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Extramembrane Control of Ion Channel Peptide Assemblies, Using Alamethicin as an Example

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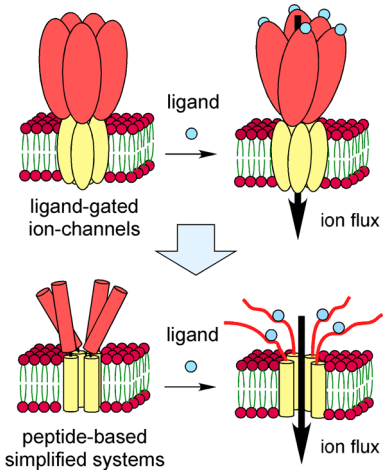
CONSPECTUS

Ion channels allow the influx and efflux of specific ions through a plasma membrane. Many ion channels can sense, for example, the membrane potential (the voltage gaps between the inside and the outside of the membrane), specific ligands such as neurotransmitters, and mechanical tension within the membrane. They modulate cell function in response to these stimuli. Researchers have focused on developing peptide- and non-peptide-based model systems to elucidate ion-channel protein functions and to create artificial sensing systems.

In this Account, we employed a typical peptide that forms ion channels, alamethicin, as a model to evaluate our methodologies for controlling the assembly states of channel-forming molecules in membranes. As alamethicin self-assembles in membranes, it prompts channel formation, but number of peptide molecules in these channels is not constant. Using planar-lipid bilayer methods, we monitored the association states of alamethicin in real time.



Many ligand-gated, natural-ion channel proteins have large extramembrane domains. As these proteins interact with specific ligands, those conformational alterations in the extramembrane domains are transmitted to the transmembrane, pore-forming domains to open and close the channels. We hypothesized that if we conjugated suitable extramembrane segments to alamethicin, ligand binding to the extramembrane segments could alter the structure of the extramembrane domains and influence the association states or association numbers of alamethicin in the membranes. We could then assess those changes by using single-channel current recording. We found that we could modulate channel assembly and eventual ion flux with attached leucine-zipper extramembrane peptide segments. Using conformationally switchable leucine-zipper extramembrane segments that respond to Fe^{3+}, we fabricated an artificial Fe^{3+}-sensitive ion channel; a decrease in the helical content of the extramembrane segment led to an increase in the channel current.

When we added a calmodulin C-terminus segment, we formed a channel that was sensitive to Ca^{2+}. This result demonstrated that we could prepare artificial channels that were sensitive to specific ligands by adding appropriate extramembrane segments from natural protein motifs that respond to external stimuli.

In conclusion, our research points to the possibility of creating tailored sensor or signal transduction systems through the conjugation of a conformationally switchable extramembrane peptide/protein segment to a suitable transmembrane peptide segment.

1. Introduction

Plasma membranes serve as a barrier between the inside and the outside of cells. Cell membrane proteins, including ion channels, transporters, and receptors, play important roles in transporting biological signals across membranes.[1,2] These proteins can contain several subunits. Given the difficulty of analyzing the three-dimensional (3D) structures of these proteins, for example by crystallography, nuclear magnetic resonance (NMR) spectroscopy, or electron microscopy, substantial uncertainty remains regarding the mechanisms for selective transmission of stimuli from the exterior of membranes into cells. Ion channels transmit signals through

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ion flux. The channel pores are paths that allow ions to pass through a membrane; in most cases, they also have filter functions that allow the penetration of specific inorganic ions (typically, Na^+, K^+, Ca^{2+}, and Cl^-). Many ion-channel proteins are equipped with gating machinery that controls ion influx and efflux in accordance with changes in membrane potential, ligand binding, and mechanical tension. For example, the nicotinic acetylcholine receptor (nAChR) channel protein comprises an assembly of five subunits that span membranes to form a pore in the center of the assembly.[3] The interaction of acetylcholine with the alpha subunit of the channel protein leads to a structural alteration in the extramembrane domain of the channel protein, opening of the channel pore, and the eventual influx of sodium ions into cells.[4] Thus, stimuli from nerve termini are transmitted to postsynaptic cells, and neurotransmission is accomplished.

