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Alamethicin−Leucine Zipper Hybrid Peptide: A Prototype for the Design of Artificial Receptors and Ion Channels

Shiroh Futaki,*,† Masayuki Fukuda,‡ Masayuki Omote,† Kayoko Yamauchi,† Takeshi Yagami,§ Mineo Niwa,‡,|| and Yukio Sugiura†

Contribution from the Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan, Institute for Medicinal Resources, The University of Tokushima, Tokushima, Tokushima 770-8505, Japan, and National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501, Japan

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Abstract: In this report, we describe a novel concept of extramembrane control of channel peptide assembly and the eventual channel current modulation. Alamethicin is a peptide antibiotic, which usually forms ion channels in various association states. By introducing an extramembrane leucine zipper segment (Alm-LeuZ), the association number of alamethicin was effectively controlled to produce a single predominant channel open state. The assembly was estimated to be a tetramer, by comparison of the channel conductance with that of the template-assembled Alm-LeuZ tetramer, which was prepared by the conjugation of a maleimide-functionalized peptide template with cysteine-derivatized Alm-LeuZ segments. Employment of an extramembrane segment of a random conformation provided higher levels of channel conductance. The result exemplified the possibility of channel current control by a conformational switch of the extramembrane segments.

Introduction

Natural receptor proteins and ion channel proteins serve in the transduction of biological signals across the cell membranes. These molecules are often constructed by the association of multiple homologous subunits to yield an organized structure. Interaction of a specific ligand may then induce a conformational switch to transmit specific ions in the cells as biological signals.

Preserving the features of natural receptor proteins in simplified peptide-based systems is a challenge in peptide engineering offering potential for creating novel molecular devices and channel protein models. Amphiphilic helical peptides derived from transmembrane segments of natural ion channel proteins[1] and from artificial design[2] have been shown to self-assemble in membranes to form channels. For example, the assembly of a mere ∼20-amino acid residue peptide can manifest a fundamental function of ion channel proteins, which are often composed of more than 10^3 amino acids. Self-assembly of the channel peptides often produces a channel of multiple open

* Corresponding author: (phone) +81-774-38-3211; (fax) +81-774-32-3038; (e-mail) futaki@scl.kyoto-u.ac.jp.
† Kyoto University.
‡ The University of Tokushima.
§ National Institute of Health Sciences.
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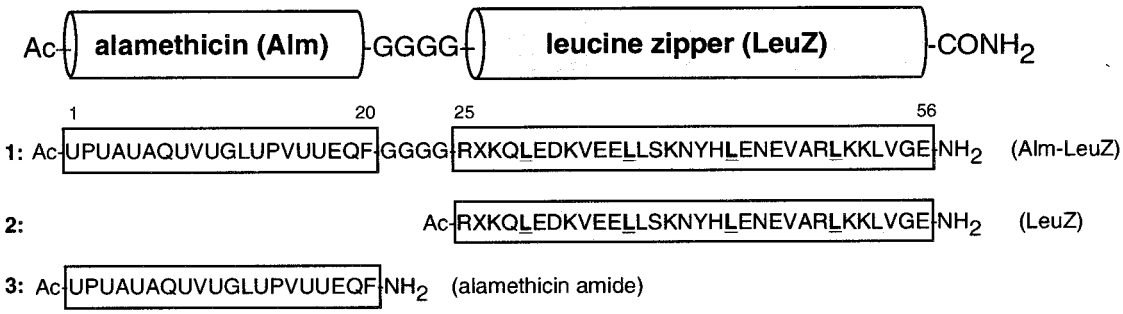

Figure 1. Amino acid sequence of the designed peptides. Residues defining the leucine repeat are underlined. Abbreviations for the amino acid residues: U, α-aminoisobutyric acid (Aib); X, norleucine (Nle); A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; V, Val; Y, Tyr.

states, where the difference in the association number (or the association state) may be detected as a difference in the channel conductance levels. To control the association number of channel peptides, template molecules have been effectively employed.[3] The application of natural pore-forming membrane proteins[4] and non-peptide-based molecules[5] as frameworks for the construction of artificial ion channels attracted our interest as a means for making designed channel pores, as well as to create ion channels in which the ion flux can be controlled by external stimuli, such as the binding of specific ligands and changes in the electrical transmembrane potential.[3c,e,f,6] In contrast to modifying a channel pore itself or the outside of a channel pore, a promising approach for the design of artificial ion channels and receptors may be developed by employing functional extramembrane segments to modify the channel peptides. The nicotinic acetylcholine receptor channel protein, for example, consists of five homologous subunits which have large extramembrane segments.[7] These extramembrane segments may be involved in the mutual recognition of the protein subunits to give the proper folding. Upon interaction with acetylcholine in an extramembrane segment, the protein switches its conformation. Subsequent opening of the channel pore results in the transduction of a neural signal in the form of an ion flux. The extramembrane segments serve to receive external signals and modulate the structure of the channels. However, few approaches have been reported on the design of artificial ion channels where extramembrane segments are utilized to play a role in the channel function.

We report here a simple system where a helical leucine zipper peptide[8] is attached to the ion channel peptide, alamethicin.[9] The leucine zipper segment plays the role of an extramembrane segment to determine the association number of the peptide in the membrane. The conformational differences in the extramembrane segment have been shown to be reflected in the channel conductance levels. This report demonstrates the potential of extramembrane segments to serve in the design of artificial channel and receptor proteins.

