Studies on the Ion Channel Properties of Transmembrane Peptide Assemblies Associated with Electrostatic Interactions

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ABSTRACT

Many structure-function relationship studies have been accumulated on helical ion channel peptides. An ultimate goal of ion channel peptide design is to construct stable and highly functionalized ion conducting pores. It is expected that specific inter-helical interactions would facilitate association of helices in phospholipid membranes and successive helix bundle formation. Several efforts have been directed for the reinforcement of the specific helix–helix associations to enhance channel formation, however, no reports examined the association of amphiphilic helices by its hydrophilic surfaces.

In the present study, we rationally designed helix bundle ion channels using the synthetic hybrid peptide K20E20, a disulfide dimer of cationic- and anionic-amphiphilic helices, Ac-CGG-(BKBA)$_5$-NH$_2$ and Ac-CGG-(BEBA)$_5$-NH$_2$. CD measurements in aqueous media implied helix stabilization in the peptide caused by the inter-helical electrostatic interactions. In addition, CD spectra recorded in the presence of DPPC liposomes and dye-leakage measurements suggested a strong association of peptide monomers in phospholipid membranes, as well as high affinities between peptide and lipid bilayers. These features allowed ion channel forming at extremely low peptide concentrations. According to electrophysiological analyses, stable helix bundles were constructed of six helices by association of three K20E20 molecules. Helix–helix association, peptide–membrane interactions, and ion channel formation of K20E20 peptides were all facilitated by intra-molecular electrostatic interactions between the hybrid peptide’s helices. Moreover, the helix–helix interactions were pH-dependently, conductance through K20E20 ion channels decreased under acidic conditions due to interruption of the salt bridges.
CHAPTER I

General Introduction

I–1. Ion Channels: Proteins and Peptides

Membrane embedded proteins are known as fundamental components of the biological membranes, they play central roles in cellular signaling, membrane transportation, cell adhesion and etc. (1,2). Ion channel proteins, classified as one of the membrane integral proteins, not only correlate with ionic homeostasis of cells but also provide electric signals in nerve systems. Structural principle of ion channel is pore (~nm) which allows ion permeation through the membranes. Excitation and electrical signaling in the nervous system involve the movement of ions through ion channels. Each channel may be regarded as an excitable molecule, as it is specifically responsive to some stimulus: a membrane potential change, a neurotransmitter or other chemical stimulus, a mechanical deformation, and so on. The channel’s response, called “gating”, is apparently a simple opening or closing of the pores. The open pore has the important property of “selective permeability”, allowing some restricted class of ions to flow passively down their electrochemical activity gradients at a rate that is very high (> $10^6$ ions per second) when considered from molecular viewpoint.
On the other hands, numerous peptides, which potent to form ion conducting pore in phospholipid membranes, have been also investigated as “ion channel peptides” (3). They are found either as natural compounds, mainly produced by microorganism, or as synthetic analogues and model molecules. These peptides have unique structures such as α-helix, β-helix, 3_{10}-helix, and can be either linear or cyclic (4–10). These peptides gathering together to induce channels in lipid bilayers may be classified in several categories according to the spatial structures involved. For example, gramicidin, isolated from Bacillus brevis, is a linear pentadecapeptide composed of alternating D- and L-amino acids (6,7). This peptide takes β-helix structures and forms monovalent cation specific channels in phospholipid membranes by head–to–head dimerization. It was also reported that similar ion channels were formed by nano-tubes of stacked artificial cyclic peptides (9,10).

I–2. Ion Channel Peptides with Helical Structures

Ion channel peptides possess diverse structure although a large number of structure-activity relationship studies have been dedicated to the naturally occurring, or synthetic α-helical ion channel peptides. The widely accepted ion conducting mechanism for the α-helical peptides is known as the “barrel-stave” model (11), which involves an assembly of α-helical monomers to form a bundle with a central ion conducting pathway (Figure 1–1). Similar helix bundle motifs are also found in the pore regions of membrane integral ion channel proteins, therefore, the bundles of the α-helical ion channel peptides have served as prototypes for ion channel proteins due to their reduced structural complexity compared to proteins (12).
Naturally Occurring Helical Ion Channel Peptides—Many organism, including fungi, insects, amphibians, and even mammals, produce hydrophobic or amphiphatic peptides which act as ion channels in lipid membranes. These bilayer openings (permeabilization) derive the affected organisms of their structurally and functionally asymmetric character of biological membranes, results in exhibiting antibiotic, fungicidal, hemolytic, virucidal, tumoricidal activities (13). These naturally occurring helical ion channel peptides are classified two families on the basis of the difference of their structure; peptaibols and non-peptaibols as shown in Table 1–1.

Peptaibols are membrane-active polypeptides isolated from fungi sources. They are characterized by the presence of an unusual amino acid, 2-aminoisobutyric acid (Aib, B), and the C-terminal α-amino alcohol (3,14). The hydrophobic characters are also common structural motif of this family. Nowadays, over 300 peptaibol sequences have been reported. Alamethicin, isolated from the fungi Trichoderma viride is one of the most investigated peptaibol, intensive studies on its ion channel properties have been concentrated (3,4,6,15).
There exist a range of channel forming peptides, isolated from toxins and venoms, which are unrelated to peptaibols. In general, these non-peptaibol ion channel peptides are less hydrophobic than the peptaibols and more amphipathic. The 26-residue peptide melittin, isolated from bee venom, has been the subject of intensive investigations (3). In addition to its ion channel forming properties, it has membrane lytic and fusogenic effects. The magainins (magainin I and II) are two closely related peptides originally isolated from *Xenopus laevis*. The “toroidal” model has been proposed as ion channel forming mechanism of those peptides (16).

**Table 1-1. Examples of Naturally Occurring Helical Ion Channel Peptides**

<table>
<thead>
<tr>
<th>peptides</th>
<th>sequence(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>peptaibols</strong></td>
<td></td>
</tr>
<tr>
<td>Alamethicin F-50</td>
<td>Ac-BPBAQBVKBVBBQQFol</td>
</tr>
<tr>
<td>Chrysospermin A</td>
<td>Ac-FBSBQLQGBBAABBBQWol</td>
</tr>
<tr>
<td>Hypelcin A</td>
<td>Ac-BPABQVLBBBPBBQQQol</td>
</tr>
<tr>
<td>Paracelsin A</td>
<td>Ac-BABABAQQBVBBBPBBQQFol</td>
</tr>
<tr>
<td>Stilboflavin A 1</td>
<td>Ac-BPBAQBBVBBBPBBQQWol</td>
</tr>
<tr>
<td>Suzukacinol</td>
<td>Ac-BABABAQQBVBBBPBBQQFol</td>
</tr>
<tr>
<td>Tricholongin BI</td>
<td>Ac-BGFBQQBBSSLBPBBQQQol</td>
</tr>
<tr>
<td>Trichorziane TA IIIc</td>
<td>Ac-BAABQBBSSLBPBBIQQQol</td>
</tr>
<tr>
<td>Trichorzin HA I</td>
<td>Ac-BGABBQBBVLBBQQQol</td>
</tr>
<tr>
<td><strong>non-peptaibol</strong></td>
<td></td>
</tr>
<tr>
<td>Melittin</td>
<td>for-GIGAVLKVLTTGLPALISWIKKRQQ-NH(_2)</td>
</tr>
<tr>
<td>δ-Toxin</td>
<td>for-MAQDIISTIGDLVKWIIDTVNKFTKK</td>
</tr>
<tr>
<td>Magainin I</td>
<td>GIGKFLHASAKFGKAFVGEIKMS</td>
</tr>
<tr>
<td>Magainin II</td>
<td>GIGKFLHASAKFGKAFVGEIMNS</td>
</tr>
<tr>
<td>Bombolitin III</td>
<td>IKIMDIALKLKVLAHV-NH(_2)</td>
</tr>
<tr>
<td>Mastoparan</td>
<td>INLKAALAKKIL-NH(_2)</td>
</tr>
<tr>
<td>Cecropin A</td>
<td>KWKLFKKIEKQGQRVDAISAGPAVAVQATQIAK-NH(_2)</td>
</tr>
<tr>
<td>Cecropin AD</td>
<td>KWKLFKKIEKQGQRVDAISAGPAVAVQATQIALAK-NH(_2)</td>
</tr>
<tr>
<td>Pardaxin</td>
<td>GFFALIPKISSLFFKTLLSAGSALSSSGQGE</td>
</tr>
</tbody>
</table>