Modeling the functions of ion channels with simplified systems is a challenge. To model ion-channel functions using synthetic molecules and to create novel signal-transmitting and sensing devices, a variety of artificial ion-channel types has been reported.[5−10] Channels and pores with gating controllable by external stimuli such as temperature, light, pH, small molecular weight ligands or metals have also been reported.[11−17] While nonpeptide/protein-based artificial ion channels may provide unique frameworks for ion channels, peptide/protein-based artificial ion channels enable the utilization of natural channel protein structural motifs. The availability of solid-phase peptide synthesis (SPPS),[18] gene manipulation, and single-channel recording using the planar lipid bilayer method[19] or the patch-clamp technique[20] may also facilitate the design, synthesis, and evaluation of these channels. We used the natural antimicrobial peptide alamethicin as a framework for artificial ion channels. Through the attachment of extramembrane segments, we endowed the original channel peptide with unique characteristics. In this Account, we describe the motivation for our interest in alamethicin channels and introduce our approach.

2. Historical Surveys of Peptide/Protein-Based Artificial Ion Channels and Their Application

Studies of the behavior of ion channels formed by antimicrobial peptides (e.g., alamethicin[21] and gramidicin[22]) were reported, along with development of an electrophysiological technique using planar lipid bilayers (black lipid membranes), as early as the 1970s.[23] In 1988, Oiki et al. reported the self-assembly of a simple, 23-residue peptide corresponding to a putative transmembrane domain of a nAChR and observed that the synthetic channel exhibited features characteristic of an authentic nAChR channel, including single-channel conductance, discrimination of cations over anions, and channel lifetimes for open and closed states in the millisecond time range.[24] At that time, only the primary structure of the channel was available, and little was known regarding its 3D structure. The study by Oiki et al. not only suggested the amino acid sequences responsible for channel pore formation but also opened avenues for constructing artificial channels with native channel-like behaviors through self-assembly of appropriate peptides. This possibility was exemplified by Lear et al., who designed a de novo amphiphilic 21-residue peptide comprising only serine (Ser) and leucine (Leu), H_2N-(Leu-Ser-Ser-Leu-Leu-Ser-Leu)$_3$-$CONH_2$, which formed channels that behaved like those observed in nAChR.[25] As alternatives to approaches that simulate channel pores by using helical peptides, methods based on forming stacks of cyclic peptides in membranes to yield nanotubes have also been developed.[26] In addition, Bayley and co-workers developed approaches that utilize natural membrane-interacting proteins (e.g., α-hemolysin) as frameworks for artificial gates in membranes.[27] Successful stochastic sensing of analytes passing through chemically modified pores of proteins has been accomplished by using ion-flux alteration by the interaction of analytes with the modified pores. Following these pioneering reports, various approaches have been developed for the creation of novel sensing devices that use ion channels. In particular, applications in DNA sequencing have become an extensively studied topic.[5−10]

3. Alamethicin as a Channel Framework

Alamethicin [Alm; acetyl-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol (Phol = phenylalaninol)] is a 20-residue helical peptide that is rich in α-aminoisobutyric acid (Aib) from the fungus *Trichoderma viride*, and is a member of the channel-forming antimicrobial peptides known as peptaibols.[21,22] The channel-forming behaviors of Alm have been studied in detail since the 1970s.[23] Alm self-associates in lipid bilayers to form channels in a voltage-dependent manner. The channel formation is understood in terms of the "barrel-stave" or "helix-bundle" model, in which channels are formed by 3−12 helical monomers to yield parallel bundles surrounding a central aqueous pore.[28] Owing to the successive association and dissociation of Alm molecules in membranes, the number of Alm molecules that form a channel is not constant and

varies continuously. Therefore, multiple conductance levels and frequent interchange are usually observed during analysis of Alm channels. Each conductance level corresponds to an associated state of different numbers or different conformations of Alm molecules.