Results

Design and Preparation of the Hybrid Peptide. The structure of the alamethicin−leucine zipper hybrid peptide (Alm-LeuZ, **1**) is shown in Figure 1. The N-terminus sequence of the peptide is taken from that of alamethicin, which is one of the most extensively studied ion channel peptides. It is well known that alamethicin self-assembles in the membrane to form ion channels comprising several alamethicin molecules.[9] The association number of the assembly interconverts very frequently, a characteristic of the peptide that was selected to assess the effect of the extramembrane segments on the control of the assembly states of the transmembrane segment. Moreover, we chose an alamethicin segment as a transmembrane segment, because previous studies have been made on the assembly state of alamethicin itself[9] as well as to stabilize particular aggregation states using the covalently cross-linked alamethicin dimer[10] and the template-assembled alamethicin tetramer.[11] On the C-terminus, a leucine zipper segment (LeuZ) derived from a yeast

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transcription regulator protein, GCN4, was placed as an extramembrane segment.[8] The leucine zipper segment was selected because it is regarded as one of the simplest motifs in protein folding. The leucine zipper peptide has been well characterized to form a stable homodimer in water[8] and to interact little with membranes.[12] A $(Gly)_4$ linker was inserted between the alamethicin and the leucine zipper segments, which were assumed to adopt helical structures. The linker was expected to separate the helices to allow ions through the channel pore. If the two helical segments were continuously and directly connected, the number of the amino acid residues comprising the helix would be more than 50. It would be doubtful that such a long helix or the assembly of the helices could keep the structure stable. Even if it were possible, the assessment of the results would be complicated because ions would have to penetrate through the helical extramembrane segments before entering the channel pore. Glycine does not have a side chain. Presumably, the array of glycines is expected to form a favorable structure for the ions to pass through the linker part to enter the pores. We thus placed the flexible tetraglycine linker between the helices.

Peptide synthesis was conducted by Fmoc-solid-phase synthesis[13] on a Rink amide resin.[14] A protected peptide corresponding to positions 21−56 of **1** was prepared using a benzotriazole-1-yloxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP)[15]/1-hydroxybenzotriazole (HOBt)/4-methylmorpholine (NMM) coupling system. The segment corresponding to alamethicin (positions 1−20) contains Aib residues. Because of the steric hindrance at the C_α position of Aib, it is difficult to construct Aib-containing peptide segments by the usual coupling method. Therefore, the peptide chain was elongated manually using Fmoc-amino acid fluorides.[16] Treatment of the protected peptide resin with 1 M trimethylsilyl bromide/thioanisole in trifluoroacetic acid[17] in the presence of *m*-cresol and 1, 2-ethanedithiol, followed by HPLC purification, gave the desired product of high purity (>98%). The fidelity of the product was ascertained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). The yield from the starting resin was 4%. The CD spectra of the peptide **1** in 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4) and in the presence of liposomes prepared from egg yolk phosphatidylcholine are shown in Figure 2a. The concentration of liposomes (∼1 mM) was chosen by referring to the reports of Woolley[6b,27] and Iwata[2d] so that alamethicin would sufficiently form a helical structure on the interaction with the membranes. The helical content of **1** in the presence of liposomes ($[\theta]_{222}$, -2.5×10^4 deg·cm^2/dmol; peptide concentration, 7 μM) was almost 5 times as large as in the absence of liposomes ($[\theta]_{222}$, -5.2×10^3 deg·cm^2/dmol; peptide concentration, 7 μM), suggesting that **1** would cause a certain structural change by the interaction with membranes. On the other hand, the CD spectra of the peptide **2** corresponding to the extramembrane LeuZ segment (positions 25−56) in the presence and absence of the liposomes (Figure 2b) were substantially identical ($[\theta]_{222}$, -3.0×10^4 deg·cm^2/dmol; peptide concentration, 23 μM), suggesting that the LeuZ sequence in Alm-LeuZ assumed an almost fully helical conformation and interacted little with the lipid membrane, as reported.[8a,12] Considering the CD spectrum of alamethicin in the presence of liposomes ($[\theta]_{222}$, $\sim -1.4 \times 10^4$ deg·cm^2/dmol),[27] the CD of Alm-LeuZ in the presence of liposomes was estimated to be almost the sum of those for alamethicin and the LeuZ peptide **2**. The result suggested that, after insertion of the alamethicin segment into the membrane, the alamethicin and LeuZ segments in Alm-LeuZ **1** would maintain their intrinsic structures without giving serious interference to each other.

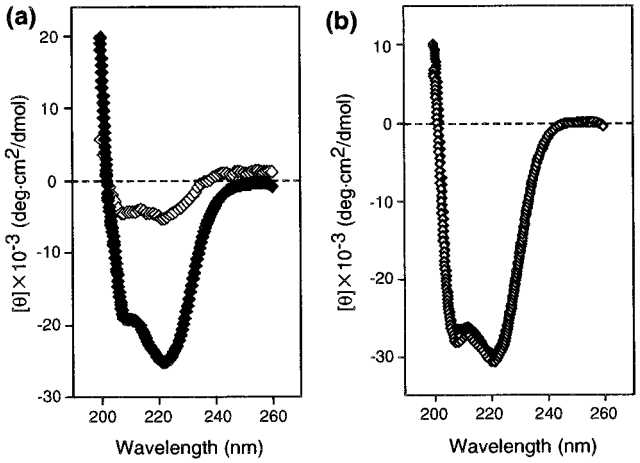

Figure 2. CD spectra of the Alm-LeuZ hybrid peptide **1** (7 μM) (a) and the LeuZ peptide **2** (23 μM) (b) in liposomes (closed diamond) and in 5 mM HEPES (pH 7.4) (open diamond), respectively. Liposomes were made from egg yolk phosphatidylcholine in 5 mM HEPES (pH 7.4). Lipid concentration, ∼1 mM.

