency and showed a significant reduction in its helical content. However, [W9]-BKBA20, which also contained a Trp residue at a similar boundary site, mostly retained the CD spectral characteristics and the helical content of BKBA20. The reason for this difference is not easily explainable, but it seems that the introduction of a Trp residue at position 11 disrupts the helical structure of BKBA20 in the aqueous environment, resulting in a loss of the helical stability of the original peptide.

Interestingly, CD spectra of [W4W16]BKBA20 exhibited a red shift of the minimum near 208 nm, a blue shift of the

Table 1. Sequence of Designed Model Peptides and Their Helix Contents

| peptide      | sequence                  | helical contents (%) | PB  | LUVs |
|--------------|----------------------------|----------------------|-----|------|
| BKBA20       | Ac–BKBA–BKBA–BKBA–BKBA–NH₂ | 42                   | 47  |
| [W8]BKBA20   | Ac–BKBA–BKBAW–BKBA–BKBA–BKBA–NH₂ | 36           | 50  |
| [W9]BKBA20   | Ac–BKBA–BKBA–WKBA–BKBA–BKBA–NH₂ | 40           | 56  |
| [W10]BKBA20  | Ac–BKBA–BKBA–BWBA–BKBA–BKBA–NH₂ | 40           | 65  |
| [W11]BKBA20  | Ac–BKBA–BKBA–BKWA–BKBA–BKBA–NH₂ | 26           | 40  |
| [W12]BKBA20  | Ac–BKBA–BKBA–BKBA–BKBA–NH₂     | 33           | 43  |
| [W4]BKBA20   | Ac–BKBA–BKBAW–BKBA–BKBA–NH₂     | 38           | 47  |
| [W16]BKBA20  | Ac–BKBA–BKBA–BKBA–BKBA–NH₂     | 37           | 45  |
| [W4W16]BKBA20| Ac–BKBA–BKBAW–BKBA–BKBA–BKBA–NH₂ | 48           |      |

*Replaced Trp residues are shown in bold. Helix contents were calculated from CD spectra in 50 mM phosphate buffer (PB) at 200 μM peptide and in the presence of large unilamellar vesicles (LUVs) of 4 mMDPPC at 20 μM peptide. In the presence of PB, the helix contents of [W4W16]BKBA20 were not estimated due to the exciton coupling derived from Trp–Trp interaction.

Figure 2. Concentration dependence CD spectra of BKBA20 and Trp-containing analogues in 50 mM PB (pH 7.4). Arrows indicate that the mean residue ellipticities, (θ), increase with increasing peptide concentration.
minimum near 225 nm, and an appearance of a small shoulder near 230 nm with increasing peptide concentration. It has been reported that the exciton coupling derived from close Trp−Trp interactions can affect the far-UV CD spectra of Trp-containing peptides and proteins, and the characteristic CD band near 230 nm in [W4W16]BKBA20 can imply Trp−Trp interactions. To investigate the influence of exciton coupling attributed to Trp pairs in intermolecular associations, we compared the subtracted difference spectra of BKBA20 from each Trp-containing analogue at a relatively high peptide concentration of 200 μM (Figure 3). Only the difference spectrum of [W4W16]BKBA20 exhibited both a positive maximum near 229 nm and a negative maximum near 218 nm, which is consistent with a typical CD signature of the exciton coupling of Trp pairs. In addition, the characteristic near-UV CD band was only observed in [W4W16]BKBA20, implying a different microenvironment for Trp residues, as well as a more specific Trp−Trp interaction (Figure S1). Both the formation of the CD exciton couplet between adjacent Trp residues and the enhanced near-UV CD band strongly support the existence of Trp−Trp interactions and that Trp residues are involved in the helix–helix interaction of [W4W16]BKBA20 monomers in the aqueous environment. However, the exciton couplet and the near-UV CD band were not observed in either [W4]-BKBA20 or [W16]BKBA20, with a Trp residue at N- and C-termini, respectively, despite the observed intermolecular associations in CD experiments. These results imply that BKBA20 and its Trp-containing analogues associate in an antiparallel helix orientation in aqueous environments.

**Interaction of Model Peptides with Membranes.** To evaluate the interaction of the peptides with phospholipid membranes, CD spectra were measured in the presence of DPPC liposomes. A comparison of CD spectra in PB and in the presence of DPPC liposomes is shown in Figure 4. The double minima in the CD spectra of all peptides in the presence of DPPC liposomes were more intense than those in the buffer, suggesting an increase in the helicity of peptides (Table 1), which can be due to peptide−membrane interactions. [W10]BKBA20 exhibited the greatest helicity in the presence of DPPC liposomes. Thus, the Trp residue on the hydrophilic face of BKBA20 plays an important role in the peptide−membrane interactions. It seems that the electrostatic interaction between lipid phosphate headgroups and the Lys side chain is a primitive interactive driving force for the peptides; among these, [W10]BKBA20 could have a secondary

![Figure 3](image-url)

*Figure 3.* Comparison of the subtracted CD spectra of the Trp-containing analogues from BKBA20 in 50 mM PB (pH 7.4); the peptide concentration was 200 μM. The subtracted spectra of [W4W16]BKBA20 (red) exhibited a typical pattern of the Trp−Trp exciton coupling.