We have been interested in developing control methods for assembly structure formation and gating of channel-forming peptides, and these methodologies may elucidate the mechanisms of the structural formation and functional exertion of natural membrane proteins, including ligand-gated ion channels.[29] A better understanding of the self-assembly process should also benefit the development of artificial ion-channel-based receptors and sensors. The synthesis of Alm is labor intensive; the steric hindrance of the Aib residue at the α-carbon in Alm necessitates that special care be taken during solid-phase synthesis, such as the employment of amino acid fluoride[30] for peptide chain elongation. Despite the labor-intensive synthesis of Alm, we have employed Alm as a framework in our research for the following reasons. (i) The characteristics of Alm channels have been studied in detail, allowing us to use the extensive information available during the design and evaluation of channel systems. (ii) Differences in the numbers of Alm molecules forming a single channel should yield different pore sizes, thereby yielding a corresponding channel current (or channel conductance) that is detectable in real time by using the planar lipid bilayer method. The assembly number and eventual channel conductance of Alm in membranes are subject to change. The association states of the channel peptide forming a single channel are detectable in real time using channel current levels in the planar-lipid-bilayer method. Therefore, the fidelity of our approach can be analyzed by studying changes in the association states of the channels. (iii) The channel conductance of Alm (typically between 0.1 and a few nanosiemens) is significantly higher than that of natural ion-channel proteins (typically $\sim$40 pS in the case of nAChR). Because analysis of a single channel at lower conductance levels requires careful removal of electrical noise, the higher conductance of Alm facilitates easier analysis of channel characteristics. (iv) Alm molecules are easily incorporated into lipid bilayers by the addition of Alm to electrolytes, which further facilitates the analysis of Alm channel characteristics. Difficulty in membrane insertion and the low probability of channel formation has often been an obstacle for channel current measurement using the planar-lipid-bilayer method. (v) Alm usually inserts into membranes from its N-terminus in a voltage-dependent manner when a negative voltage is applied to the *trans* side of the membrane (compared with the peptide addition, or *cis*, side), thereby enabling one-directional insertion of Alm molecules into membranes. Characteristics (i)−(v) of Alm are reviewed in detail in references 21, 22, and 28.

4. Assembly Control of Alm Using Extramembrane Segments

As described above, assembly of a 23-residue peptide corresponding to a transmembrane segment of a nAChR channel protein exhibited conductance and kinetics characteristics very similar to those of a nAChR channel.[24] In a nAChR channel, conformational alteration of the extramembrane segments caused by interaction with its ligand acetylcholine is transmitted into the transmembrane segments to stabilize the open state of the channel.[4] We hypothesized that if we attached an appropriate extramembrane segment to a channel-forming peptide and if the extramembrane segment could induce conformational alteration by some stimuli, the conformational alteration in the extramembrane segment would affect the assembly state of the channel-forming peptide and thus its channel current. To evaluate this working hypothesis, we prepared hybrid peptides of Alm and leucine-zipper peptides as a model.[31]

The leucine-zipper motif is one of the simplest protein motifs formed by the association of two amphiphilic helical segments of about 30−40 residues.[32] In this motif, hydrophobic leucine residues occur every seven residues. The leucine residues form hydrophobic cores in the presence of additional hydrophobic amino acids. Hydrophobic interactions between two helices stabilize the coiled-coil dimer structure. One of the best-studied leucine-zipper peptides that forms a stable homodimer is derived from the yeast transcription factor GCN4.[33] We employed this segment as the extramembrane segment of Alm and evaluated the influence of its presence on peptide assembly. Various approaches have been reported to create novel channel systems based on Alm.[34,35] Most of these approaches were designed to modify the pore linings or orifices. In addition, several approaches have been reported that utilize cross-linking of ion-channel-forming peptides and other molecules to stabilize membrane assembly.[36−42] Before our study, few reports had evaluated the influence of extramembrane segments on channel assembly and whether they effectively utilize the conformational alteration of extramembrane segments in channel assembly.

A hybrid peptide composed of Alm and a GCN4 leucine zipper (Alm-LeuZ)[29,31] was prepared using Fmoc-SPPS (Fmoc = fluorenylmethyloxycarbonyl). While peptide segments