a) The sequences are shown using the standard single letter code for amino acids. The following abbreviations are used for terminal blocking groups: Ac=acetyl; for=formyl; ol=C-terminal amino alcohol; NH\(_2\)=C terminal amide.
Synthetic Helical Ion Channel Peptides—Various synthetic ion channel peptides have been studied to obtain the knowledge upon essential structural principles of the ion channel formation. Their sequences have been either designed de novo or have been derived from the sequence of ion channel proteins (Table 1–2). Montal and co-workers have synthesized transmembrane segments of ion channel proteins and performed reconstruction of ion conduct pores on artificial phospholipid bilayers (17–21). However, in comparison to more complex sequences of natural ion channel peptides or transmembrane segments derived from natural ion channel proteins, de novo designed helical ion channel peptides provide a simple and straightforward model for studying on the relationships between the peptide structures and ion channel properties. Menestrina et al. and Otoda et al. synthesized hydrophobic peptides Boc-(Ala-Aib-Ala-Aib-Ala)$_n$-OMe ($n = 1 – 4$) and Boc-(Ala-Aib)$_8$-OMe, respectively (22,23). Although these ion channel peptides are classified as model peptides with hydrophobic character, a great majority of the synthetic $\alpha$-helical ion channel peptides are amphiphilic with a distribution of hydrophilic and hydrophobic surfaces on opposite sides of the helix (5,24,25). For example, Lear et al. reported that ion channel forming of 21-residue amphiphilic Leu-Ser-Ser-Leu-Leu-Ser-Leu repeat motif (5). The channels are modestly cation selective and exhibit asymmetric current–voltage curves which reflect voltage–dependent conductances. Lee et al. described ion channel forming of amphiphilic peptides, (Leu-Ala-Arg-Leu)$_n$-(Leu-Arg-Ala-Leu)$_n$ (4$_n$), (Leu-Ala-Glu-Leu)$_n$, and ( Ala-Arg-Leu)$_n$ (8,25,26). Higashimoto et al. designed Aib rich peptide sequence, Ac-(Aib-Xxx-Aib-Ala)$_n$-NH$_2$ (BXBA20), as template of amphiphilic helix (27). Polar or nonpolar amino acids were incorporated into the Xxx position of the sequence and utilized for various SAR studies of ion channel forming (28,29).
Table 1–2. Examples of Synthetic Helical Ion Channel Peptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synthetic Transmembrane Segments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pep-8</td>
<td>GFLLMITLILFSQFPMILR-NH$_2$</td>
<td>(17)</td>
</tr>
<tr>
<td>S3</td>
<td>DPWNWLDFTVIFAYVTEFVDL</td>
<td>(18)</td>
</tr>
<tr>
<td>S4</td>
<td>RVIRLARIARVLRLIRAAGIR</td>
<td>(19)</td>
</tr>
<tr>
<td>M2δ</td>
<td>EKMSTASVLLAQAVFLLLTQR</td>
<td>(20)</td>
</tr>
<tr>
<td>M2GlyR</td>
<td>PARVGLGITTVLTMTTQSSGSRA</td>
<td>(21)</td>
</tr>
<tr>
<td><strong>De novo Designed Model Peptides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P5 – P20</td>
<td>Boc-(ABABA)$_n$-OMe ($n = 1 – 4$)</td>
<td>(22)</td>
</tr>
<tr>
<td>AB16</td>
<td>Boc-(Ala-Aib)$_8$-OMe</td>
<td>(23)</td>
</tr>
<tr>
<td>Pep-P</td>
<td>LLLALLQLLFGALLLLE</td>
<td>(30)</td>
</tr>
<tr>
<td>LS-I</td>
<td>(LSLLSL)$_3$-NH$_2$</td>
<td>(5,24)</td>
</tr>
<tr>
<td>LS-II</td>
<td>(LSLLSL)$_2$-NH$_2$</td>
<td>(5)</td>
</tr>
<tr>
<td>LS-IIb</td>
<td>(LSBLSL)$_3$-NH$_2$</td>
<td>(31)</td>
</tr>
<tr>
<td>4$_3$</td>
<td>Ac-(LARL)$_3$-NHMe</td>
<td>(25)</td>
</tr>
<tr>
<td>4$_6$</td>
<td>(LARL)$_5$-(LRAL)$_3$</td>
<td>(25)</td>
</tr>
<tr>
<td>ARL$_8$</td>
<td>(Ala-Arg-Leu)$_8$</td>
<td>(8)</td>
</tr>
<tr>
<td>VRL$_8$</td>
<td>(Val-Arg-Leu)$_8$</td>
<td>(8)</td>
</tr>
<tr>
<td>LRL$_8$</td>
<td>(Leu-Arg-Leu)$_8$</td>
<td>(8)</td>
</tr>
<tr>
<td>LAEL</td>
<td>(Leu-Ala-Glu-Leu)$_3$</td>
<td>(26)</td>
</tr>
<tr>
<td>BXBA20</td>
<td>Ac-(BXBA)$_5$-NH$_2$ ($X = K, E, G, S$)</td>
<td>(27–29)</td>
</tr>
</tbody>
</table>

I–3. Appropriate Helix–Helix Interactions in Phospholipid Bilayers

Ion channel forming by helix peptides involves association of helices in phospholipid bilayer. Thus, understanding of helix packing and the interactions between helices in the membranes is therefore key to clarify the channel forming. Strong and appropriate helix–helix interactions occurred in phospholipid bilayer would be required for advanced ion channel peptide designs. The coiled-coil model peptides, developed by Hodges and co-workers (32–36), have widely used to investigate helix–helix interactions and association. Several noncovalental interactions involved
in the helix association have been evaluated in aqueous environments (e.g. hydrogen bonds, ionic interactions, van der Waals interactions, hydrophobic interactions, helix dipole, and so on). Among them, hydrophobic interactions between nonpolar residues are thought to be the major driving force in the peptide association in aqueous environments (34,35). However, in the situation of the bundle forming of amphiphilic helices, the hydrophobic interaction would be occurred between the hydrophobic surfaces of helices and the hydrophobic core of phospholipid bilayers. Therefore, enhancement of helix–helix interactions via hydrophobic surfaces is not appropriate for peptide association in hydrophobic environments and structural principles of the coiled-coil model peptides could not be appropriate for association of ion channel helices in phospholipid membranes.

Interestingly, despite their rare presence in transmembrane helices, strongly polar residues are highly conserved, especially in multi-spanning transmembrane proteins, suggesting molecular interactions that either functionally or structurally favor these residues (37,38). Inter-helical polar interactions have been observed in some integral membrane protein structures available at high resolution (39). In addition, genetic and biophysical studies of transmembrane proteins have identified critical polar residues that may participate in electrostatic interactions (40,41). Recently, interactions between the side chains of polar residues have been evaluated through various model systems and have been utilized for promotion of helix association in lipid bilayers or detergent micelles (e.g. hydrogen bond (42–47), and cation–π interactions (48)). These interactions have been incorporated into coiled-coil peptides (42,43), protein transmembrane segments (44), as well as polyleucine (45,46) and helix–loop–helix peptide analogs (47). Consequently, in recent years, substantial
information has been accumulated on the appropriate arrangements of amino acids to promote helical peptides associations.

**I-4. Concept and Aims of This Study**

*De novo* synthesis of model ion channels composed of $\alpha$-helical peptides has provided groundwork for design of novel synthetic molecules with desired ion channel properties (24,49). In this approach, construction of stable ion conducting pores at low peptide concentrations has been considered as one of the primary tasks in developing synthetic ion channel scaffolds. In addition, controlling the size of the helical bundle construct is also required for favorable modifications of the channel functions such as a regulation of ion conductance and ion selectivity. To address these issues, it is necessary to facilitate channel forming efficiency, pore stability, and to clarify its pore structures. These factors should correspond with peptide concentration, ion channel lifetime, and ion conductance patterns, respectively, in the channel activity evaluations.

On the basis of “barrel-stave” model (11), the helix bundle formation is involved binding of helices to lipid membranes, their successive insertion and formation of higher order structures by contacts between helices. The helix bundle would need to contain more than two monomeric helices if they were to provide an ion conducting pathway, and two-stranded coils might occur as intermediates in the bundle assembly process. DeGrado et al. reported on ion channels formed from a 21-meric amphiphilic $\alpha$-helix peptide composed of Ser and Leu residues and noted that the peptide could form two-stranded or higher ordered coiled-coil like structures (5,24). Chung et al. reported that dimeric form of the peptide is a major component of the peptide-lipid membrane equilibrium (50). Woolley and Wallace also suggested the spontaneous dimerization of alamethicin in lipid bilayers using CD techniques (51). Hence, it is expected that
stabilizing the two-stranded helical peptides would directly promote the formation of helix bundle assemblies. Moreover, appropriate tertiary structure contact of helices should facilitate the bundle formation with desired structures and physicochemical properties. Covalently oligomerization of helical ion channel peptides is one of the practical approaches to enhance the helix–helix interactions (52,53). The techniques should facilitate native helix–helix associations by favored long-range peptide–peptide interactions. In fact, some oligomeric helices further stabilized their secondary structures and favorable pore formation and stabilization were reported. However, the technique did not reinforce native driving forces of helix–helix interaction. It is expected that to reinforce the helix–helix interactions in oligomeric ion channel helices would further facilitate pore forming.

In the present study, two-stranded association of amphiphilic helices via hydrophilic surfaces was performed to facilitate helix–helix interactions in the phospholipid bilayer, and successive forming of helix bundle with ion channel activities. The hetero-dimeric peptides of cationic and anionic amphiphilic helices were designed to address the investigation. When ion pairs are formed between hydrophilic surfaces of the cationic and the anionic helices, enhancement of helix association and subsequent channel formation in the phospholipid bilayers are expected without the interference of the peptide–bilayer hydrophobic interactions.

Alternative purpose of this study is to control the channel functions with the electrostatic interaction. One of the goals in the design of model synthetic peptide ion channel constructs is to transmit signals from external stimuli as ionic currents across the membrane through peptide ion channels. Futaki et al. utilized specific molecular recognition of the host–guest chemistry (e.g. protein metal binding domain versus metal ion (54), biotin–avidin interaction (55)) for ion channel activation. These approaches
are classified as ligand-gating models of ion channels. In the case of present study, since ion channel formation has been facilitated through inter-helical salt bridge formation, the associated helix bundle stability would be influenced by pH conditions. Influence of pH on conformation and ion channel properties of chimeric helices are therefore studied to examine the effect of disruption of the intra-molecular electrostatic interactions on ion transport activities.
CHAPTER II

Design and Structural Properties of Dimeric Model Peptides

II–1. Introduction

Numerous artificial helical ion channel peptides, especially possessing amphiphilic characters, have been designed and synthesized for the studies upon relationships between structures and ion channel properties of helix peptides (see Chapter I–1). BXBA20 series peptides, 20-residual amphiphilic helices, have been developed as de novo designed ion channel molecules since a decade ago. Its structural and ion channel properties were evaluated in previous researches (27–29). One of the present issues in the ion channel design is to construct specific and stable pore structures with high efficiency. “Barrel-stave model”, most widely accepted ion conduct mechanism of helical peptides, involves construction of helix bundle assemblies (11). According to the model, helix bundles are constructed via two major processes composed of peptide–bilayer interaction and association of helices in phospholipid membranes. Thus, it is expected that the appropriate helix–bilayer, as well as helix–helix interactions in phospholipid bilayer would enable (i) high efficiency of ion channel forming, (ii) construction of rigid ion conducting pores, and (iii) assembling of pore with specific structures. The two-stranded coils of ion channel
helices have been presumed as an intermediate in the helix bundle assembling processes (50,51). Therefore, it is expected that to facilitate the forming of two-stranded helices in phospholipid bilayer could be an appropriate approach to address the issues and association of amphiphilic helices via hydrophilic surfaces have a potential to be an advanced method for artificial ion channel designs.