![Figure 4](image-url)

*Figure 4.* CD spectra of BKBA20 and Trp-containing analogues in 50 mM PB (black) and in the presence of DPPC LUVs (red). Spectra were collected at neutral pH conditions (pH 7.4). The peptide concentration was 20 μM, and the DPPC concentration was 4 mM to give P/L ratios of 1/200.
helix stabilizing effect by Trp anchoring. A red shift of the $\pi - \pi^*$ transition band near 208 nm and an increase in the $\frac{[\theta]_{n-\pi^*}}{[\theta]_{\pi-\pi^*}}$ ratio were reported as indicators of a two-stranded $\alpha$-helical coiled-coil structure. In the presence of DPPC liposomes, the $\frac{[\theta]_{n-\pi^*}}{[\theta]_{\pi-\pi^*}}$ ratio of [W4W16]BKBA20 was 1.21 while the ratio was 1.00 in buffer. Compared with [W4W16]BKBA20, no significant change in the $\frac{[\theta]_{n-\pi^*}}{[\theta]_{\pi-\pi^*}}$ ratio was observed for other peptides. In addition, a red shift in the minimum from 207.5 to 209.5 nm was observed for [W4W16]BKBA20 in the presence of DPPC liposomes. These results indicate that the existence of two-stranded (or higher order) $\alpha$-helical aggregates of [W4W16]BKBA20 was more preferable in the lipid bilayers than in the aqueous environment.

**Ion Conductance Property of Peptides.** To investigate the effect of Trp residues on the peptide association states in the membranes and the channel-forming properties, the ion conducting activities of peptides were measured in the diphytanoylphosphatidylcholine (DPhPC) bilayer. The peptides were added to the cis-side of the membranes, and a transmembrane voltage of +80 mV was applied on this side. Figure 5 shows the single-channel current recordings and the relative frequency of the observed channel currents. BKBA20 formed channels with single level conductance of approximately 260 pS, consistent with our previous reports. According to the helical bundle model, the pore size of BKBA20 was estimated to be 0.19 nm, which corresponded to a tetrameric helix bundle. With the exception of [W4W16]-
BKBA20, clear open-close transitions of ion channels were observed for all Trp-containing peptides, as shown in Figure 5.

Four peptides, [W8]BKBA20, [W9]BKBA20, [W10]-BKBA20, and [W12]BKBA20, predominantly exhibited open states with conductance of approximately 280, 400, 420, and 250 pS, respectively. The calculated pore diameter of these peptides ranged from 0.19 to 0.22 nm and is comparable to that of BKBA20. These results indicated that the substitution of Trp residues at those positions scarcely affects the association status of BKBA20 in the membranes. Although less frequent, [W8]BKBA20, [W10]BKBA20, [W12]BKBA20, and [W16]BKBA20 exhibited higher conductance levels. These higher levels are most probably due to the simultaneous opening of two channels because the higher conductance level was approximately twice that of the lower conductance. However, [W4]BKBA20, [W11]BKBA20, and [W16]BKBA20 peptides formed channels with a higher conductance of 750, 730, and 710 pS, respectively, suggesting an increase in pore diameter to approximately 0.33 nm. Among the previously stated peptides, [W10]BKBA20 and [W16]BKBA20 significantly increased the lifetime of channel-opening state as compared with other peptides. Interestingly, [W4W16]-BKBA20 did not show a clear-cut open–close state but rather caused membrane perturbation.

From previous spectral observations, we hypothesized that channels formed by [W4W16]BKBA20 were highly stable at 100 nM peptide concentration. To verify this hypothesis, we examined the concentration dependency of ion channel activities of selected peptides (Figure 6). At a peptide concentration of 10 nM, there was no apparent ion channel activity with BKBA20, whereas [W10]BKBA20 and [W16]-BKBA20 formed well-defined ion channels with a single conductance level. Furthermore, [W4W16]BKBA20 not only exhibited open–close states but also multi-state conductance even at 10 nM. Interestingly, single conductance channels comparable to those of 100 nM BKBA20 were observed when the concentration of [W4W16]BKBA20 was lowered to 1 nM. This ion channel activity with well-resolved open–close states at 1 nM peptide concentration was not observed in other Trp-containing peptides (data not shown). From these results, it can be concluded that the introduction of a Trp residue at positions 10 or 16 substantially stabilizes the lifetime of the channel open state, and the introduction of two Trp residues at both termini, positions 4 and 16, stabilizes the aggregated state required for channel function.