Properties of the Channel Formed by the Alm-LeuZ Hybrid Peptide. The association state of the transmembrane peptides was assessed by observing ion channel current using the planar lipid bilayer method.[18] This technique is equivalent to the "patch-clamp" system[19] for monitoring the ion flux going through a single-channel pore in real time. A difference in the association state of channel-forming peptides would cause a difference in the channel pore structure, which should be reflected in the channel current. Figure 3a shows a single-channel current recording of the alamethicin amide **3** (applied voltage, +100 mV; peptide concentration, 10 nM). Besides the current level corresponding to the closed state (close), multiple levels of channel current (open 1−4) were observed. Such multiple current levels are typically observed in the channels of the alamethicin-related peptides, which are attributed to the existence of various channel pore structures with different association numbers or different conformations of the alamethicin molecules in the membranes.[9] A channel with a larger association number of the alamethicin molecules would create a larger pore size and an eventual higher channel current level. The frequent fluctuation in the current levels and the repetitiveness of several particular current levels were indicative of continual interconversion between the several metastable assembly states of alamethicin in the membrane. Although the channel recording of alamethicin amide **3** in Figure 3a appears to show several channel pores simultaneously open, the channel

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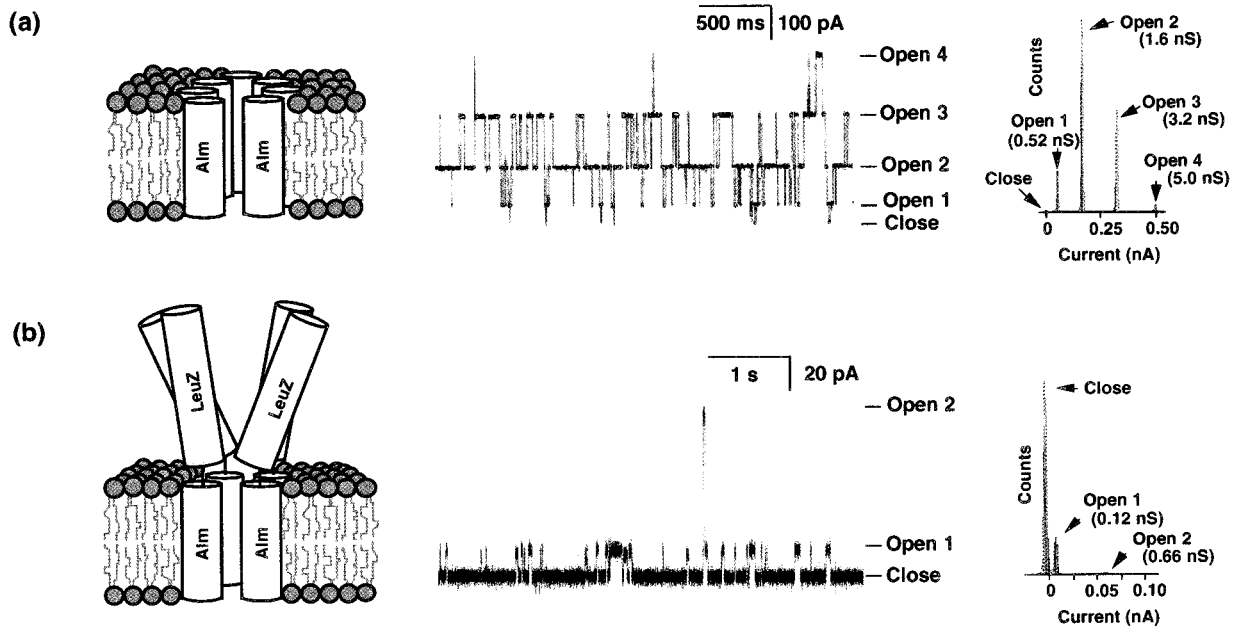

Figure 3. Schematic representation of a possible association state of alamethicin amide **3** (a) and Alm-LeuZ **1** (b) (left) and single-channel currents from lipid bilayers containing the respective peptides [**3**, 10 nM (a); **1**, 50 nM (b)] at +100 mV (center). The current histograms are shown at the right of the respective records. Electrolyte, 1 M KCl. Number of experiments (*n*) was 9 and 21 for **3** and **1**, respectively.

current is attributed to a single-channel pore. If the channel currents were those going through more than a one-channel pore, we would see current levels twice as high as the respective channel levels, especially that for open 1. This is not the case; therefore, the observed current was deduced to be of the peptide assembly comprising a single-channel pore.

On the other hand, the single-channel recording of the Alm-LeuZ hybrid peptide **1** is shown in the Figure 3b. With a marked difference from the case of alamethicin amide **3**, a channel recording of **1** (50 nM) showed a single predominant open state designated as open 1. Association of a different number of pore-forming peptides should result in different channel current levels. Therefore, by introdution of the leucine zipper extramembrane segment, the association number of the alamethicin segment in the membrane was effectively controlled and a particular association state of the hybrid peptide was stabilized. A higher current level (open 2) was also observed, albeit less frequently, and may suggest the presence of a channel state of a higher association number or alternative conformation. No channel current was observed when the peptide corresponding to the LeuZ segment **2** was added to the electrolyte (peptide concentration, 100 nM), suggesting that the LeuZ segment cannot form an ion channel and that the current observed for Alm-LeuZ was attributed to the transmembrane alamethicin molecules.