In the present study, ion pairs were incorporated into synthetic dimers of amphiphilic helical ion channel peptides to enhance helix–helix interactions and subsequent helix bundle formation. This chapter describes structural properties of the dimeric model peptides designed based on the BXBA20. The intra- and inter-molecular helix–helix interactions of the dimeric model peptides, as well as interactions between peptides and phospholipid bilayer, including membrane perturbation activities, were also described in this chapter for understanding of the electrostatically effects. In addition, availability of the electrostatic inter-helical interactions were concerned using the chimeric dimer of modified alamethicin helices.
II–2. Results

Peptide Design

It have been reported on syntheses, structural and ion channel properties of the amphiphilic helical peptide series, Ac-(Aib-Xxx-Aib-Ala)₂-NH₂ (BXBA20) (27,28). The N- and C-terminals of this sequence were acetylated and amidated, respectively, to cancel termini charges. The unusual amino acid, Aib residues in the peptide sequence are potent inducers of helical structures (56). Higashimoto et al. concluded that at least 20 amino acid residues are required for the α-helical peptide to span lipid bilayers (27), and helix forming propensities and ion channel properties of BKBA20 and BEBA20, containing cationic Lys and anionic Glu residues at the Xxx positions, respectively are also reported (28). On the basis of these two monomeric parent peptides, two homo- and two hetero-dimeric model peptides were designed (Figure 2–1). Helical peptides were cross-linked via disulfide bond at termini Cys side chains and two Gly residues were inserted between the individual helices and Cys residues as spacers (57). K20E20 and anti-K20E20 have been designed as hetero-dimeric model peptides. K20E20 and anti-K20E20 are cross-linked analogs with parallel and anti-parallel orientations of helices, respectively. The anti-K20E20 was synthesized to estimate the effect of helix orientation for ion channel activities. K20K20 and E20E20, the respective homo-dimeric peptides of BKBA20 and BEBA20, were used as reference peptides to estimate the effects of electrostatic interactions. The chimeric dimer of cationic and anionic modified alamethicin was designed to test the applicability and availability of the electrostatic interaction. Details of the peptide design are described following section in this chapter.
FIGURE 2–1: Primary structures of model peptides. K20K20 and E20E20 are homo-dimers of BKBA20 and BEBA20, respectively. K20E20 and anti-K20E20 are hetero-dimeric model peptides; the two monomeric helices, BKBA20 and BEBA20, are cross-linked in parallel or anti-parallel orientations.

**Structures of Model Peptides and Inter-Helical Interaction**

Conformations and molecular interactions of model peptides in aqueous solution were evaluated by CD spectroscopy in 50 mM phosphate buffer (pH 7.4). CD spectra of all peptides, with exception of K20E20 at higher concentrations, showed typical α-helical conformations with characteristic double minima around 207 nm and 224 nm, and a maximum around 195 nm. K20E20 also showed typical α-helix structures under lower peptide concentrations, although spectra gradually changed to the patterns with single minima around 231 nm and single maxima around 197 nm as
increase of peptide concentration (Figure 2–2a). It seems that the spectral change reflected inter-molecular association of K20E20 since the changes were concentration-dependent. The anti-K20E20 did not show this spectral change, typical patterns of α-helix were conserved at all examined peptide concentrations (Figure 2–2b). Thereby, it seems that the concentration-dependent changes in CD spectra of these two peptides were influenced by the helix orientation. Negative band around 224 nm derived from n–π* transition ([θ]_n–π*) correlates to the helical content of overall secondary structure (58).

FIGURE 2–2: Concentration dependence of CD spectra of hetero-dimeric peptides. The (a) and (b) represent spectra of K20E20 and anti-K20E20, respectively. All measurements were carried out in 50 mM phosphate buffer at neutral pH conditions (pH 7.4). The α-helical patterns of K20E20 spectra are gradually transformed with the incremental increase in the peptide concentration.
FIGURE 2–3: Concentration dependence of $[\theta]_{n-\pi^*}$ of model peptides. Symbols represent K20E20 (open circle), anti-K20E20 (closed circle), K20K20 (open triangle), BKBA20 (closed triangle), E20E20 (open square), and BEBA20 (closed square). $[\theta]_{n-\pi^*}$ in K20E20 decreases at net helix concentrations above 20 µM.

Figure 2–3 shows the relationship of concentration and helical content (ellipticity at $[\theta]_{n-\pi^*}$) of model peptides. With exception of K20E20, the helical contents of all model peptides did not show significant concentration dependence. According to the concentration dependent CD measurements, the inter-molecular association of K20E20 was negligible at concentrations lower than 10 µM (net helix concentration of 20 µM). Subsequent CD measurements were carried out at this
concentration to avoid the inter-molecular association of K20E20. In the absence of the inter-molecular interactions leading to peptide association, at the 20 μM net helix concentration, helix contents of K20E20, anti-K20E20, K20K20, E20E20, BKBA20 and BEBA20 were 64, 64, 52, 29, 44, and 24%, respectively (Table 2–1). Helix contents of cationic peptides are larger than those of anionic peptides and homo-dimeric peptides showed larger helix contents than their respective monomers. Hetero-dimeric model peptides exhibited highest helix contents among model peptides. These results suggested that the helical conformations in hetero-dimeric peptides were stabilized by intra-molecular interactions. Figure 2–4 shows the pH titration CD spectra of K20E20 at 100 μM peptide concentration. pK_α of ε-amino groups of Lys is 10.5 and pK_α of γ-carboxyl groups of Glu is 4.1, respectively. The CD spectrum of associated K20E20 (at 100 μM) was converted to a typical α-helix spectrum at low pH (uncharged Glu; pH \( \leq 4.0 \)) or high pH (uncharged Lys; pH \( \geq 11 \)) conditions. These results suggest that the inter-helical electrostatic interaction between side chains strongly affects the inter-molecular association of K20E20 at high peptide concentrations.

According to the results of concentration dependent CD measurements and pH titration CD measurement, K20E20 showed electrostatic dimer–dimer interaction. At low peptide concentrations, the electrostatic dimer–dimer interaction of K20E20 is possibly limited to intra-molecular ion pair formation. In order to assess helix–helix interactions between cationic and anionic monomeric peptides, mixing ratios of BKBA20 and BEBA20 were gradually changed and CD spectra were measured to monitor the conformational change (Figure 2–5a). The spectral conversion was constantly against the mixing ratio of monomeric peptides. The relationship of the \([\theta]_{\text{in-π}}\) versus the monomeric peptide mixing ratios was linear and therefore no measurable inter-helical interactions between the monomeric peptides were detected
Mixing of K20K20 and E20E20 also did not show any increase in overall helical contents, implying the absence of inter-molecular interaction between the dimeric peptides. The \([\theta]_{n-\pi^*}\) ellipticities in the CD spectra of K20E20 and anti-K20E20 were decreased to comparable levels to those of monomeric peptides after cleavage of disulfide linkage with 1 mM DTT treatments. These results further confirmed that electrostatic interactions existed in hetero-dimeric peptides and covalent dimerization of helices was a requisite for this interaction.

**FIGURE 2–4:** pH-dependence of the K20E20 CD spectra. Peptide concentration is 100 \(\mu\text{M}\). (a) A typical \(\alpha\)-helical pattern is observed in Glu or Lys side chain charged or uncharged conditions. (b) pH dependence of \([\theta]_{n-\pi^*}\) (open circles) and \([\theta]_{\pi-\pi^*}\) (closed circles). Spectra show drastic change at pK\(_a\) of Lys (pK\(_a\) of \(\varepsilon\)-NH\(_2\) = 10.5) and Glu (pK\(_a\) of \(\gamma\)-COOH = 4.1) side chains.
FIGURE 2–5:  (a) CD spectra of various mixing ratio of BKBA20 and BEBA20.  (b) The mean residue ellipticities at \(n-\pi^*\) transitions ([\(\theta\)\(n-\pi^*\)]) of model peptides. The spectra were corrected in 50 mM phosphate buffers (pH 7.4). Net helix concentrations are 20 \(\mu\)M. Open circles represents [\(\theta\)\(n-\pi^*\)] at variable mixing ratios of BKBA20 and BEBA20. The transition of [\(\theta\)\(n-\pi^*\)] is linear: no interactions are observed between the monomeric helices. Mixing of K20K20 and E20E20 also does not show increase in [\(\theta\)\(n-\pi^*\)]. The helical contents and helix-helix interactions of hetero-dimeric peptides were decreased by reduction of disulfide linkage with 1 mM DTT treatments.