**DISCUSSION**

In natural membrane proteins that form dimers by helix–helix association, interactions between helices are stabilized by close contact van der Waals packing. In the present study, substituting Ala8 and Ala12 on the hydrophobic face of BKBA20 with a Trp residue abolished the aggregation of BKBA20 helices in an aqueous environment. This implies that the helix association of BKBA20 in the aqueous environment is stabilized by hydrophobic interactions due to close packing between the side chains of small residues such as Ala or Aib on the hydrophobic face and was interrupted by the presence of Trp with a relatively bulky side chain. Therefore, the overall steric complementarity of the hydrophobic region is crucial for the helix–helix interaction in these peptides. However, based on its CD spectra, [W4W16]BKBA20 (with two Trp residues in both termini) exhibited Trp–Trp interactions that implied self-association of the helices. Generally, the termini of helical peptides tend to fray and given that the helical content of BKBA20 is 42% in aqueous environments, the helix termini are expected to be flexible (less structured). Thus, it can be suggested that due to a more flexible peptide backbone at both termini, Trp residues were able to contact closely and form interhelical Trp–Trp interactions without affecting the close packing in the middle zones of the helices. Several studies have suggested that the initial formation of the two-stranded α-helical structure is an important process in constructing the channel structure. In the present study, [W4W16]BKBA20 formed a stable ion channel even at 100-fold lower concentration (1 nM) than BKBA20. Hence, two Trp residues at both termini of [W4W16]BKBA20 can contribute to stabilizing a two-stranded α-helical structure via Trp–Trp interaction in an aqueous environment, which in turn may act as an intermediate structure toward more stable multimeric channel structures in the lipid membranes. On the other hand, [W4W16]BKBA20 caused membrane perturbation at 100 nM. It has been reported that apoA-I mimetic peptides, amphipathic peptides with aromatic residues can solubilize lipid vesicles to form nanoparticulate peptide–lipid complexes. The membrane-perturbing [W4W16]BKBA20 may also disrupt lipid vesicles, a mechanism similar to the apoA-I mimetic peptides. To further explore this case, the turbidity of peptide–lipid dispersions was measured at high P/L ratios (Figure S2). Unlike apoA-I mimetic peptides, Trp-containing BKBA20 analogues hardly cleared lipid vesicles. Therefore, it seems that the Trp-containing BKBA20 series interact with lipid vesicles as a concentration-dependent mixture of monomeric/dimeric conformations and form ion conducting complexes, which are essentially different from membrane solubilizing peptide–lipid complexes formed by apoA-I mimetic peptides. However, at extremely low peptide concentrations required for ion channel studies, it is not feasible to determine the accurate molecularity of the peptide structures or their detailed mode of interaction with the lipid vesicles by CD measurements.

The helical contents of all designed peptides increased in the presence of the liposomes as compared with aqueous milieu, suggesting that these peptides interacted with the lipid bilayers. In particular, the helical contents of [W10]BKBA20 were significantly increased in the presence of liposomes. In general, the indole group in the Trp side chain possesses a micro-dipole and has an amphipathic character with a relatively high affinity for the lipid bilayer surface through both hydrophobic and electrostatic interactions. The Trp residue of [W10]-BKBA20 is, therefore, located at a critical site for the peptide–membrane interaction, and the high affinity of Trp side chain for the lipid bilayer surface may promote the peptide–membrane interaction and subsequent partitioning of peptides into the lipid bilayer. This may be the preferable situation for channel formation, as Huang et al. reported that the first step in the formation of ion channels was peptide–membrane interactions that aligned the peptides parallel to the bilayer surface and resulted in their subsequent accumulation on the membrane surfaces. One possible explanation for the prolonged stabilization of the ion channel observed with [W10]BKBA20 is that the Trp residue on the hydrophilic face promoted the peptide–membrane interaction due to the high affinity for the membrane surface, leading to the formation of multimeric associated structures of the peptide necessary for ion channels.