The current−voltage relationship for the open 1 of the hybrid peptide **1** is shown in Figure 4. A significant channel current has been observed only at the positive voltages as is observed in the natural alamethicin channel.[9b] This fact strongly suggested that the hybrid peptide **1** was incorporated into the membrane from the N-terminal side (alamethicin side) of the molecule. The modulation of assembly was both voltage- and concentration-dependent. When higher voltages or higher peptide concentrations were applied, the more frequently open 2 or higher current levels were observed (Figure 5). In these cases, the observable association or conformation states were more organized compared to alamethicin amide **3**, which lacked the extramembrane segment (Figure 3a).

Consideration of the Association Number. The above results suggested that the association number of alamethicin was

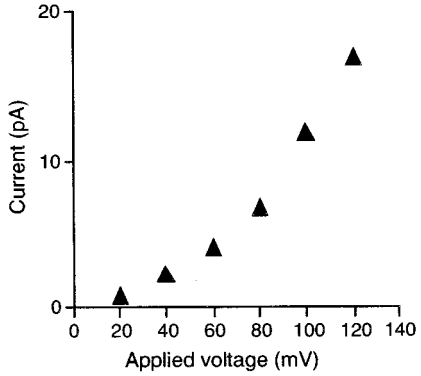

Figure 4. Current−voltage relationship of the Alm-LeuZ channel (open 1) in 1 M KCl. The peptide was added to the solution at one side of the membrane (cis side).

effectively controlled by introducing an α-helical extramembrane segment. How many molecules of the hybrid peptides are then assembled to form a channel structure? The concept of a template-assembled synthetic protein (TASP) proposed by Mutter[20] would be a useful approach to obtain a peptide assembly of a predefined association number. On the basis of this approach, Matsubara et al. synthesized an alamethicin tetramer using an aminobenzoate-based template molecule.[11] The channel conductance of the tetramer corresponded well to that for the open 1 of the Alm-LeuZ hybrid peptide **1**. This was also comparable to the conductance that is attributed to the tetramer assembly of natural alamethicin.[9b,11] Based on these comparisons, the association number of the Alm-LeuZ was deduced to be a tetramer. However, the possibility remains that the extramembrane segment might influence the channel conductance, even though the $(Gly)_4$ linker should diminish the influence of the extramembrane segment on the channel conductance. To answer this question, we synthesized a template-assembled Alm-LeuZ tetramer **6**.

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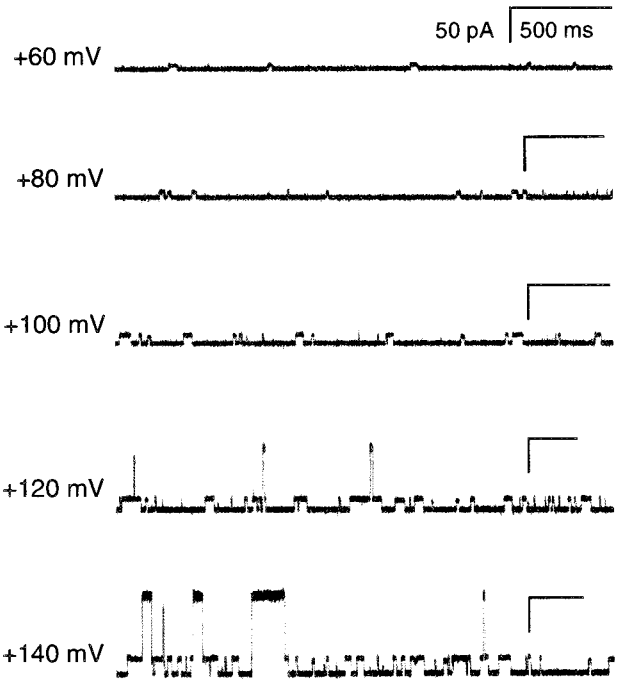

Figure 5. Effect of voltage on the modulation of the assembly of **1** (peptide concentration, 50 nM). Electrolyte, 1 M KCl.

The synthetic scheme of the Alm-LeuZ tetramer is shown in Figure 6. A peptide segment bearing an extra Gly-Gly-Cys linker on the C-terminus of the Alm-LeuZ peptide (Alm-LeuZ-GGC **1′**) was prepared by solid-phase synthesis. The peptide **1′** consisted of 59 residues and had a molecular weight of 6226. To tether four molecules of such a large peptide on a template would be difficult without using an efficient cross-linking chemistry.[21] The reaction of maleimide with thiol was selected for the tethering because the chemistry is quite often employed in the cross-linking between proteins.[22] Application of this chemistry to the attachment of an artificial four-helix-bundle protein on a gold surface was also reported.[23] A template having four maleimide moieties **5** was then prepared by modifying template **4** (Ac-Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-Gly-NH_2)[20,21] with a heterobifunctional cross-linking agent, *N*-(4-maleimidobutyryloxy)succinimide (GMBS). The mass of **1′** and maleimide-functionalized template **5** were ascertained by MALDI-TOFMS. Incorporation of Alm-LeuZ-GGC segments on **5** was conducted using 4 equiv of the segment **1′** in 6 M guanidine hydrochloride (GnHCl) containing 50 mM 2-morpholinoethanesulfonic acid (MES; pH 6.0) under an argon atmosphere. Even using the highly reactive chemical species, it was difficult to bring the reaction to completion by incubation at room temperature. Most of the products in this case were molecules where only two or three Alm-LeuZ segments were introduced on the template. By incubation of the segment and template at 37 °C for 120 h, we were successful in carrying the reaction almost to completion. HPLC purification of the product gave pure (>98%) Alm-LeuZ tetramer **6** in a yield of 37%.