Peptide–Membrane Interactions and Peptide Structures in Membrane

In order to explore the structural changes of model peptides in phospholipid bilayers and to assess the membrane affinities of these peptides, CD spectra were measured in the presence of DPPC lipid vesicles (Figure 2–6). A red shift of the \(\pi-\pi^*\) transition band (the minimum around at 207 nm), and an increase in the
\([\theta]_{n-\pi^*}/[\theta]_{\pi-\pi^*}\) ratio reflect two-stranded helix association (34,59). It is worth mentioning that the \([\theta]_{n-\pi^*}/[\theta]_{\pi-\pi^*}\) ratios of the two hetero-dimeric peptides were significantly increased in the presence of DPPC SUVs. The \([\theta]_{n-\pi^*}/[\theta]_{\pi-\pi^*}\) ratios of K20E20 and anti-K20E20 in 50 mM phosphate buffers were both 1.15 and these ratios were increased to 1.56 and 1.84, respectively, in the presence of 4 mM DPPC SUVs. In addition, minimum ellipticity of the \(\pi-\pi^*\) transitions in hetero-dimer peptides shifted to 211 – 212 nm in presence of DPPC SUVs. Other peptides did not exhibit significant changes either in their ellipticity ratios and/or \(\pi-\pi^*\) minimum ellipticities. These results strongly suggested the two-stranded helix association of the hetero-dimeric peptides in phospholipid bilayers. Moreover, the spectral change did not depend on lipid concentrations. Indistinguishable spectra were obtained at all measured lipid concentrations, implying high membrane affinities of hetero-dimeric peptides for phospholipid bilayers (60). CD spectra of BKBA20 and K20K20 were increased in a lipid concentration dependent manner. Isodichroic points found around 205 nm in the spectra of these peptides indicated equilibrium between membrane-bound and free cationic peptides. Relative affinities and helix association propensities of cationic peptides were less than those of hetero-dimeric peptides. BEBA20 and E20E20 did not show any spectral change by addition of SUVs, thus no peptide–lipid interactions were detected.
FIGURE 2–6: Liposome titration CD measurements. Net helix concentrations are 20 µM. Dotted lines and solid lines represent spectra recorded in the absence or presence of DPPC SUVs, respectively. Concentrations of DPPC are 0.5 mM, 1 mM, 2 mM, 3 mM and 4 mM. Cationic peptides (left) exhibit successive spectral changes corresponding to the lipid concentration. Anionic peptides (center) do not show any spectral change in all conditions. Spectra of hetero-dimeric peptides (right) show significant increase of the $[\theta]_{\pi-\pi^*}/[\theta]_{\pi^*-\pi^*}$ ratios and red shifts of $\pi-\pi^*$ band in the presence of DPPC SUVs. The spectral changes of the hetero-dimeric peptides did not depend on the measured lipid concentrations.
Table 2–1: Circular Dichroism Data of Model Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>([\theta]_\text{n-\pi^*})</th>
<th>% Helix</th>
<th>([\theta]<em>\text{n-\pi^*}/[\theta]</em>\text{\pi-\pi^*})</th>
<th>(\Delta [\theta]<em>\text{n-\pi^*}/[\theta]</em>\text{\pi-\pi^*})</th>
<th>Wavelength at ([\theta]_\text{n-\pi^*})</th>
</tr>
</thead>
<tbody>
<tr>
<td>BKBA20</td>
<td>-15584</td>
<td>44</td>
<td>1.10</td>
<td>1.14</td>
<td>0.04</td>
</tr>
<tr>
<td>K20K20</td>
<td>-18192</td>
<td>52</td>
<td>1.16</td>
<td>1.21</td>
<td>0.05</td>
</tr>
<tr>
<td>BEBA20</td>
<td>-9583</td>
<td>24</td>
<td>1.01</td>
<td>1.01</td>
<td>0</td>
</tr>
<tr>
<td>E20E20</td>
<td>-11264</td>
<td>29</td>
<td>1.02</td>
<td>1.08</td>
<td>0.06</td>
</tr>
<tr>
<td>K20E20</td>
<td>-21677</td>
<td>64</td>
<td>1.15</td>
<td>1.56</td>
<td>0.41</td>
</tr>
<tr>
<td>anti-K20E20</td>
<td>-21615</td>
<td>64</td>
<td>1.15</td>
<td>1.84</td>
<td>0.69</td>
</tr>
</tbody>
</table>

a) \([\theta]_\text{n-\pi^*}\) were measured at room temperature in 50 mM phosphate buffer (pH 7.4).

b) Helix contents were estimated from \([\theta]_\text{n-\pi^*}\) using Chen’s equation (58).

**pH Effects for the K20E20 Structure**

It was expected that electrostatically facilitated helix–helix and helix–bilayer interactions degraded in the ion-pairs absent conditions. To investigate the validity of this assumption, liposome titration CD spectra of K20E20 were measured in acidic media (pH 3.0) to assess whether the neutralization of the negative charge of Glu side chains affected the helix association and the high membrane affinity of K20E20 (Figure 2–7). No significant difference in the \([\theta]_\text{n-\pi^*}/[\theta]_\text{\pi-\pi^*}\) ratio and the red shift of the \(\pi-\pi^*\) band was detected in the absence or presence of DPPC vesicles. However, spectra were dependent on the lipid concentration and an isodichroic point appeared at ~205 nm. These results indicated that the helix association propensities, as well as the membrane affinities of K20E20 were diminished by decrease and disruption in the electrostatic inter-helical interactions at low pH. Same experiments were carried out for monomeric peptides. Both BKBA20 and BEBA20 showed lipid concentration dependent spectral changes. It is notable that the membrane interaction of BEBA20 in neutral pH conditions was also altered under acidic conditions.
FIGURE 2–7: Liposome concentration dependent CD spectra recorded under acidic conditions. Measurements were carried out in 50 mM phosphate buffers (pH 3.0), in the presence and absence of DPPC SUVs. Net helix concentrations are 20 µM. Spectral changes of all model peptides are lipid concentration dependent. Isodichroic points are found around 205 nm in each spectrum. Significant differences of the $\theta_{n-\pi^*}/\theta_{\pi-\pi^*}$ ratios and $\pi-\pi^*$ band in the absence or presence of DPPC SUVs were not observed.

<table>
<thead>
<tr>
<th>peptide</th>
<th>buffer $\theta_{n-\pi^<em>}/\theta_{\pi-\pi^</em>}$</th>
<th>4 mM DPPC $\Delta \theta_{n-\pi^<em>}/\theta_{\pi-\pi^</em>}$</th>
<th>wavelength at $\theta_{n-\pi^*}$</th>
<th>buffer $\theta_{n-\pi^<em>}/\theta_{\pi-\pi^</em>}$</th>
<th>4 mM DPPC $\Delta \theta_{n-\pi^<em>}/\theta_{\pi-\pi^</em>}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>K20E20</td>
<td>1.14</td>
<td>1.08</td>
<td>0.06</td>
<td>209</td>
<td>209</td>
</tr>
<tr>
<td>BKBA20</td>
<td>1.07</td>
<td>1.16</td>
<td>0.09</td>
<td>207</td>
<td>210</td>
</tr>
<tr>
<td>BEBA20</td>
<td>1.19</td>
<td>1.13</td>
<td>0.06</td>
<td>209</td>
<td>210</td>
</tr>
</tbody>
</table>

All measurements were carried out in acidic environment (pH 3.0).
**Antimicrobial Activities of Model Peptides**

The interactions between the model peptides and bacterial cellular membranes were estimated by antimicrobial activity measurements. Minimum inhibitory concentration (MIC) values of model peptides against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* were summarized in Table 2–3. Only cationic peptides, BKBA20 and K20K20, exhibited antimicrobial activities against *B. subtilis* and *S. aureus*. Heterodimeric peptides and anionic peptides showed none or negligible antimicrobial activities.

<table>
<thead>
<tr>
<th>peptides</th>
<th>MICs/µg mL⁻¹</th>
<th><em>S. aureus</em></th>
<th><em>B. subtilis</em></th>
<th><em>E. coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>K20E20</td>
<td>&gt;100</td>
<td>100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>anti-K20E20</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>K20K20</td>
<td>50</td>
<td>25</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>E20E20</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>BKBA20</td>
<td>25</td>
<td>6.3</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>BEBA20</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
</tbody>
</table>

**Membrane Perturbation Activities of Model Peptides**

Membrane perturbation activities of model peptides were evaluated by dye-leakage measurements. Figure 2–8 shows dye-leakage activities of model peptides against zwitterionic DPPC or acidic DPPC/DPPG (3:1) SUVs. Anionic peptides, BEBA20 and E20E20, did not possess significant activities, whereas both cationic peptides, BKBA20 and K20K20, showed unignorable activities against the both SUVs (20 – 50% Leakage). Hetero-dimeric peptides also showed comparable activities against DPPC/DPPG SUVs, however, the leakage activities against DPPC SUVs significantly increased.
Concentration dependent dye-leakage measurements were carried out to assess membrane perturbation activities of model peptides against zwitterionic DPPC membranes (Figure 2–9). BKBA20 and K20K20 exhibited distinct membrane perturbation (~50 and ~30%, respectively) at 2 µM net helix concentrations. K20E20
and anti-K20E20 showed higher leakage activity than those of cationic peptides; both peptides showed over 90% activities at comparable concentrations. None or negligible membrane perturbation activities were observed for the anionic peptides under any peptide concentrations.

**FIGURE 2–9:** Concentration dependent dye-leakage measurements. DPPC concentrations of calcein trapped SUVs are 100 µM in all measurements. Symbols represent K20E20 (open circle), anti-K20E20 (closed circle), K20K20 (open triangle), BKBA20 (closed triangle), E20E20 (open square), and BEBA20 (closed square). Hetero-dimeric peptides exhibit higher leakage activities compared to those of cationic peptides.
Electrostatic Interactions for Natural Ion Channel Peptides

The electrostatic helix–helix interactions and correlated changes of structural and biophysical properties were experimentally evaluated using the dimeric model peptides of BXBA20. In order to investigate the applicability and availability of the electrostatic interactions, similar modification was applied to representative natural ion channel peptide, alamethicin, (see Chapter 1–2). The modification was carried out based on the structural properties of K20E20, Lys and Glu residues were incorporated into individual alamethicin helices. As shown in Table 2–4, specific amino acids in alamethicin are known as important residues for ion channel activities. Schematic drawing of the peptide design is showing in Figure 2–10. Specific amino acid residues playing important roles for the ion channel activities were conserved. AlamKE is designated as heterodimer peptide of the cationic and the anionic modified alamethicin. AlamK and AlamE were designed as monomeric components of AlamKE.

Table 2–4: Important Residues in Alamethicin F50 for Ion-Channel Activity

<table>
<thead>
<tr>
<th>residues</th>
<th>roles for peptide functions</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro2</td>
<td>helix stabilization, membrane insertion</td>
<td>(61)</td>
</tr>
<tr>
<td>Gln7</td>
<td>inter molecular hydrogen bond formation</td>
<td>(4,6,15,61)</td>
</tr>
<tr>
<td>Aib10</td>
<td>helix disorder, hydrophilicity</td>
<td>(61)</td>
</tr>
<tr>
<td>Gly11</td>
<td>helix disorder, hydrophilicity</td>
<td>(6,61)</td>
</tr>
<tr>
<td>Pro14</td>
<td>helical distortion with Aib10 or Gly11, voltage-dependence(?)</td>
<td>(4,6,61)</td>
</tr>
<tr>
<td>Gln18</td>
<td>ion conduction, inter-molecular hydrogen bond</td>
<td>(15,61)</td>
</tr>
<tr>
<td>Gln19</td>
<td>ion conduction, inter-molecular hydrogen bond</td>
<td>(15,61)</td>
</tr>
<tr>
<td>PheOl20</td>
<td>stabilization in lipid bilayer</td>
<td>(61)</td>
</tr>
</tbody>
</table>
FIGURE 2–10: Peptide design of dimeric alamethicin analog. (a) Substituted positions in alamethicin on the basis of polar residues in BXBA20. (b) Sequences of the synthetic alamethicin analog peptides.