[W10]BKBA20, [W16]BKBA20, and [W4W16]BKBA20 exhibited distinct channel activities at lower concentrations.
than other peptides, which showed that the channel structure is stabilized by incorporating the Trp residue. Cation–π interactions between the transmembrane domains have been reported to be involved in stabilizing the channel structure and controlling the functions in natural ion channel proteins such as the influenza M2 protein and chloride intracellular channel protein.54,55 Similar to these reports, activities of the Trp-containing peptides that exhibited channel activity with longer lifetimes compared with that of BKBA20 could be explained by the additional stabilization via cation–π interactions, as these peptides have plausible interactions of the Trp residue with the Lys residue between adjacent α-helices. Figure 7 displays a schematic model of the channel structure stabilized by the intermolecular cation–π interaction. According to the cylindrical bundle model,6 the pore of BKBA20 consists of tetrameric helices; exposure of charged residues to the hydrophobic core of the membranes is generally considered to be energetically disadvantageous, and thus the five positively charged Lys residues could face inwardly in the pore’s interior in the lipid bilayers, and thereby be exposed to the aqueous environment in which the ions pass through the membranes. In addition, under experimental conditions where a positive transmembrane voltage was applied to the cis-side of the membrane are oriented parallel to each other. The adjacent helices; the cation–π interactions from these residues are expected to occur inside of the pore and are thought to contribute to channel stability. Similar interactions can be expected with the C-terminal W16 and N-terminal W4 at the pore circumference, and a slightly enlarged channel would explain the higher conductance values. While the conductance values were comparable, the open-state lifetimes were different in these analogues. Thus, the C-terminal W16 may stabilize the channel structure by the cation–π interaction with the Lys residue at position 18 of the adjacent helix. In contrast, the N-terminal W4 had a minimal effect on channel activity. Futaki et al. reported that higher voltage is required for channel activation of alamethicin analogues with a polar residue at the N-terminus compared with alamethicin.56 In this given condition under cis-positive voltages, the insertion of [W4]-BKBA20 into the membrane could be sterically or energetically unfavorable for the Trp side chain to cross the hydrophobic core of the membrane to contribute to the channel structure. Surprisingly, [W4W16]BKBA20 exhibited ion channel activity with well-resolved open—close states even at extremely low peptide concentrations (1 nM). As described previously, the [W4W16]BKBA20 dimer, an intermediate of ion channel structures, is stabilized through Trp–Trp interactions in aqueous solution. Furthermore, in the case of [W4W16]-BKBA20, there could be two potential cation–π interactions per helix at both termini. These cumulative contributions to the stabilization of the channel structure may be sufficient to compensate for the energy penalty required for Trp residues to cross the hydrophobic core of the membranes. Furthermore, the introduction of the Trp residue at the terminus of the helix led to an increase in pore diameter as explained in Figure 7. This increase in pore diameter can be attributed to the presence of the Trp residue with bulky side chains at the helix–helix interaction interface in the channel structure.

Several viroporins include domains rich in basic or aromatic residues that play certain functional roles.10 Among them, the delta peptide of EBOV, a 40-residue peptide encoded by the GP gene through RNA editing, has strong membrane-permeabilizing activity and is believed to be involved in EBOV pathogenesis.57–59 Gallacher and Garry proposed a putative tetrameric pore structure of the EBOV delta peptide where the positively charged residues are facing inside of the pore to form a cationic pore,50 which is consistent with our Trp-containing BKBA20 model peptides. Furthermore, the C-terminal Trp residue of the EBOV delta peptide has been reported to be essential for membrane permeability activity.59 Thus, interactions of aromatic residues, especially Trp residues, may contribute to the stabilization of the ion channel structure of several viroporins and thereby can promote the membrane perturbation and high fusogenic activity required for the viral life cycle.

Our peptides were based on Aib-containing peptaibols, which do not usually contain Trp residues. We demonstrated a synergistic ion channel stabilization effect by using systematically designed peptides containing specifically inserted Trp residues. The overall original structure of peptaibols is predominantly helical because of their high Aib content, and thus these may support the formation of the helix bundle without the aid of additional side chain interactions. This may be the sufficient reason to exclude Trp residues from naturally occurring peptaibols as excessive stabilization may result in toxic effects, such as membrane disruption, rather than the desired antimicrobial action against invading microorganisms.

**CONCLUSION**

In this study, amphipathic α-helical peptides were designed on the basis of the Aib-containing channel forming peptide BKBA20, as a model, to evaluate the stabilization effect of Trp residues on ion channels. This demonstrated that Trp residues

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**Figure 7.** Hypothetical model of association states of Trp-containing analogues stabilized through cation–π interactions in the lipid bilayers. (A) Top view of the channel structure formed by [W10]BKBA20; putative intermolecular cation–π interactions may be present between W10 and K6/K14. (B) Top view of the channel structure formed by [W16]BKBA20. A putative intermolecular cation–π interaction is present between W16 and K18. (C) Side view of the channel structure. Peptides with their C-termini facing the cis-side of the membrane are oriented parallel to each other.
can modulate the conductance, lifetime, and effective concentration of these peptides. The stabilization effects can be explained by mostly cation–π interactions and π–π interactions contributed by the Trp side chain. These findings on the contribution of aromatic residues to ion channel stabilization are crucial for improvement of the artificial ion channel peptide design for various biological activities such as antimicrobials, drug delivery systems, and cell lysis for cancer treatment.