The protein was detected as a single band by SDS−PAGE with an apparent molecular weight comparable to the theoretical value (26 489). No bands corresponding to the trimer and dimer of Alm-LeuZ attached on the template were observed on the gel. Unfortunately, attempts to determine the precise molecular weight of the final product **6** using MALDI-TOFMS or electrospray-ionization mass spectrometry were unsuccessful, probably due to the poor ionization tendency of peptides and proteins with stable secondary structures[24] or high hydrophobicity.[25] Only a broad peak between 26 500 and 25 000 was observed in the MALDI-TOFMS spectrum. The detected broad peak may be ascribed to the strong laser power required for ionization. The integrity and purity of the final product **6** was, however, sufficiently supported by the following facts: (i) **1′** and the maleimide-template **5** were pure (>98 and >97%, respectively, as determined by analytical HPLC) and characterized by TOFMS, respectively, (ii) cross-link formation between a maleimide and a thiol is a well-established reaction, and (iii) no byproducts, including those from insufficient introduction of the segment **1′** on the template (monomer, dimer, and trimer of Alm-LeuZ), were recognized in the final product **6** by HPLC,

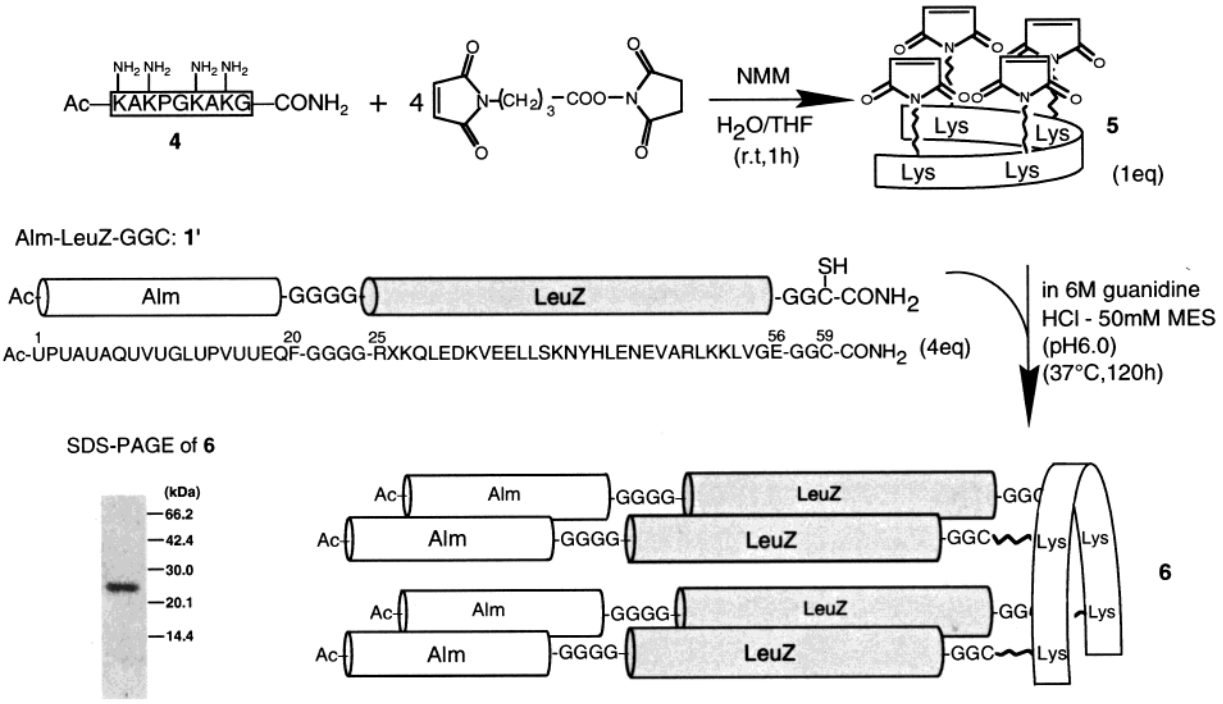

Figure 6. Design and synthesis of the template-assembled Alm-LeuZ tetramer **6**.

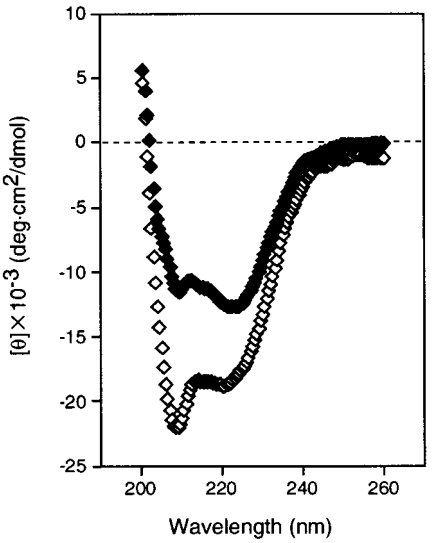

Figure 7. CD spectra of the template-assembled Alm-LeuZ tetramer **6** (2.5 μM) in methanol (open diamond) and liposomes (closed diamond). Liposomes were made from egg yolk phosphatidylcholine in 5 mM HEPES (pH 7.4). Lipid concentration, ~1 mM.