The CD spectra of the alamethicin analogs collected in 50 mM phosphate buffer and in the presence of DPPC liposome were shown in Figure 2–11. Spectra of AlamKE and mixtures of AlamK and AlamE showed random-coil like spectral patterns in both conditions. The spectra of AlamKE and mixtures of its monomeric components were overlapped, any helix–helix interactions in AlamKE were not
detected as the secondary structural changes. In addition, significant spectral change of AlamKE was not detected between aqueous environment and in the presence of DPPC liposomes. The reduction of the number of Aib residues by the residual substitutions might cause degradation of the helical propensities of alamethicin.

**FIGURE 2–11:** CD spectra of monomeric and dimeric alamethicin analogs collected in (a) 50 mM phosphate buffer (pH7.4) and (b) the buffer in the presence of 2mM DPPC SUVs (pH7.4). The opened circles represent AlamKE. The crosses represent the mixture of AlamK and AlamE (1:1). Closed circle is spectra of alamethicin. Net helix concentrations of the model peptides are 20 µM.
II–3. Discussion

Structures of Model Peptides and Inter-Helical Interaction

In the present study, a series of ion channel model peptide dimers, composed of covalently-linked amphiphilic helices with cationic or anionic characters, were designed and synthesized. Sequences of the domains employed as a monomeric peptide, Ac-(Aib-Xxx-Aib-Ala)₅-NH₂, have been developed as templates for amphiphilic channel forming peptides (27–29). BKBA20 and BEBA20, possessing Lys and Glu residues at Xxx positions, respectively, exhibited helical structures with amphiphilic character. The helix components in the dimers should be possessing amphiphilicities since CD spectra of all dimeric model peptides also showed typical patterns of α-helix structure.

Concentration and pH dependences of K20E20 CD spectra implied an existence of electrostatic interactions between the K20E20 molecules at high peptide concentrations. It was assumed that cationic and anionic helices in K20E20 molecules were associated via ion pair forming between the polar residues on hydrophilic surfaces of their amphiphilic helices. It is difficult to distinguish the contributions of electrostatically inter- or intra-molecular interactions at high peptide concentration, although the interactions should be limited in intra-molecular interaction under lower peptide concentration (< 20 µM net helix concentration) and it caused stabilization of helix structures. Since the stabilization of secondary structure of hetero-dimeric peptides degraded by cleavage of disulfide linkage, the dimerization of helices is requisite for the inter-helical interaction, i.e., degradation of the electrostatic inter-helical interactions caused by peptide dilution could be avoided by covalent peptide dimerization. So far, Baldwin and co-workers have performed stabilization of
secondary structure of helical peptides by intra-helical ion pairs (62). In the present case, the helix stabilizations were performed by inter-helical electrostatic interactions. Several types of long-range interactions can act cooperatively to stabilize secondary structures although hydrophobic interactions can play a dominant role in secondary structure formation (34,35). Contribution of intra-molecular hydrophobic interactions in the stabilization of hetero-dimeric peptides could not be easily evaluated in the presence of electrostatic interactions. Nevertheless, it is undoubtable that electrostatic interactions, especially ion-pair forming, contributed helix stabilization.

**Peptide–Membrane Interactions and Peptide Structures in Membrane**

Dimerization of cationic helices and anionic helices provided significantly differences not only helix–helix interactions of model peptides but also peptide–bilayer interactions. The orders of peptide–membrane affinities and membrane perturbation activity are hetero-dimeric peptides, cationic peptides and anionic peptides. Overall, peptide–membrane interactions and membrane perturbation activities of anionic peptides were not detected, whereas, cationic peptides significantly increased those peptide–membrane interactions. Cationic feature of peptides have been considered as one of the primary factors to interact with phospholipid bilayers, thus, this observations implied importance of peptide cationic features. More noteworthy is considerably higher degrees of those interactions (activities) observed in the case of hetero-dimeric peptides. Hydrophobicity has been widely accepted as an alternatively important factor for interaction of peptides and proteins with zwitterionic phospholipid bilayers (13). As a result of ionic and/or polar interactions between the hydrophilic surfaces of amphiphilic hetero-dimeric peptides, the hydrophobic surfaces of these peptides are externalized and exposed. Significant dye-leakage activities and high membrane
affinities of hetero-dimeric peptides against DPPC membranes also imply higher peptide hydrophobicity; rather than remaining in aqueous environments, the hetero-dimeric peptides prefer to accumulate on membrane surfaces and partition into them.

According to antimicrobial activity measurements, two cationic peptides, BKBA20 and K20K20 exhibited antimicrobial activity against Gram-positive bacteria. Cationic features of an antimicrobial peptide allow them to interact more effectively with the negatively charged lipopolysaccharide (LPS) (63) and/or cytoplasmic bacterial cellular membranes (64). Antimicrobial activity of the cationic peptides further supported the importance of cationic properties of antimicrobial peptides as a general feature of their activity against bacteria. Hetero-dimeric peptides were not active against bacteria; although both dimers contained the cationic BKBA20 domains. These results could confirm the shielding of the cationic characteristics of these peptides caused by intra-molecular electrostatic interaction of negatively-charged Glu side chains with Lys positively-charged side chains. Relatively lower leakage activities of hetero-dimeric peptides against acidic DPPC:DPPG (3:1) SUVs than those of zwitterionic DPPC SUVs also supports this assumption.

Two-stranded helix association of hetero-dimeric peptides was facilitated in the presence of DPPC SUVs. Generally, relative intensity of ion pair is weaker (≤ 0.5 kcal/pair) in aqueous solutions than those in hydrophobic environments due to higher permittivity. Inter-helical ion pairs might be reinforced in hydrophobic environment of phospholipid bilayer and associations of helices further stabilized. The cooperatively insertion of the voltage-sensor domains of Shaker and related ion channel proteins have been reported (65,66). S4 segment helix (voltage sensor domain) possessing many cationic residues is inserted into biological membranes after formation
of ion pairs with other helix domains (S2 and S3 segments). The electrostatic interactions play a critical role not only in stabilizing the voltage sensor in the intact proteins but also during the process of membrane integration.

**Applicability and Availability of Electrostatic Interactions**

According to CD spectra of K20E20, recorded in acidic conditions indicated degradation of peptide–membrane affinity and helix association in the phospholipid bilayer. These results supported that the importance of inter-helical electrostatic interactions for peptide–membrane interactions. Moreover, it was also expected that the pH sensitivities upon helix association and peptide–membrane interactions allow controlling of ion channel activities.

In order to test the applicability of the electrostatic helix–helix interaction, dimeric analog of electrostatically modified alamethicin, AkamKE, was designed and its structure was evaluated. K20E20 exhibited larger degree of the spectrum than that of the mixture of BKBA20 and BEBA20. However, any effects on the electrostatic interactions were not observed in AlamKE. The dimeric alamethicin analog did not take secondary structures because of poor conservation of Aib residues (50% conservation). This observation implying that one of essential structural requirements for the inter-helical electrostatic interaction is helix forming propensity of individual ion channel helices in the dimer.
CHAPTER III

Channel Forming Properties of Dimeric Model Peptides

III–1. Introduction

In the consequence of several biophysical experiments described in chapter II, the chimeric dimers of amphiphilic cationic and anionic helices showed high helix forming propensities, high peptide–membrane affinities and high degrees of two-stranded association of helices in the phospholipid bilayer. All these natures, expected as favorable factors for channel forming, were accelerated by the inter-helical electrostatic interactions.

In this chapter, details of the electrophysiological properties of the model peptides were described. Ion channel properties among the dimeric model peptides were compared to assess the effects of inter-helical electrostatic interactions for ion channel activities. Application of the electrostatic interaction for regulation of channel function was also concerned, thus, ion channel properties of K20E20 under acidic condition were evaluated. The ion channel forming of K20E20, facilitated by the inter-helical electrostatic interaction of ionic pairs, suggested the possibility of modulation of ion channel characteristics by the pH change. Several reports have
mentioned that the pH alteration caused conformational disorder or unfolding of helices stabilized by intra- or inter-helical salt bridges (62,67). Thus, the assembly of helical bundles assisted by salt bridges should be destabilized by a decrease in electrostatic interactions resulting in possible ion channel inactivation. It was expected that modulation of the helical bundle formation by modification of pH or ionic strength could be therefore utilized as a general method to control the activation or inactivation of the engineered synthetic ion channels.
III–2. Results

**Ion Channel Properties of Monomeric Model Peptides**

Ion channel activities of the model peptides were evaluated by tip-dip technique on zwitterionic and mechanically stable DPhPC bilayers (Chapter IV). Due to the small dimensions of bilayers, the technique permits low noise levels (68). Channel conductance (G) is expressed as the reciprocal resistance with units of siemens (S). BKBA20 has shown single level ion conductance of 228 pS (27). According to the cylindrical bundle model (61), the pore was composed of tetrameric helices with 0.35 nm pore diameter. BEBA20 showed multi-level conductance patterns with large conductance values (28).

** Ion Channel Properties of Homo-Dimeric Model Peptides**

Typical conductance patterns of homo-dimeric model peptides were illustrated in Figure 3–1. K20K20 exhibited single state conductance patterns including spiky current fluctuation at 50 nM peptide concentration. Single-level conductance pattern with ca. 230 pS conductivity was observed occasionally at 5 nM peptide concentration (net helix concentration of 10 nM). Ion channel forming of K20K20, detected at lower peptide concentrations than those of BKBA20 (27), implying favorable entropic factors of covalent dimerization in the former peptide. Similarly, the conductance patterns of E20E20 at 50 nM were also different from those of BEBA20. However, small and single state conductance patterns were only occasionally observed in E20E20. A clear interpretation of the differences of conductance patterns between dimeric and monomeric anionic peptides could not be obtained.
**K20K20**

peptide concentration: 50 nM  
potential: +60 mV

**E20E20**

peptide concentration: 50 nM  
potential: +200 mV

**FIGURE 3–1:** Representative single-channel conductance patterns of the dimeric model peptides. Measurements were carried out under neutral (pH 7.4) conditions. (upper and middle traces); Conductance patterns of K20K20 at 50 nM and 5 nM peptide concentrations, respectively. K20K20 occasionally exhibited clear open–close transitions of ion channel at 5 nM peptide concentrations. (bottom trace); Conductance patterns of E20E20 at 50 nM peptide concentration.