**MATERIALS AND METHODS**

**Materials.** Fmoc-Ala-OH, Fmoc-Lys-OH, Fmoc-Trp-OH, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uroniumhexafluorophosphate (HBTU), and NovaPEG Rink amide resin LL (low loading) were obtained from Novabiochem (Tokyo, Japan). N,N-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole (HOBT) were purchased from the Peptide Institute (Osaka, Japan). DPhPC was obtained from Avanti Polar Lipids. DPPC was purchased from Sigma-Aldrich (Tokyo, Japan). All other reagents were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

**Peptide Synthesis.** Peptides were synthesized with the solid-phase method using the Fmoc strategy on NovaPEG Rink Amide resin LL (0.19 mmol/g, 25 mmol scale as 1 equiv). A 10-fold excess of Nα-Fmoc-protected amino acids on a 25 mmol scale was activated with HBTU/HOBt and 1-hydroxybenzotriazole (HOBT) were purchased from the Peptide Institute (Osaka, Japan). DPhPC was obtained from Avanti Polar Lipids. DPPC was purchased from Sigma-Aldrich (Tokyo, Japan). All other reagents were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

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**CD Measurements.** CD spectra were measured by a Jasco J-820 CD spectropolarimeter (Jasco, Tokyo, Japan) in the far-UV CD wavelength range (320–190; 1.0 nm bandwidth at a step resolution of 0.2 nm and a scan speed of 20 nm/min) at room temperature, in a cylindrical quartz cell of 0.1 cm pathlength. The reported spectra are the average of 4–12 scans with blanks (spectra without peptides) subtracted. Peptide solutions were prepared 30 min before measurements. Ellipticity was converted as the MRE [θ]. The helix contents (helicities) of the peptides were estimated by the formulas

 \[ [\theta]_{322,\infty} = \frac{(\theta - 40,000)(n - 4.6)}{n} \]  

\[ \text{Helix contents} \% = \frac{[\theta]_{322}}{[\theta]_{322,\infty}} \times 100 \]  

where [θ]322 is the MRE at 222 nm, [θ]322,∞ is the MRE at 222 nm when a peptide adopts 100% helical structure, and n is the number of residues in the peptide. For the 20-meric peptide used in the present study, the [θ]322,∞ is estimated to be \(-30,800 \text{ deg cm}^2 \text{ dmol}^{-1}\) using eq 1.

**Preparation of Large Unilamellar Vesicles.** DPPC (4.4 mmol) was dissolved in 100 mL of chloroform. The chloroform stock solutions were then evaporated into thin films using a nitrogen stream and further dried in vacuo overnight. The thin lipid film was then suspended in 1 mL of 50 mM PB (pH 7.4) and vortexed for 30 min at 60 °C. LUVs were prepared using a LiposoFast extruder (Avestin, Canada) through a 100 nm filter. Lipid dispersions were passed through a filter for a total of 21 times.

**Single-Channel Measurements.** Single-channel measurements were conducted using the tip-dip patch-clamp method. Glass pipettes were prepared using hemocrit hard grass capillaries (Narishige, Tokyo, Japan). The pipette tips with approximate 1 μm diameters were prepared by two-pull method with a microelectrode puller (Narishige) and were used without being heat polished. Electrolyte solutions consisted of 500 mM KCl solutions buffered with 5 mM HEPES at pH 7.4. DPhPC monolayers were formed on the surface of the dish by adding 2 μL of 10 mM DPhPC solution dissolved in hexane. Single-channel currents were analyzed by pClamp 6 software (Axon Instruments, Inc., Union City, CA). Using the helical bundle model, the pore size and the number of helices comprising the bundle were calculated on the basis of the following formulas

\[ G = \frac{\pi r^2}{\rho(1 + \frac{r}{2})} \]  

\[ r = R \left( \frac{1}{\sin \left( \frac{\pi}{N} \right)} - 1 \right) \]  

where G is the conductance of the pore, r is the radius of the pore, and N is the number of helix monomers participating in the bundle. The helix radius (R) was assumed to be 0.5 nm; the pore length (l) of the 20-meric helical peptide was 3.0 nm; and buffer resistivity (ρ) of 500 mM KCl solution estimated from limiting ion conductivity at 25 °C was 0.13 Ω m. 

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05254.

Near-UV CD spectra of BKBA20 and Trp-containing analogues and lipid solubilization assay (PDF)