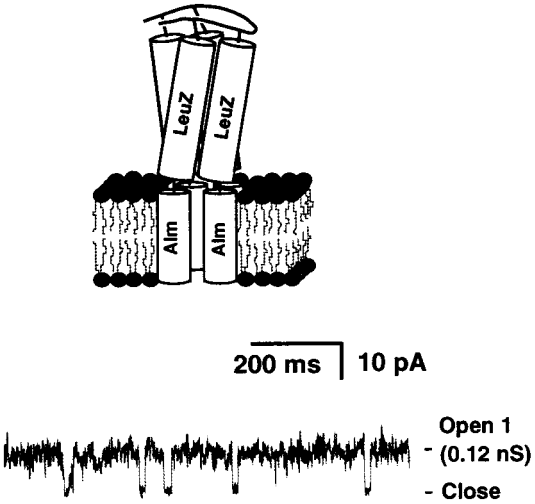

Figure 8. Schematic representation and a single-channel recording of the template-assembled Alm-LeuZ tetramer **6** (1 nM) at +100 mV (n = 5). Electrolyte, 1 M KCl.

SDS−PAGE, and MALDI-TOFMS. The CD spectra of protein **6** in the liposomes and methanol suggested that the Alm-LeuZ segments in tetramer protein **6** formed a helical structure (Figure 7). It is not clear at this stage why the helical content of the template-assembled Alm-LeuZ tetramer **6** is smaller than Alm-LeuZ **1**. One possible explanation is that the close disposition of the LeuZ segments by the template may give some steric hindrances among the segments to influence on their structures.

Figure 8 shows a typical channel recording of the Alm-LeuZ tetramer **6**. Though burstlike channel currents were sometimes observed, presumably because the template may tether the four Alm-LeuZ peptides too rigidly, a long durable open state (open 1) corresponding well to that of untethered **1** was predominantly observed. We thus concluded that the assembly of Alm-LeuZ corresponding to the open 1 would be most probably that of a tetramer.

Reflection of Conformational Differences in the Extramembrane Segment in the Channel Current. If a conformational change in the extramembrane segment could affect the channel current, such systems may be developed as artificial receptor systems. To examine whether such an idea is possible, we designed another two hybrid peptides, Alm-[cFos]LeuZ (**7**) and Alm-[Gly]LeuZ (**9**) (Figure 9). The former peptide has a cFos leucine zipper peptide at the C-terminus of alamethicin as the extramembrane segment. cFos is a cancer-related protein that does not form a stable homodimer and preferably forms heterodimers with other proteins such as cJun.[26] The molecular ellipticity at 222 nm of the leucine zipper peptide **8** derived from the cFos sequence in the presence of liposomes turned out to be about one-third that of **2** ($[\theta]_{222}$, -0.8×10^4 deg·cm^2/dmol) (Figure 10), which was in contrast to the extramembrane segment of **1** that exhibited an almost fully helical structure. The peptide **8** in 5 mM HEPES (pH 7.4) gave a CD spectrum slightly different from that in the presence of liposomes, suggesting that the segment may have some interaction with membranes (Supporting Information).

In **9**, leucine residues, which play a crucial role in the formation of the α-helical coiled-coil structure in the heptad repeat of the zipper segment ("d" positions[8]), were replaced with glycines. The replacement of leucines with glycines destabilized the helical conformation. The CD spectrum of peptide **10**, which corresponds to the extramembrane segment of **9**, in the presence of liposomes was suggestive of a random structure (Figure 10). The CD spectrum of **10** in 5 mM HEPES (pH 7.4) was also suggestive of a random structure (Supporting Information).

A typical channel current record of **7** at the applied voltage of +100 mV is shown in Figure 11a. Although the extramembrane segment exhibited a less ordered structure than that of **1** and was not expected to form a stable dimer between the extramembrane segments, the assembly was still organized to produce one predominant open state (open 1) at +100 mV. This current level corresponded well to that of **1**. Channel current for [cFos]LeuZ peptide **8** was not observed at a peptide concentration of 100 nM.

Compared with **8**, the modulation of the assembly was less efficient in the case of **9** at the same applied voltage. A larger channel current level corresponding to the open 2 of **1** or sometimes higher levels was prominently observed (Figure 11b). The result exemplified that the conformational alternation in the extramembrane segments between helix and random coil can be reflected in the ion channel current, which strongly suggested the probability of switching ion channel current by extramembrane conformational alternation.

Discussion

Although considerable attention has been directed toward the construction of artificial receptors and ion channels, few approaches have focused on the effect of the extramembrane segment on the stabilization of channel structure and channel current regulation. In this paper, we have demonstrated that the assembly of membrane peptides can be effectively controlled with the use of a suitable extramembrane segment. Conformational differences in the extramembrane segments were associ-

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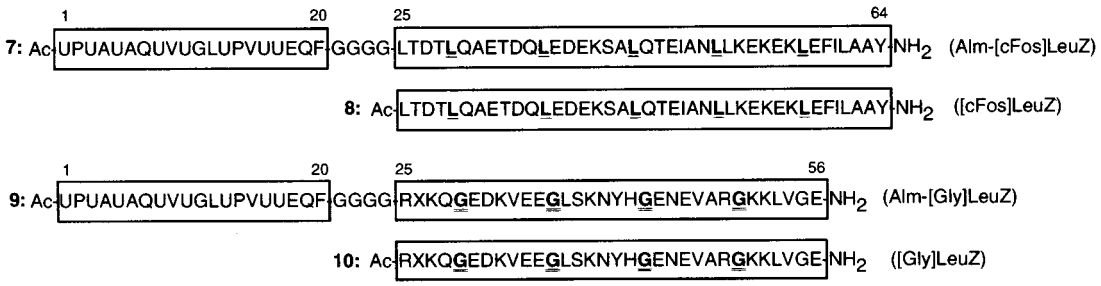

Figure 9. Amino acid sequences of Alm-[cFos]LeuZ **7**, Alm-[Gly]LeuZ **9**, and the peptides corresponding to the respective extramembrane segments (**8** and **10**). Residues defining the leucine repeat are underlined. Glycine residues substituting for the leucine residues are double-underlined. Abbreviations for the amino acid residues: U, α-aminoisobutyric acid (Aib); X, norleucine (Nle); A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr.