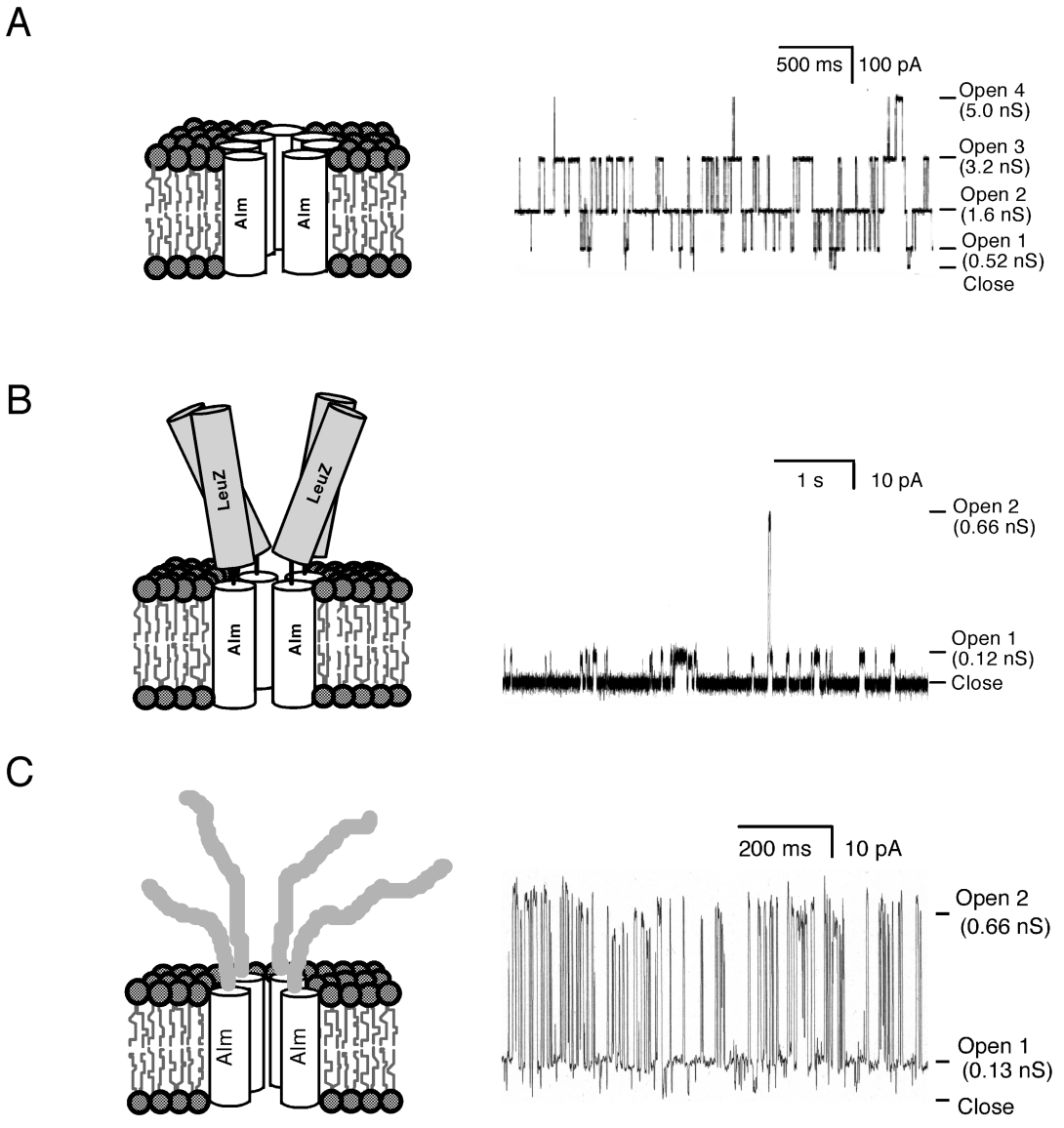

FIGURE 1. Schematic representation of a possible association state and the single channel records of alamethicin amide (Ac-UPUAUAQUVUGL-UPVUUEQF-amide, without an extramembrane segment) (A), Alm-LeuZ (Ac-UPUAUAQUVUGLUPVUUEQF-GGGG-RXKQLEDKVEELLSKNYHLENEVA-RLKKLVGE-amide, bearing an extramembrane segment having an α-helical structure) (B), Alm-[Gly]LeuZ (Ac-UPUAUAQUVUGLUPVUUEQF-GGGG-RXKQGEDKVEEGLSKNYHGENEVARGKKLVGE-amide, bearing an extramembrane segment having a random-coil structure) (C). Ac = acetyl; U = Aib; X = norleucine (Nle). Voltage, +100 mV; electrolyte, unbuffered 1 M KCl. Reprinted with permission from ref 31. Copyright 2001 American Chemical Society.