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helical model peptides of various chain lengths. J. Biol. Chem. 1991, 266, 20218–20222.
(14) Juuvadi, P.; Vunnan, S.; Merrifield, E. L.; Boman, H. G.; Merrifield, R. B. Hydrophobic effects on antibacterial and channel-forming properties of cecropin A-melittin hybrids. J. Pept. Sci. 1996, 2, 223–232.
(15) Li, W.; Nicol, F.; Szoka, F. C., Jr GALA: a designed synthetic pH–responsive amphipathic peptide with applications in drug and gene delivery. Adv. Drug Deliv. Rev. 2004, 56, 967–985.
(16) Ellerby, H. M.; Lee, S.; Ellerby, L. M.; Chen, S.; Kyotota, T.; del Rio, G.; Sugihara, G.; Sun, Y.; Bredesen, D. E.; Arap, W.; Pasqualini, R. An artificially designed pore–forming protein with anti–tumor effects. J. Biol. Chem. 2003, 278, 53311–53316.
(17) Ben-Tal, N.; Honig, B. Helix–helix interactions in lipid bilayers. Biophys. J. 1996, 71, 3046–3050.
(18) Zhou, F. X.; Cocco, M. J.; Russ, W. P.; Brunger, A. T.; Engelman, D. M. Interhelical hydrogen bonding drives strong interactions in membrane proteins. Nat. Struct. Biol. 2000, 7, 154–160.
(19) Ruan, W.; Lindner, E.; Langosch, D. The interface of a membrane–spanning leucine zipper mapped by asparagine–scanning mutagenesis. Protein Sci. 2004, 13, 555–559.
(20) Dawson, J. P.; Weinger, J. S.; Engelman, D. M. Motifs of serine and threonine can drive association of transmembrane helices. J. Mol. Biol. 2002, 316, 799–805.
(21) Dawson, J. P.; Melnyk, R. A.; Deber, C. M.; Engelman, D. M. Sequence context strongly modulates association of polar residues in transmembrane helices. J. Mol. Biol. 2003, 331, 255–262.
(22) Summerman, H. K. B.; Kobayashi, Y. M.; Autry, J. M.; Jones, L. R. A leucine zipper stabilizes the pentameric membrane domain of phospholamban and forms a coiled–coil pore structure. J. Biol. Chem. 1996, 271, 5941–5946.
(23) Melnyk, R. A.; Partridge, A. W.; Deber, C. M. Retention of native–like oligomerization states in transmembrane segment peptides: Application to the Escherichia coli aspartate receptor. Biochemistry 2001, 40, 11106–11113.
(24) Russ, W. P.; Engelman, D. M. The GxxG motif: A framework for transmembrane helix–helix association. J. Mol. Biol. 2000, 296, 911–919.
(25) Johnson, R. M.; Hecht, K.; Deber, C. M. Aromatic and cation–π interactions enhance helix–helix association in a membrane environment. Biochemistry 2007, 46, 9208–9214.
(26) Johnson, R. M.; Heslop, C. L.; Deber, C. M. Hydrophobic helical hairpins: Design and packing interactions in membrane environments. Biochemistry 2004, 43, 14361–14369.
(27) Sal-Man, N.; Gerber, D.; Bloch, I.; Shai, Y. Specificity in transmembrane helix–helix interactions mediated by aromatic residues. J. Biol. Chem. 2007, 282, 19753–19761.
(28) Chung, L. A.; Lear, J. D.; DeGrado, W. F. Fluorescence studies of the secondary structure and orientation of a model ion channel peptide in phospholipid vesicles. Biochemistry 1992, 31, 6608–6616.
(29) Cory-Wright, J.; Alqaqazaz, M.; Wroe, F.; Jeffreys, J.; Zhou, L.; Lumnis, S. C. R. Aromatic residues in the fourth transmembrane–spanning helix M4 are important for GABA receptor function. ACS Chem. Neurosci. 2018, 9, 284–290.
(30) Garneau, L.; Klein, H.; Lavoie, M.-F.; Brochiero, E.; Parent, L.; Sauvé, R. Aromatic–aromatic interactions between residues in KCh3.1 pore helix and SS transmembrane segment control the channel gating process. J. Gen. Physiol. 2014, 143, 289–307.
(31) Sato, H.; Feix, J. B. Peptide–membrane interactions and mechanisms of membrane destruction by amphipathic α–helical antimicrobial peptides. Biochim.Biophys.Acta, Biomembr. 2006, 1758, 1245–1256.
(32) Jelokhani-Niaraki, M.; Nakashima, K.; Kodama, H.; Kondo, M. Interaction and orientation of an α–aminosobutyric acid– and tryptophan–containing short helical peptide pore–former in phospholipid vesicles, as revealed by fluorescence spectroscopy. J. Biochem. 1998, 123, 790–797.

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS
We are grateful to Analytical Research Center for Experimental Sciences, Saga University for the use of MALDI–TOF MS. This work was also partly supported by the Nanotechnology Platform Program (Molecule and Material Synthesis) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