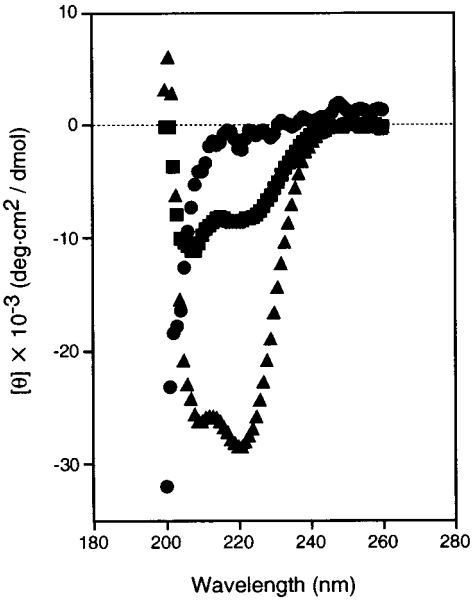

Figure 10. CD spectra of the peptides corresponding to the extramembrane segments of the hybrid peptides in the presence of liposomes: LeuZ **2** (15 μM), closed triangle; [cFos]LeuZ **8** (18 μM), closed square; [Gly]LeuZ **10** (24 μM), closed circle. Liposomes were made from egg yolk phosphatidylcholine in 5 mM HEPES (pH 7.4). Lipid concentration, ~1 mM.

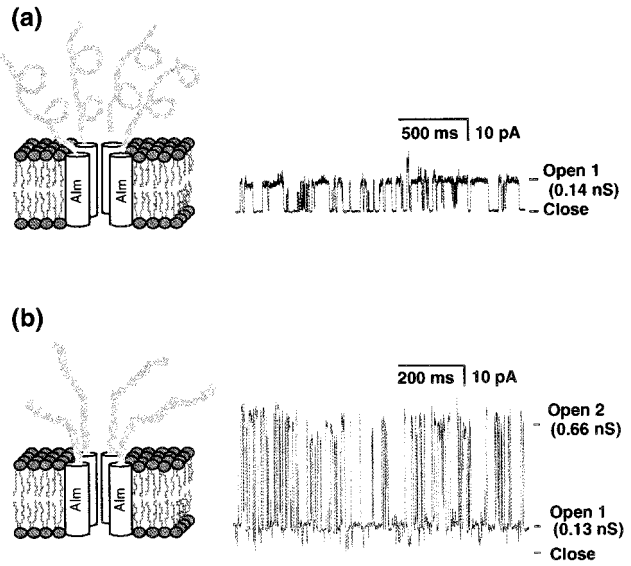

Figure 11. Schematic representation of a possible association state and a single-channel recording of Alm-[Fos]LeuZ **7** (10 nM) ($n = 5$) (a) and Alm-[Gly]LeuZ **9** (3 nM) ($n = 6$) (b) at +100 mV. Electrolyte, 1 M KCl.

ated with modifications in channel current. Because it may be possible to design peptide segments that switch conformation on receiving external stimuli, the potential exists to regulate transmission of signal through the membrane as channel current. By controlling extramembrane assembly, supramolecular protein structures may be constructed that function as artificial receptors.

The GCN4-derived leucine zipper was reported to form a stable homodimer[8a] and interact little with membranes.[12] Because the assembly of Alm-LeuZ was deduced to be that of a tetramer, it would be plausible that two dimers of Alm-LeuZ formed a channel. Natural alamethicin was reported to form channels comprising up to 10 molecules.[9] The association of the transmembrane segments of **1** is thus regulated by the extramembrane domain, which, because of the GCN4-derived leucine zipper segment, has a strong tendency to form a dimer. When the dimers come close together in the tetramer formation, a certain steric hindrance may arise that prevents complete assembly of the hybrid peptide and creates a metastable tetramer assembly. The stabilization of the tetramer assembly may therefore be contingent on dimer formation and the associating nature of the transmembrane segments. In the association of **9**, steric hindrance between the extramembrane segments may contribute to assembly modulation.

You et al. reported that a covalently cross-linked alamethicin dimer predominantly formed a hexamer (three dimers),[10] whereas **1** was suggested to form a tetramer. The alkyl chain linkers used to tether the alamethicin molecules are much less hindered than extramembrane segments and may account for the difference in association number.

The helical content of the peptide corresponding to the extramembrane segment of **7** was about one-third in the presence of liposomes (Figure 10), but the predominant channel conductance corresponded well with that of **1**. A helical content of ~30% may be the minimum criterion for the above association control. Whether the dimer formation is indispensable for assembly stabilization is not clear at this point. O′Shea reported that the cFos leucine zipper peptide can form a homodimer with a dissociation constant of ~6 μM.[26] It may be possible that the leucine zipper segment of Alm-[cFos]LeuZ becomes concentrated on the membrane to form a dimer in accordance with alamethicin molecules assembling in the membrane. Further study using, for example, ultracentrifuge or size exclusion chromatography may shed light on the mechanism of assembly control by the extramembrane segments. However, consideration should be necessary on the structural alternation of Alm-LeuZ in the presence and absence of lipids as shown in Figure 2a. The effect of the possible aggregation of the alamethicin segment in aqueous buffer should also be taken into account.