corresponding to the GCN4 leucine-zipper segment were prepared using a standard Fmoc-SPPS protocol, introduction of the Aib residues and any amino acids next to Aib residues was accomplished using a Fmoc-amino-acid fluoride (e.g., Fmoc-Aib-F).[30] The circular dichroism (CD) spectrum of Alm-LeuZ in the presence of liposomes suggests that this peptide forms a helical structure in the membrane and that Alm and LeuZ segments independently form helical structures without interacting with each other. For the channel-current analysis of Alm-LeuZ, the planar-lipid-bilayer method was employed, which allows analysis of the ion-flux through a single channel pore in real time (i.e., single-channel current measurement) with sensitivity comparable to that of the patch-clamp technique. As is typical in Alm channels, Alm without an extramembrane segment formed channels with several levels of channel conductance (Figure 1A). On the other hand, introduction of the LeuZ segment into Alm as an extramembrane yielded a single conductance level corresponding to that of the Alm-LeuZ tetramer assembled on a peptide template,[29] suggesting that the introduction of a helical peptide segment significantly affected the assembly state (Figure 1B). We designed two analogues of Alm-LeuZ

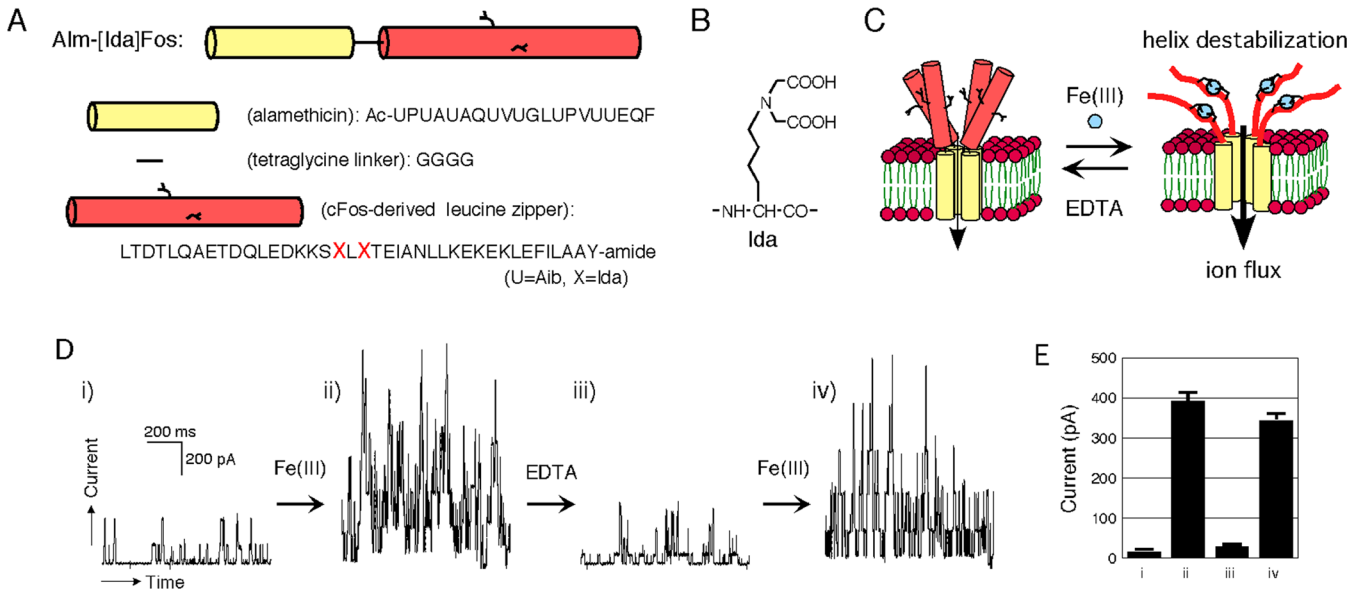

FIGURE 2. (A) Design of artificial metal-gated ion-channel peptide, Alm-[Ida]Fos. (B) Structure of Ida. (C) Schematic representation of the artificial receptor channel that transmits outside stimuli (metal) to inside the membrane as an increase in the ion flux. (D) Channel current records of Alm-[Ida]Fos in the absence of Fe(III) (i); after the addition of 2 μM Fe(III) (ii); after the addition of 10 μM EDTA (iii); and after the addition of 12 μM Fe(III) (iv). Peptide concentration, 2.5 nM; voltage, +160 mV; electrolyte, 1 M KCl containing 10 mM HEPES and 1 μM EDTA (pH 7.0). (E) Average channel current going through the membranes and the standard errors of 25 recordings of the channel states corresponding to (i)−(iv) in (D). Reprinted with permission from ref 43. Copyright 2006 American Chemical Society.

with leucine-zipper segments with reduced helical contents. The decrease in the helical content in the extramembrane segments led to a higher peptide aggregation number (or a different conformation) that produced higher channel conductance (Figure 1C). The assembly modulation in Alm-LeuZ may be attributable to formation of metastable pseudotetramer assemblies facilitated by frequent exchange of the pairs of GCN4 dimers that are proximately located on the membranes after assembly of the transmembrane Alm segments. However, even when extramembrane segments do not show strong affinity, steric hindrance among the extramembrane segments prevents excess assembly of Alm molecules in the membrane, leading to a reduced association number.