**Concentration Dependence and Single Channel Properties of K20E20**

Concentration dependence of ion channel activities of K20E20 is illustrated in Figure 3–2. The erratic conductance patterns were detected in the 10 – 100 nM peptide concentration range with high frequencies. As peptide concentration decreased, the current fluctuations also decreased. Finally, at 1 nM concentration the ion channel activity of K20E20 was well resolved with distinct open–close states. The
channel forming under extremely low peptide concentration reflects high channel forming ability of K20E20. Overall, K20E20 showed distinct ion channel activities, whereas anti-K20E20 did not exhibit ion channels with well-resolved open–close states under comparable conditions (data not shown).

**FIGURE 3–2:** Conductance patterns of K20E20 recorded under various peptide concentrations. Erratic patterns were dominantly observed at 10 – 100 nM peptide concentration range.
Figure 3–3 exhibits voltage dependence of conductance patterns of 1 nM K20E20 and its amplitude histograms. Time scales of the conductance patterns and the histograms are 500 milli-seconds and 20 seconds, respectively. This measurement was reproducible under similar conditions. Both conductance patterns and those histograms exhibit that the conductance states of K20E20 are single-level through any examined membrane potentials. Moreover, mean channel opening duration increased correspond to applied membrane potentials. However, individual channel opening durations of K20E20 (at 1 nM) were at milli-second order time scales, the channel lifetime was comparable to those of other model peptides recorded at higher peptide concentrations.

An ohmic linear I–V relationship for the open state of the main channel of K20E20 is exhibited in Figure 3–4. The ion conductance in this channel is constant and equal to the slope of the line. The mean conductivity of K20E20 was approximately 1.5 nS under all membrane potentials. Observed currents, conductance values, estimated pore diameters, and number of helices in bundle structures were summarized in Table 3–1. Calculated pore diameter was ca. 1.0 nm, which corresponded to a hexameric helix bundle.
FIGURE 3–3: Representative single-channel conductance patterns of K20E20 and amplitude histogram. Measurements were carried out under neutral (pH 7.4) conditions. Ion channel properties of K20E20 at 1 nM peptide concentrations. Conductance patterns recorded under variable membrane potentials (+90 mV to +190 mV).
**FIGURE 3–4:**  \( I-V \) plot for 1 nM K20E20 at variable membrane potentials. A linear \( I-V \) relationship is observed.

**Table 3–1:**  Observed Ion Conductivities and Calculated Pore Structures of K20E20

<table>
<thead>
<tr>
<th>potential/mV</th>
<th>current/pA</th>
<th>conductance/nS</th>
<th>pore diameter/nm(^{a})</th>
<th>number of helices(^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>+90</td>
<td>143</td>
<td>1.59</td>
<td>1.0</td>
<td>6.0</td>
</tr>
<tr>
<td>+100</td>
<td>160</td>
<td>1.60</td>
<td>1.0</td>
<td>6.0</td>
</tr>
<tr>
<td>+120</td>
<td>173</td>
<td>1.44</td>
<td>0.95</td>
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</tr>
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<td>+130</td>
<td>183</td>
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<td>5.9</td>
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<tr>
<td>+190</td>
<td>330</td>
<td>1.74</td>
<td>1.1</td>
<td>6.2</td>
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</tbody>
</table>

\(^{a}\) Pore diameter and number of helices were calculated by equation 6 and 7, respectively (Chapter IV) (1,61). The radius of helix (R) is estimated as 0.5 nm; the helix length (l) for the 20-residue peptide is 3.0 nm; the resistivity (\( \rho \)) of 500 mM KCl solution was calculated from limiting ionic conductance at 25 °C is 0.13 \( \Omega \)m (69).
**pH Effects for the K20E20 Ion Channel Activities**

If the formation of K20E20 ion channels was assisted by the existence of inter-helical ion-pairs, structural disorder and perturbation of channel activity should occur by any disruption in the ion-pair network. Liposome titration CD spectra of K20E20, collected under acidic media (Chapter II–2), showed degradation of both peptide–bilayer interaction and two-stranded helix association in the bilayer. Ion channel activities of K20E20 were also measured under acidic conditions (pH 3.0) to examine the effects of disappearance of inter-helical electrostatic interaction (Figure 3–5). Conductance patterns of 50 nM K20E20 recorded in neutral pH exhibited erratic patterns, while the patterns were converted to single states with clear open–close transition under acidic conditions. Relative ion conductivities at neutral pH were quite large but, in comparison, the ion conductivity under acidic conditions was considerably smaller. Under acidic conditions, the mean conductivity was 174 pS, which corresponded to tri- or tetramerical helix bundles.
FIGURE 3–5: Comparison of K20E20 conductance patterns recorded under neutral (pH 7.4) and acidic (pH 3.0) conditions. Concentration of the dimeric peptide in both conditions is 50 nM. The applied potential is +50 mV in both traces. Erratic conductance patterns with large ion conductivity were recorded in the neutral pH conditions and the patterns were converted to single state conductance patterns in the acidic conditions.
III–3. Discussion

**Ion Channel Forming of Hetero-Dimer Peptides**

The various biophysical studies performed in the chapter II indicated the facilitated helix–helix and helix–bilayer interactions of hetero-dimers of cationic and anionic helices. This high membrane affinity and high degrees of two-stranded helix forming propensity of the hetero-dimeric peptides should therefore allow the penetration and association of these peptides in membrane interiors leading to formation of appropriate homomeric ion conducting channels even at considerably low peptide concentrations (as low as 1 nM).

The two hetero-dimeric peptides, K20E20 and anti-K20E20, possess comparable membrane affinities and helix association propensities, however, anti-K20E20 did not exhibit well-resolved ion channel activities. In the presence of a transmembrane potential, macrodipole moments of helical structures interact with the external electric field and as a result of this interaction are oriented perpendicular to the membrane surface \((3)\). In the case of anti-K20E20, it seems that as a result of the anti-parallel orientation of the macrodipoles of its helical components, stable ion conducting assemblies could not be formed for this peptide in lipid membranes. The parallel orientation of helices is therefore a necessary requirement for the ion channels formed of helical bundles.

**Structures and Electrophysiological Properties of K20E20 Pore**

In accordance with the barrel-stave model \((II)\), the pore size is dependent on the number of incorporated helices in the bundle. Consequently, increase or decrease in the number of helices results in various conductance levels. Therefore, helix
bundles consisted of specific number of helices have a good potential to be employed as scaffolds for various functional modifications of the pores. Comparison of ion conductance patterns between K20E20 and alamethicin were exhibited in Figure 3–6. Alamethicin showed multi-state ion conductance pattern which reflect various pore size. On the other hands, patterns of K20E20 showed single-state conductance patterns. Thus far, many efforts have been directed to control helix association, especially chemical cross-linking techniques have been utilized to regulate pore structures. The technique of template-assembled synthetic protein (TASP), proposed by Mutter, has been employed to control helix numbers and orientations, as well as the helix components in a bundle structure (70). Several investigations have previously reported on the construction of controlled pore sizes using this approach (71). Single-state conductance of 1 nM K20E20 is a specific case for stabilization of helix bundle with hexameric helices. No sub-conductance levels were observed in this simple ion channel, which was selectively stabilized without possessing an elaborate molecular design.

A common feature of the mechanism of cation/anion selectivity is that the chemical nature and structure of the amino acid side chains located in proximity of the pore region can act as selective filters by selecting ions on the basis of their polarity and charge density (72,73). We have already reported the mild cation selectivity of BEBA20 and the nonspecific ion selectivity of BKBA20 (28), however the cation/anion selectivity concepts could not be easily applied in the case of K20E20, since equal number of cationic and anionic amino acid side chains coexist in this peptide.

The mean opening duration of K20E20 ion channels was voltage dependent, however, duration of individual channel openings were not significantly elongated. One possible interpretation for this observation is that the dimer–dimer inter-molecular interactions were only weakly enhanced by ion pair formation between cationic and anionic helices. You et al. and Okazaki et al. reported that the channel life-times of alamethicin were drastically increased by helix dimerization (52,53). The glutamine at position 7 of alamethicin lining the channel’s interior is assumed to stabilize the helix bundles by hydrogen bonding networks that could promote helix–helix interactions in the ion channel helical bundle (4,6,15,61,74). In order to obtain longer pore opening durations, further stabilization of pores with emphasis on dimer–dimer interactions in the bundle will be required in future studies.

**pH Dependence of K20E20 Ion Channel Function**

The erratic but large conductivity of K20E20 channels under neutral pH conditions was switched to a single level with relative low conductivity under acidic conditions. Figure 3–7 exhibits a schematic drawing of the bundle forming process by K20E20 under neutral and low pH conditions. As shown in this scheme, the ion pairs in K20E20 provide the intermediates of helix bundles under neutral pH conditions,
however, under acidic conditions the ion pairs cease to interact due to protonation of Glu side chains. Interestingly, the ion conductance patterns and conductivities of K20E20 recorded in the acidic medium are similar to those of BKB20 (27–29), therefore, it seems plausible that the ion conductance activity is caused by channels composed of only the BKBA20 domains of the hetero-dimeric peptides. The BEBA20 domain should not be capable of forming ion channels under acidic conditions due to the considerable reduction of helix amphiphilicity. Nevertheless, membrane interaction of BEBA20 observed under acidic conditions cannot be completely ignored. Some helical peptides, containing carboxyl groups, can interact with lipid membranes especially under acidic conditions by neutralization of the carboxyl groups (75,76). Whether the BEBA20 segments are involved in the ion conducting structures of K20E20 pores cannot be verified at the present stage.