■ REFERENCES
(1) Unwin, N. The structure of ion channels in membranes of excitable cells. Neuron 1989, 3, 665–676.
(2) Spach, G.; Ducloher, H.; Molle, G.; Valleton, J.-M. Structure and supramolecular architecture of membranes channel forming peptides. Biochimie 1989, 71, 11–21.
(3) Lear, J. D.; Wasserman, Z. R.; DeGrado, W. F. Synthetic amphiphilic peptide models for protein ion channels. Science 1988, 240, 1177–1181.
(4) Woolley, G. A.; Wallace, B. A. Model ion channels: Gramicidin and alamethicin. J. Membr. Biol. 1992, 129, 109–136.
(5) Isawa, T.; Lee, S.; Oishi, O.; Aoyagi, H.; Ohno, M.; Anzai, K.; Kirino, Y.; Sugihara, G. Design and synthesis of amphipathic 3(10) helical peptides and their interactions with phospholipid bilayers and ion channel formation. J. Biol. Chem. 1994, 269, 4928–4933.
(6) Sansom, M. S. P. Alamethicin and related peptibols–model ion channels. Eur. Biophys. J. 1993, 22, 105–124.
(7) Payne, J. W.; Jakes, R.; Hartley, B. S. The primary structure of alamethicin. Biochem. J. 1970, 117, 757–767.
(8) Fox, R. O., Jr; Richards, F. M. A voltage–gated ion channel model inferred from the crystal structure of alamethicin at 1.5–A resolution. Nature 1982, 300, 325–330.
(9) Nieva, J. L.; Madan, V.; Carrasco, L. Viroporins: Structure and biological functions. Nat. Rev. Microbiol. 2012, 10, 563–574.
(10) Gonzalez, M. E.; Carrasco, L. Viroporins. FEBS Lett. 2003, 552, 28–34.
(11) Oki, S.; Danho, W.; Montal, M. Channel protein engineering: synthetic 22–mer peptide from the primary structure of the voltage–sensitive sodium channel forms ion channels in lipid bilayers. Proc. Natl. Acad. Sci. U.S.A. 1988, 85, 2393–2397.
(12) Oki, S.; Danho, W.; Madison, V.; Montal, M. M2 δ, a candidate for the structure lining the ionic channel of the nicotinic cholinergic receptor. Proc. Natl. Acad. Sci. U.S.A. 1988, 85, 8703–8707.
(13) Agawa, Y.; Lee, S.; Ono, S.; Aoyagi, H.; Ohno, M.; Taniguchi, T.; Anzai, K.; Kirino, Y. Interaction with phospholipid bilayers, ion channel formation, and antimicrobial activity of basic amphipathic α–
(33) Situ, A. J.; Kang, S.-M.; Frey, B. B.; An, W.; Kim, C.; Ullner, T. S. Membrane anchoring of α-helical proteins: Role of tryptophan. J. Phys. Chem. B 2018, 122, 1185−1194.
(34) Yau, W.-M.; Wimley, W. C.; Gawrisch, K.; White, S. H. The Preference of tryptophan for membrane interfaces. Biochemistry 1998, 37, 14713−14718.
(35) Whitmore, L.; Wallace, B. A. Analysis of peptibol sequence composition: implications for in vivo synthesis and channel formation. Eur. Biophys. J. 2004, 33, 233−237.
(36) Hibgashimoto, Y.; Kodama, H.; Jelokhani-Niaraki, M.; Kato, F.; Kondo, M. Structure−function relationship of model Aib−containing peptides as ion transfer intermembrane templates. J. Biochem. 1999, 125, 705−712.
(37) Hara, T.; Kodama, H.; Hibgashimoto, Y.; Yamaguchi, H.; Jelokhani-Niaraki, M.; Ehara, T.; Kondo, M. Side chain effect on ion channel characters of Aib rich peptides. J. Biochem. 2001, 130, 749−755.
(38) Formaggio, F.; Crisma, M.; Rossi, P.; Scrimin, P.; Kaptein, B.; Broxterman, Q. B.; Kamphuis, J.; Toniolo, C. The first water-soluble 3,3′,3″-trihelic peptides. Chem.—Eur. J. 2000, 6, 4498−4504.
(39) Manning, M. C.; Woody, R. W. Theoretical CD studies of polypeptide helices: examination of important electronic and geometric factors. Biopolymers 1991, 31, 569−586.
(40) Grishina, I. B.; Woody, R. W. Contributions of tryptophan side chain to the circular dichroism of globular proteins: exciton couplets and coupled oscillators. Faraday Discuss. 1994, 99, 245−262.
(41) Ladokhin, A. S.; Selsted, M. E.; White, S. H. CD spectra of indolcinium antimicrobial peptides suggest an amphiphilic helix. Biochemistry 1999, 38, 12313−12319.
(42) Zhou, N. E.; Kay, C. M.; Hodges, R. S. Synthetic model protein. Positional effects of interchain hydrophobic interactions on stability of two-stranded α-helical coiled−coles. J. Biol. Chem. 1992, 267, 2664−2670.
(43) Lau, S. Y. M.; Taneya, A. K.; Hodges, R. S. Synthesis of a model protein of defined secondary and quaternary structure. Effect of chain length on the stabilization and formation of two-stranded α-helical coiled−coles. J. Biol. Chem. 1984, 259, 13253−13261.