The above results suggest that employment of conformationally switchable extramembrane segments that are sensitive to external stimuli might influence the assembly states of Alm molecules in the membranes and the eventual channel current. In another words, these systems may be useful as artificial sensor systems, in which stimuli are transmitted as alterations in channel current levels. To establish proof of concept, we attached a modified c-Fos-derived leucine-zipper segment to Alm (Alm-[Ida]Fos)[43] (Figure 2A). The c-Fos-derived leucine-zipper segment preferably forms coiled-coil heterodimers with cJun-derived leucine-zipper segments but has a very low tendency to form homodimers. We incorporated a pair of iminodiacetic acid derivatives of lysine (Ida)[44] (Figure 2B) via n and $n + 2$ positioning (i.e., one Ida was placed two amino acid residues from the other Ida) in the c-Fos segment so that chelation of Ida with metals such as Fe^{3+} yielded structural destabilization (i.e., conformational alteration) in the extramembrane segment. Eventually, addition of Fe^{3+} activated the Alm-[Ida]Fos channel, which was then deactivated by the removal of Fe^{3+}. Repetitive on/off switching of the channel by the addition and removal of Fe^{3+} was possible, and thus, an artificial Fe^{3+}-receptor ion channel had been established (Figure 2C).

The robustness of our results is promising for the design of channel current control using conformationally switchable extramembrane segments. To confirm the applicability, we prepared a hybrid channel protein of Alm with a calmodulin C-terminal-derived peptide (CaMc)[45] in the hope of establishing an artificial Ca^{2+}-sensitive channel. Ca^{2+} plays an important physiological role as a ubiquitous intracellular mediator. Calmodulin is a small, acidic Ca^{2+}-binding protein with a dumbbell-like structure and is composed of N- and C-terminal domains.[46] Each domain contains two EF-hand Ca^{2+}-binding loops that independently bind Ca^{2+}. Because the C-terminal domain (CaMc) has a higher affinity for Ca^{2+} and induces a greater structural change than the N-terminal domain,[47−50] we selected CaMc as an extramembrane segment. Considering that the extramembrane CaMc is more