Natural ion channel proteins transmit extracellular stimuli such as binding of ligand molecules or change of membrane potentials via allosteric change of protein structures resulting in ionic conductance (77). Electrostatic interactions play important roles in the biological functions of ion channel proteins. An X-ray crystallographic analysis of ASIC (acid sensing ion channel), typical pH-sensing ion channel proteins, has been recently published (78). The pH-sensing domain of ASIC was identified at a far distance from the pore region, and an electrostatic carboxyl–carboxylate interaction acted as a trigger for ion channel activation/deactivation.
FIGURE 3–7: Suggested scheme for the pore forming mechanism and pH-sensitive behavior of K20E20. Equilibriums of two-stranded helix association and peptide membrane interactions are favored by inter-helical electrostatic interactions and ion channel formation is facilitated. The channel forming ability is degraded in the acidic conditions due to the disordered electrostatic interactions. Note that the pore forming in acidic conditions may require higher peptide concentrations than neutral conditions.
 CHAPTER IV

Experimental Procedures

Materials

N-α-Fmoc protected amino acids, HBTU, and Rink amide resin were purchased from Novabiochem (Tokyo, Japan). DCC, HOBr and TFA were available from Peptide Institute (Osaka, Japan). Polypropylene vessels for solid phase peptide synthesis were purchased from Pierce (Rockford, IL). DPhPC was obtained from Avanti Polar Lipids (Alabaster, AL) as 50 mg/mL chloroform solutions. DPPC were purchased from Sigma (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan), and used as received.

Peptide Synthesis

The peptide elongations of C-terminal amidated model peptides (BXBA20 model peptides) were carried out by Fmoc solid-phase peptide synthesis on Rink amide resin (0.61 mmol/g, 50 mmol scale as 1 equivalent). The peptide elongations of the alamethicin analogs were carried out by Fmoc solid phase synthesis on phenylalaninol (Pheol) pre-loaded 2-chloro trityl resins (0.77 mmol/g, 50 mmol scale as 1 equivalent). Ten equivalents of N-α-Fmoc protected amino acids were pre-activated with
HBTU-HOBt in the presence of DIEA for 20 minutes. Then coupling reaction was carried out for 40 minutes at room temperature. Fmoc groups were removed by 20% piperidine/DMF treatments for 10 minutes. After the peptide elongation, N-termini were acetylated by AcOH; pre-activated with DCC/HOBt for 3 hours. Crude linear products were cleaved from the resins by TFA treatments containing 5% TIS and 5% H₂O for 90 minutes. The filtrates were concentrated under N₂ breezing, and then crude products were obtained from ether precipitation. The crude linear peptides were purified by RP-HPLC on a Wakosil 5C4–200 column (φ 10.0 x 250 mm). HPLC was performed on a system comprised of 807-IT integrator (Jasco, Tokyo, Japan), two pumps PU-980 (Jasco), a UV-970 detector (Jasco), and Rheodyne 7125 injector (Rheodyne Inc., CA). Peptide dimerizations via disulfide bond formation were performed by air oxidation of termini Cys side chains. Monomeric peptides were dissolved in 50% MeOH or aqueous solution and then bubbled for 12 – 24 hours. Progress of the air oxidation was monitored by analytical RP-HPLC on a Wakosil 5C4–200 column (φ 4.0 x 150 mm) using the following solvent systems: (A) 95% water/5% CH₃CN/0.05% TFA; and (B) 5% water/95% CH₃CN/0.04% TFA. After air oxidation, dimeric peptides were repurified by the semi-preparative RP-HPLC. The purities of the peptides were confirmed by the analytical RP-HPLC. HPLC chromatograms of purified peptides were shown in Figure 5–1. The structures of peptides were verified by MALDI-TOF MS analyses. Mass spectra were measured on a Voyager-DERP (PerSeptive Bioscience, Framingham, MA) with α-cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) as matrix. Amino acid compositions and peptide concentrations of stock solutions used in various assays were determined by quantitative amino acid analysis. Purified peptides were hydrolyzed in a 6N hydrochloric acid at 110°C for 24 hours. After hydrolysis, the residual HCl
removed in vacuo and then neutralized with TEA. Amino acids in the hydrolysate were labeled with phenylisothiocyanate (PITC) treatments in the presence of TEA for 20 minutes. Obtained phenylthiocarbamyl (PTC) derivatives were analyzed by HPLC on a LiChrospher 100 column (ϕ 4.0 x 250 mm) using the following solvent systems: (A) 60 mM CH₃COONa buffer (pH 6.0)/CH₃CN (94:6); and (B) 60 mM CH₃COONa buffer (pH 6.0)/CH₃CN (40:60).

K20E20. Amino acid ratios in acid hydrolysate: Ala 10.0 (10), Lys 4.8 (5), Glu 5.6 (5), Gly 4.6 (4). MALDI-TOF MS: 4249.7 [expected for (M+H)+ 4248.3]. HPLC: R.T. 16.1 min.

anti-K20E20. Amino acid ratios in acid hydrolysate: Ala 10.0 (10), Lys 5.0 (5), Glu 4.6 (5), Gly 4.0 (4). MALDI-TOF MS: 4249.4 [expected for (M+H)+ 4248.3]. HPLC: R.T. 18.0 min.


E20E20. Amino acid ratios in acid hydrolysate: Ala 10.1 (10), Glu 10.0 (10), Gly 4.7 (5). MALDI-TOF MS: 4276.2 [expected for (M+Na)+ 4275.0]. HPLC: R.T. 20.8 min.

AlamKE. Amino acid ratios in acid hydrolysate: Ala 4.1 (4), Lys 5.4 (5), Glu 12.8 (11), Gly 6.3 (6), Pro 3.9 (4), Val 1.8 (2), Leu 2.0 (2). MALDI-TOF MS: 4766.3 [expected for (M+H)+ 4764.5]. HPLC: R.T. 10.4 min.

AlamK. Amino acid ratios in acid hydrolysate: Ala 2.0 (2), Lys 5.7 (5), Glu 3.8 (3), Gly 1.2 (1), Pro 2.0 (2), Val 1.1 (1), Leu 1.1 (1). MALDI-TOF MS: 2165.6 [expected for (M+H)+ 2164.3]. HPLC: R.T. 7.7 min.
Ala**mE**. Amino acid ratios in acid hydrolysate: Ala 2.0 (2), Glu 9.4 (7), Gly 1.1 (1), Pro 1.9 (2), Val 1.0 (1), Leu 1.0 (1). MALDI-TOF MS: 2192.0 [expected for (M+Na)\(^+\) 2191.1]. HPLC: R.T. 11.6 min.

**FIGURE 5–1:** HPLC profiles of synthetic model peptides.
Circular Dichroism Measurements

CD spectra were recorded on a Jasco J-720 spectropolarimeter (Jasco) at room temperature. Cylindrical cells with 0.02 cm or 0.2 cm path lengths were used for measurements. The CD cuvettes were washed with a concentrated NaOH aqueous solution between measurements to remove any peptide adhering to its inner surface. Far-UV CD experiments were carried out in the wavelength range between 190 to 260 nm. Four scans were averaged for each sample and blank was subtracted from all spectra. Prepared sample solutions were left at room temperature for 30 minutes before measurements. The concentrations of stock solutions were determined on the basis of the quantitative amino acid analysis data. All staffs for preparing and transferring the solution were siliconized to prevent nonspecific adsorption of the hydrophobic peptides. The results are expressed as the mean residue ellipticity \([\theta]\) with units of degrees cm\(^2\) dmol\(^{-1}\) and calculated from equation 1:

\[
[\theta] = \theta_{obs}/(10lcn)
\]  

(Eq. 1)

where \(\theta_{obs}\) is the ellipticity measured in millidegrees, \(c\) is the molar peptide concentration, \(l\) is the optical path length of the cell in cm, and \(n\) is the number of amino acid residues in peptides. Number of amino acid residues of dimeric model peptide was presumed to be 40. The helix contents of model peptides were given according to the Chen’s method (58).

Preparation of Small Unilamellar Vesicles (SUVs)

DPPC (8.8 µmol) was dissolved in chloroform (100 µL), the chloroform was evaporated by stream of \(N_2\) gas and the sample was then dried in vacuo overnight. The
lipid film was hydrated with 55 mM phosphate buffer (2 mL) and vortexed at 50 °C for 30 minutes. SUVs were prepared by probe sonication for 20 minutes using a Branson 250 Sonifier (Branson, Danbury, CT). Calcein entrapped SUVs used for dye-leakage measurements were prepared by a similar method. The lipid film (88 µmol) was hydrated by 2 mL of 100 mM tris-HCl buffer (pH 7.4) containing 70 mM calcein and then the suspension was sonicated. Untrapped calcein was removed by gel filtration chromatography on a Sephadex G-25 column. The lipid concentration was determined by the phosphorous analysis (79).

**Antimicrobial Activity Assay**

The minimum inhibitory concentration (MIC) of model peptides against *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* were determined by dilution method in MuellerHinton agar medium. Plates were incubated at 30 °C for 12 hours. Gramicidin S was used as reference compound. The MIC of the each peptide is reported as the average of triplicate measurements.

**Dye-leakage Measurements**

Dye-leakage measurements were carried out using a Jasco FP-720 spectrofluorophotometer (Jasco) at room temperature. The fluorescence intensity data were collected 40 minutes after the addition of peptides to SUV-containing buffers. Final lipid concentrations of SUVs were 100 µM. Calcein was excited at 490 nm, and emission at 530 nm was monitored. To determine the fluorescence intensity for 100% dye release, 5 µL of Triton X-100 was added to dissolve and break apart the vesicles. The Triton X-100 treatments were carried out after each measurement. The percentage of dye release caused by the peptides was evaluated by equation 2:
Leakage % = \[(F_t - F_0)/(F_{100} - F_0)\] x 100 \hspace{1cm} \text{(Eq. 2)}

where \(F_t\) is the fluorescence intensity achieved by the peptides, \(F_0\) and \(F_{100}\) are fluorescence intensities observed in the absence of the peptides and after Triton X-100 treatment, respectively.