(44) Rath, A.; Tulumello, D. V.; Deber, C. M. Peptide models of membrane protein folding. Biochemistry 2009, 48, 3036−3045.
(45) Creamer, T. P.; Rose, G. D. α-Helix−forming propensities in peptides and proteins. Proteins 1994, 19, 85−97.
(46) DeGrado, W.; Wasserman, Z.; Lear, J. Protein design, a minimalist approach. Science 1989, 243, 622−628.
(47) Woolley, G. A.; Wallace, B. A. Temperature dependence of the interaction of alamethicin helices in membranes. Biochemistry 1993, 32, 9819−9825.
(48) Zhou, N. E.; Kay, C. M.; Hodges, R. S. Synthetic model proteins. Positional effects of interchain hydrophobic interactions on stability of two-stranded α-helical coiled−coles. J. Biol. Chem. 1992, 267, 2664−2670.
(49) Iau, S. Y. M.; Taneya, A. K.; Hodges, R. S. Synthesis of a model protein of defined secondary and quaternary structure. Effect of chain length on the stabilization and formation of two-stranded α-helical coiled−coles. J. Biol. Chem. 1984, 259, 13253−13261.
(50) Creamer, T. P.; Rose, G. D. α-Helix−forming propensities in peptides and proteins. Proteins 1994, 19, 85−97.
(51) DeGrado, W.; Wasserman, Z.; Lear, J. Protein design, a minimalist approach. Science 1989, 243, 622−628.
(52) Woolley, G. A.; Wallace, B. A. Temperature dependence of the interaction of alamethicin helices in membranes. Biochemistry 1993, 32, 9819−9825.
(53) Zhou, N. E.; Kay, C. M.; Hodges, R. S. Synthetic model proteins. Positional effects of interchain hydrophobic interactions on stability of two-stranded α-helical coiled−coles. J. Biol. Chem. 1992, 267, 2664−2670.
(54) Williams, J. K.; Zhang, Y.; Schmidt-Rohr, K.; Hong, M. pH-dependent conformation, dynamics, and aromatic interaction of the gating tryptophan residue of the influenza M2 proton channel from solid−state NMR. Biophys. J. 2013, 104, 1698−1708.
(55) Peter, B.; Polansky, A. A.; Fanucchi, S.; Dirr, H. W. A. Lys−Trp cation−π interaction mediates the dimerization and function of the chloride intracellular channel protein 1 transmembrane domain. Biochemistry 2014, 53, 57−67.
(56) Noshiro, D.; Asami, K.; Futaki, S. Metal−assisted channel stabilization: Disposition of a single histidine on the N−terminus of lamichetin yields channels with extraordinarily long lifetimes. Biophys. J. 2010, 98, 1801−1808.
(57) Sanches, A.; Trappier, S. G.; Mahy, B. W.; Peters, C. J.; Nichol, S. T. The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. Proc. Natl. Acad. Sci. U.S.A. 1996, 93, 3602−3607.
(58) Volchkov, V. A.; Dolnik, O.; Martinez, M. J.; Reynard, O.; Volchkov, V. E. RNA editing of the GP gene of Ebola virus is an important pathogenicity factor. J. Infect. Dis. 2015, 212, S226−S233.
(59) He, J.; Melnik, L. I.; Komin, A.; Wiedman, G.; Fuseler, T.; Morris, C. F.; Starr, C. G.; Searson, P. C.; Gallaher, W. R.; Hristova, K.; Garry, R. F.; Wimley, W. C. Ebola virus delta peptide is a viroporin. J. Virol. 2017, 91, e00438−e00417.
(60) Gallaher, W.; Garry, R. Modeling of the Ebola virus delta peptide reveals a potential lytic sequence motif. Viruses 2015, 7, 305−305.
(61) Macdonald, R. C.; Macdonald, R. I.; Menco, B. P. M.; Takeshita, K.; Subbarao, N. K.; Hu, L.-r. Small−volume extrusion apparatus for preparation of large, unilamellar vesicles. Biochim.Biophys. Acta 1973, 1061, 297−303.
(62) Chen, Y.-H.; Yang, J. T.; Chau, K. H. Determination of the helix and β form of proteins in aqueous solution by circular dichroism. Biochemistry 1974, 13, 3350−3359.
(63) Gans, P. J.; Lyy, P. C.; Manning, M. C.; Woody, R. W.; Kallenbach, N. R. The helix−coil transition in heterogeneous peptides with specific side−chain interactions: Theory and comparison with CD spectral data. Biopolymers 1991, 31, 1605−1614.
(64) MacDonald, R. C.; Macdonald, R. I.; Menco, B. P. M.; Takeshita, K.; Subbarao, N. K.; Hu, L.-r. Small−volume extrusion apparatus for preparation of large, unilamellar vesicles. Biochim.Biophys. Acta 1991, 1061, 297−303.
(65) Coronado, R.; Latorre, R. Phospholipid bilayers made from monolayers on patch−clamp pipettes. Biophys. J. 1983, 43, 231−236.
(66) Robinson, R. A.; Stokes, R. H.Electrolyte solutions, 2nd ed.; Butterworths: London, U.K., 1970.

NOTE ADDED AFTER ISSUE PUBLICATION

Equations 1−4 were missing in the original publication on December 29, 2020 and were added on February 2, 2021.