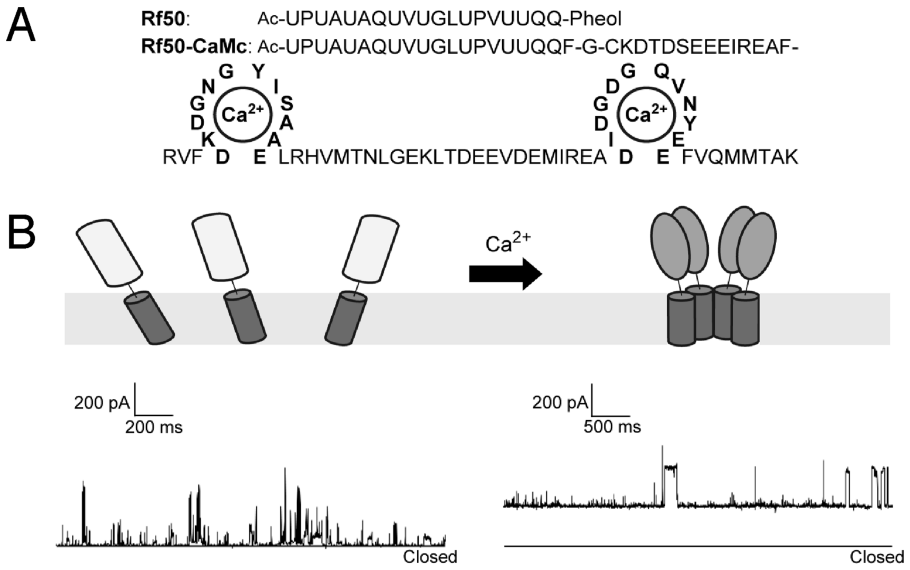

FIGURE 3. (A) Structure of Rf50 and Rf50-CaMc. U = Aib; Pheol = phenylalaninol; Ac = acetyl. Two Ca^{2+}-binding loops are highlighted in bold. (B) Schematic representation of Ca^{2+}-stimulated gating of the Rf50-CaMc channel, and channel current records of Rf50-CaMc in the absence (left) or presence (right) of 5 mM $CaCl_2$. Voltage, +180 mV; electrolyte, 1 M KCl containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.2 mM EDTA, and 10 mM dithiothreitol (DTT) (pH 5.4). Reprinted with permission from ref 45. Copyright 2013 American Chemical Society.

hydrophilic than a LeuZ segment, we employed the more hydrophobic Alm (Rf50) as a transmembrane segment to stably retain the hybrid protein in the membranes. Alm (Rf50) has a glutamine (Gln) at position 18 (Figure 3A), whereas Alm has a glutamic acid (Glu) in that location.[21,22]

Preparation of Rf50-CaMc was accomplished by using native chemical ligation, since the CaMc segment has more than 70 amino acid residues and is thus difficult to synthesize chemically. Therefore, a CaMc segment bearing an extra cysteine on the N-terminus (Cys-CaMc) was prepared by recombinant expression using intein-mediated purification with an affinity chitin-binding tag (IMPACT) system for ligation with the Rf50 thioester.[51,52] Because the N-terminal region of the CaMc segment (which is proximal to the transmembrane Rf50 segment) is rich in the acidic amino acids aspartic acid (Asp) and Glu, we performed the channel current recording of Rf50-CaMc under acidic conditions (pH 5.4) to reduce electrostatic repulsion, which would have prevented Rf50-CaMc from self-assembling in membranes to form channels.

In the absence of Ca^{2+}, Rf50-CaMc yielded burstlike channel currents with no discrete channel conductance levels.[45] The addition of Ca^{2+} significantly stabilized the channel open state (corresponding to a 6-mer assembly) and increased the mean channel current by 6-fold (Figure 3B). Conversely, Mg^{2+} produced no significant changes in the channel current. Thus, successful employment of a natural protein motif as the extramembrane gating segment for Alm was achieved, while maintaining a simple structure and the intrinsic functions of the extramembrane and channel-forming segments. The addition of Ca^{2+} led to an increase in the surface hydrophobicity of the extramembrane segment, which may also have influenced assembly modulation together with steric hindrance between extramembrane segments in the Rf50-CaMc channel. In summary, use of a natural ligand-binding peptide/protein segment in an Alm extramembrane segment may lead to a tailored ligand-gated channel.

5. Assembly Control of Alm by Metal Chelation

As described above, formation of Alm channels is voltage-dependent, and a helix dipole interaction between Alm and membranes' electric fields leads to insertion of the N-terminus into membranes.[21−23] Therefore, the absence of a membrane potential should lead to cancellation of both the membrane insertion state of Alm and channel assembly. Many natural membrane proteins have hydrophilic regions that are exposed to the aqueous environment on either side of transmembrane segments, which prevent the liberation of the transmembrane segments from the membranes. Disposition of hydrophilic amino acids on both sides of Alm may contribute to the stabilization of its insertion state and assembly in membranes. Alm has a negatively charged Glu at position 18, which has been reported to hamper the membrane insertion of the C-terminus.[22] Disposition of charged residues on the N-termini would prevent the

inserted Alm segment from turning back to the C-terminus side, therefore extending the lifespan of the channel. Therefore, we designed an Alm analogue with a histidine-glycine (His-Gly) extension on its N-terminus (HG-Alm).[53] HG-Alm formed a channel with multiple conductance states, as observed in Alm, in an electrolyte containing ethylenediaminetetraacetic acid (EDTA) as a metal chelating agent. A higher voltage (250−300 mV) was necessary to activate the HG-Alm channel compared with Alm (100−150 mV). However, once channels were formed, longer channel lifetimes were obtained, even at lower voltages than the voltage necessary for Alm channel activation.