In conclusion, the importance of extramembrane structure on assembly and signal transmittance has been demonstrated using the artificial receptor protein Alm-LeuZ. Steric hindrance between the extramembrane domains has also been suggested to influence supramolecular structural formation of membrane proteins. These results offer a novel concept for designing artificial sensor systems and for studying the mechanisms of natural channel proteins. Therefore, Alm-LeuZ represents a prototype for the construction of artificial receptors.

Experimental Section

Preparation of Alamethicin−Leucine Zipper Hybrid Peptides. Peptide synthesis was conducted by Fmoc-solid-phase synthesis[13] on Rink amide resin.[14] A protected peptide corresponding to positions 21−56 of **1** was prepared by a Shimadzu PSSM-8 synthesizer using a PyBOP/[15]HOBt/NMM coupling system. The peptide chain was then elongated manually using the Fmoc-amino acid fluoride method[16] to construct an alamethicin segment. N-Terminal acetylation was conducted using acetic anhydride and NMM (10 equiv, each) in DMF at room temperature for 30 min. The obtained protected peptide resin for **1** was treated with 1 M trimethylsilyl bromide/thioanisole in trifluoroacetic acid[17] in the presence of *m*-cresol and 1,2-ethanedithiol at 0 °C for 1.5 h, followed by HPLC purification on a YMC protein-RP column (yield from the starting resin, 4%). As judged by the analytical HPLC, the purity was estimated to be >98%. The HPLC condition and retention time together with those for other peptides are shown in the Supporting Information. The fidelity of the product was ascertained by MALDI-TOFMS using a Voyager RP spectrometer (Applied Biosystems) [calcd for $(M + Na)^+$ 6014.9; found, 6014.3]. Other peptides were prepared basically as stated above. The purity of each peptide was estimated to be at least more than 96% from analytical HPLC. MALDI-TOFMS for Alm-LeuZ-GGC **1′**, LeuZ **2**, alamethicin amide **3**, Alm-[cFos]LeuZ **7**, [cFos]LeuZ **8**, Alm-[Gly]LeuZ **9**, and [Gly]LeuZ **10**: 6227.4 [calcd for $(M + H)^+$ 6227.1], 3866.1 [calcd for $(M + Na)^+$ 3868.4], 1998.7 [calcd for $(M + Na)^+$ 2000.3], 6777.9 [calcd for $(M + Na)^+$ 6777.6], 4607.9 [calcd for $(M + Na)^+$ 4608.2], 5786.2 [calcd for $(M + H)^+$ 5785.4], and 3639.7 [calcd for $(M + H)^+$ 3640.0], respectively.

Maleimide Template 5. GMBS (7.6 mg, 27 μmol) (purchased from Dojindo, Kumamoto, Japan) in tetrahydrofuran (200 μL) was added to an aqueous solution (100 μL) of Ac-Lys-Ala-Lys-Pro-Gly-Lys-Ala-Lys-Gly-NH_2[21] (**4**; 5.0 mg, 5.4 μmol) containing NMM (2 μL, 22 μmol). The mixture was stirred at room temperature for 30 min and then purified by HPLC to give the maleimide template **5** (2.5 mg, 29%) (purity, >97% as estimated from HPLC analysis). MALDI-TOFMS 1586.7 [calcd for $(M + H)^+$ 1586.0].

Construction of the Template-Assembled Alm-LeuZ Tetramer 6. Alm-LeuZ-GGC **1′** (760 μg, 0.12 μmol) and maleimide template **5** (50 μg, 0.031 μmol) were reacted in 6 M GnHCl/50 mM MES (pH 6.0) (40 μL) under an argon atmosphere at 37 °C for 120 h. HPLC purification of the product using a column of TSKgel Octadecyl-4PW (4.6 × 150 mm) and lyophilization afforded a pure template-assembled Alm-LeuZ tetramer **6** (300 μg, 0.011 μmol; 37%) (purity, >98% as estimated from HPLC analysis). The protein was detected as a single band on SDS−PAGE with an apparent molecular weight comparable to the theoretical value (26 489) (Figure 6).

CD Measurements. CD spectra were recorded on a Jasco J-600 spectrometer using a 2-mm cuvette at 20 °C. Liposomes were prepared from egg yolk phosphatidylcholine in the presence of 5 mM HEPES (pH 7.4) as reported [final concentration of the lipid, 0.84 mg/mL (~1 mM)].[2d]

Channel Activity Measurements. Planar lipid bilayers were formed by the painting method.[18b] Diphytanoylphosphatidylcholine dissolved in decane (20 mg/mL) was used as a bilayer-forming lipid. Solutions were unbuffered KCl, and all the measurements were done at room temperature (~22 °C). A small quantity of the peptide in methanol (usually 1−5 μL) was added to the electrolytes at one side of the membrane (designated as the cis side). The applied voltage was defined as the voltage of cis with respect to the compartment of the other side (trans). Channel conductance is defined as the membrane current divided by the applied voltage. The membrane current was measured under voltage clamp conditions using 1-kHz filtering and sampling at 5 kHz.

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Supporting Information Available: HPLC conditions and retention times for the synthesized peptides and CD spectra of **8** and **10** in the presence and absence of liposomes. This material is available free of charge via the Internet at http://pubs.acs.org.

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