**Single-Channel Measurements**

Single channel measurements were performed by tip-dip technique at room temperature (Figure 5–2) \((27, 68)\). Briefly, the patch clamp pipettes were prepared (~1 \(\mu\)m inner diameter) by the two-pulls method using a microelectrode puller (Narishige, Tokyo, Japan) from hematocrit hard grass capillaries (Narishige) without fire polishing. The electrolyte solutions were comprised of 500 mM KCl solutions buffered with 5 mM HEPES (pH 7.4) or 500 mM KCl 5mM HEPES buffer (pH 3.0) which pH adjusted by 1N HCl. Pipettes were filled with the buffer solutions containing peptides. The pipette tip was immersed in a disposable petri dish filled with the buffer solution. After immersion of the pipette, DPhPC phospholipid monolayer was spread on the surface of the dish solution by careful addition of 1 to 2 \(\mu\)L of a 10 mg/mL DPhPC solution in hexane. Ten minutes were allowed for the evaporation of the hexane and the bilayer was then formed on the pipette tips after reimmersion of pipettes. Seals of 2 to 20 G\(\Omega\) were formed at the tip of pipettes. Single-channel currents were amplified using an Axopatch 1D patch-clamp amplifier (Axon Instruments Inc., Union City, CA) controlled by pClamp 6 software (Axon Instruments Inc.). Data was filtered at 1 or 2 kHz frequency, stored directly on a hard disk, and analyzed with an AxoGraph 3.5 (Axon Instruments Inc.).

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FIGURE 5–2: Schematic presentation of the experimental apparatus for the pipette-dipping (tip-dip) technique.

**Helix Bundle Model (Cylindrical-Bundle Model)**

In the helix bundle model, a transmembrane channel is assumed to be a bundle of helices which long enough to span the membrane. The model considers the diffusion of ions through an inflexible rigid structure of the helix-bundle. Assuming the cylindrical pore as a conducting structure (surrounded by bulk solution electrolytes on the two sides of a membrane) which obeys Ohm’s law, we can calculate the pore’s resistance by integrating the resistance along the path of the current flow through it. In the case of a cylinder the resistance is

\[
R_{\text{pore}} = \frac{\rho l}{\pi r^2} \quad \text{(Eq. 3)}
\]
where $\rho$ stands for the bulk solution resistivity, $l$ is for the length of the pore and $r$ is for its radius. A further resistance of the pore is termed as “access resistance”, which is the resistance along the convergent paths from the bulk medium to the mouth of the pore, is calculated to be

$$R_{\text{access}} = \frac{\rho}{2r} \quad (\text{Eq. 4})$$

$$R_{\text{total}} = R_{\text{pore}} + R_{\text{access}} \quad (\text{Eq. 5})$$

considering these equations the conductance ($G$) of the pore (or channel) is:

$$G = \frac{1}{R_{\text{total}}} = \frac{\pi r^2}{\rho(l + \pi r/2)} \quad (\text{Eq. 6})$$

Through geometrical calculations the radius ($r$) of the pore can easily calculated (see the cross-section of the pore in Figure 5–3). By taking $R$ for the radius of the monomeric helix, the pore radius can be calculated as

$$r = R[1/\sin(\pi/N) - 1] \quad (\text{Eq. 7})$$

where $N$ is the number of helix monomers in the bundle.

The approximations made in this treatment of channel conductance are radical, as they ignore all interaction between the ion and channel, but they allow one to obtain an upper limit for the conductance of a channel of dimensions equivalent to the cylindrical volume occupied by a single $\alpha$-helix crossing a bilayer. Channel–ion interactions would thus be expected to results in a conductance of less than above $R_{\text{pore}}$. 
Theoretical conductances for various values of N were calculated for channel-forming peptide in accordance with the above considerations. To our understanding, following parameters and bulk solution resistivities commonly used for this model.

The radius of helix (R) is estimated as 0.5 nm; the helix length (l) for the 20-residue peptide is 3.0 nm; the resistivity (ρ) of 500 mM KCl solution was calculated from limiting ionic conductance at 25 ºC is 0.13 Ωm (69).

FIGURE 5–3: Schematic presentation of the helix-bundle model: profile of a hexameric pore (left) and its cross-section (right).
CHAPTER V

Overview and Conclusion

In the present study, ion pairs were incorporated into synthetic ion channel molecules to facilitate channel formation. The electrostatic helix–helix interactions were expected to be useful technique for artificial ion channel designs. A series of the covalently-linked homo- and hetero-dimers of cationic and anionic helices were designed and synthesized. Biophysical and electrophysiological experiments were carried out and observations were compared among these model peptides to assess contribution of the electrostatic interaction. The electrostatically effects on ion channel forming are overviewed as well as availabilities of the electrostatic interactions in a peptide design are also concerned in this chapter.

Electrostatically Assisted Helix Association and Ion Channel Formation

Numerous SAR studies have been accumulated on helical ion channel forming peptides (Chapter I–2). Essential natures and structural requirements of the ion channel molecules have been identified. Ion channel forming ability can be related to the following factors, the reinforcement of these factors would help ion channel formation. (i) The helicity of peptide: The α-helix dipole moment originates from the
particular alignment of the peptide units (3.5 D/units) in this secondary structure (80). Since stiff and long α-helix possess large dipole moment, a stable helix could strongly interact with electric field and stabilize the helix bundle structure in the lipid bilayer (3).

(ii) The self-association of peptide: Channel of helical peptide is assumed to be transmembrane peptide bundle, therefore self-association of peptides in membrane environments, is concerned as essential factor. (iii) The affinity of the peptide to membrane: The probability of channel formation decrease with reducing the binding affinity, which can be understood by postulating that the fraction of arranged peptides in channel is a small among the bound peptides (81). (iv) The amphipathicity of the peptide: Helical peptides in the helix-bundle channel are believed to have an interior (pore lumen) face which is more hydrophobic than the face of exterior (bilayer) face. The capability of forming amphipathic helix is a common feature of several channel-forming peptides (3,82).

BKBA20 and BEBA20 employed as monomeric helices in present study possessing amphipathic characters (27,28). According to CD measurements, all model peptides exhibited characteristic α-helix conformations, thus, helical components in dimeric peptides should have amphiphilic characters. Helicity, self-association property, and affinity of peptide to membrane were all facilitated in covalently connected hybrid cationic and anionic interacting helices. Increase in the helical contents of these chimeric peptides, in comparison to monomeric or homo-dimeric peptides, was a strong indication of the contribution of electrostatic interactions in helix–helix interaction and stabilization. The packing of helices, supported by electrostatic interaction, would contribute the stabilization of secondary structures. The tight helix–helix interaction of hetero-dimeric peptides, supported by inter-helical salt bridges, could also directly promote the formation of helical bundle assemblies in
phospholipid bilayers by bypassing the formation of double-stranded associated intermediates. Moreover, association of helices upon the hydrophilic cores of amphiphilic peptides enhanced peptide–membrane interactions by increments of peptides hydrophobicity. Popot and Engelman proposed a two-stage folding model of \( \alpha \)-helical membrane proteins, composed of insertion of helices into the membrane and interaction and association of the inserted transmembrane helices \((83,84)\). On the basis of this model, both high membrane affinity and self-association properties of the hetero-dimeric peptides facilitate helix bundle formation in lipid bilayers. In fact, K20E20 had clear open–close transition of its ion channel even under extremely low peptide concentrations and this observation further supports the above assumption. Design of amphiphilic helical constructs with hydrophilic oppositely-charged surfaces is therefore seems to be an appropriate method to promote and facilitate the formation of helical bundle assemblies in lipid membranes. In addition, the electrophysiological experiments of K20E20 implied construction of pore with specific architectures. Construction of specific pore structure was also achieved by utilizing of electrostatic helix–helix interactions.

*Modulation of Ion Channel Activity by Electrostatic Interactions*

One of the goals in artificial ion channel peptide design is to construct pore equips the functions correlate to outer stimuli sensing and pore activation. It was expected that the utilization of electrostatic helix–helix interactions would be an appropriate approach to address the functional modifications. Generally, many proteins unfold at pH values less than about 5 or greater than 10. Salt bridges between ionizing groups can contribute to the stability of the folded state of some proteins and can be disrupted by extreme pH values, at which one of the interacting group is no
longer ionized. The salt bridge should correlate to protein functions since the biological function of proteins is related to how they fold and stabilize.

CD measurements performed under low pH conditions implied lowering affinity of K20E20 against phospholipid membrane, as well as degradation of helix association propensity of K20E20 in phospholipid bilayer, in comparison to the neutral pH conditions. Moreover, ion channel activities of K20E20 recorded in acidic condition, compared to the activities in neutral pH conditions, also showed significantly degradation of its ion conductivity. In consequence of those observations, we concluded that K20E20 formed pH-sensitive ion channels in phospholipid bilayers and responded to the changes in pH by modifying its conductance patterns. Since the conductance in K20E20 ion channels could be modulated by changes in pH, this peptide can serve as a minimalist model for pH-sensing ion channel proteins. The K20E20 peptide model and its selectively modified analogs can also have applications in studying the voltage-gating and voltage-dependence mechanisms of the protein ion channels.
ABBREVIATIONS

The abbreviations according to biochemical nomenclature proposed by *IUPAC-IUB Joint Comission, Eur. J. Biochem.*, **138**, 9–37 (1984), are used throughout. Unless otherwise specified. Additional abbreviations are as follows:

Aib (B): 2-aminoisobutyric acid
CD: circular dichroism
DCC: N,N-dicyclohexylcalbodiimide
DIEA: N,N-diisopropyl-ethylamine
DMF: N,N-dimethylformamide
DPhPC: diphytanoylphosphatidylcholine
DPPC: dipalmitoylphosphatidylcholine
DPPG: dipalmitoylphosphatidylglycerol
DTT: dithiothreitol
Fmoc: 9-fluorenylmethoxycarbonyl
HBTU: 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate
HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid
HOBt: 1-hydroxybenzotriazole
MALDI-TOF MS: matrix assisted laser desorption ionization time-of-flight mass spectrometry
Rink amide resin: 4-(2’,4’-dimethoxyphenyl-Fmoc-aminomethyl) phenoxy resin
RP-HPLC: reversed phase high-performance liquid chromatography
SUVs: small unilamellar vesicles
TFA: 2,2,2-trifluoro acetic acid
TIS: triisopropylsilane
REFERENCES


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