Unexpectedly, we also found that the channel open lifetime of HG-Alm was considerably longer in the presence of metals, such as Zn^{2+}, Ni^{2+}, and Co^{2+}, with stabilization of specific conductance states corresponding to the 8-, 10-, and 12-mer assemblies (Figure 4).[53] Although we prepared other analogues of Alm bearing a lysine (Lys) or Glu at the N-terminus instead of a His, no similar stabilization effect by metals was observed. These results suggest that metal chelation with the His forms a metastable dimer assembly of HG-Alm molecules. Similarly, blocking the N-terminus amino group of His or protonation of the imidazole nitrogen at low pH also inhibited stabilized assembly. Utilizing the significant difference in the conductance levels and lifetimes of HG-Alm channels in the absence and presence of metal ions, we showed the feasibility of reversible metal switching of the HG-Alm assembly and the channel current. Additionally, we found that substitution of Aib in HG-Alm with other aliphatic amino acids significantly influenced channel gating.[54]

6. Scope and Limitations of Using Extramembrane Segments for Channel Gating

As exemplified by the use of leucine-zipper and CaMc segments, the strategy of utilizing appropriate extramembrane segments is promising for channel assembly control or gating. Although approaches using modification of channel pores of natural proteins and those formed with assembled peptides have been applied for practical sensing (e.g., for DNA sequencing or small molecules),[55−57] such techniques are successful only when the channel pores are appropriately sized for proper sensing. The analytes must penetrate the pores, and the interaction between the pores and the analytes must accelerate or inhibit the ion flux in the membrane. Sensing cannot be accomplished if the pores are too small or too large for the analytes, and adapters, including cyclodextrins, are often employed to adjust pore size.[55,58]

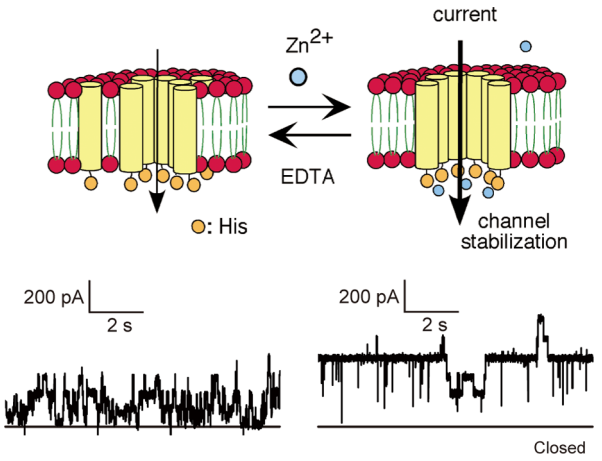

FIGURE 4. Schematic representation and typical channel current records of HG-Alm in the absence and presence of $ZnCl_2$. Voltage, +80 mV; electrolyte, 1 M KCl containing 10 mM HEPES (pH 7.0). Reprint from ref 53.

When the pore sizes are suitable, these approaches may attain very sensitive analyte detection. These detection methods are often based on stochastic sensing, and very sensitive analysis of the channel current is required. Alternatively, approaches that use extramembrane segments as gating machinery are less limited with respect to channel pore size. Depending on the availability of appropriate membrane segments that specifically interact with ligands (or analytes) and lead to structural alteration in extramembrane segments and eventually in channel pores, there is much more room for channel design. Alternatively, in Alm-based channels, since the probability of channel formation is voltage-dependent, the numbers of peptides that form a pore, and the eventual channel conductance can vary. These characteristics may be disadvantages of Alm-based approaches in terms of reproducibility. Thus, employment of suitable extramembrane segments that yield fixed numbers of segments and the embedding of relatively large numbers of channel peptides in the membrane to average the current may be necessary for future application of this approach to practical sensors.

To analyze the channel current of artificial channel peptides/proteins, the planar lipid bilayer[19] and the tip-dip[59] methods have been used. The former approach employs two chambers connected by a pinhole (typically, having a diameter of a few hundred micrometers), where a lipid bilayer is formed. The latter approach uses glass pipettes, as in the patch-clamp technique. With repeated dipping of the pipet tips into buffers with surface monolayer membranes, single bilayers are formed on the ends of the glass pipettes. These procedures have sensitivities equivalent to that of the patch-clamp technique and allow single-channel analysis.

However, because difficulties often arise in the formation of membranes, further development is needed. Given that the chambers used for analysis by these methods are usually in the milliliter range, downsizing of the system is desired to facilitate easier and less-sample-consuming analysis. Recently, methods using droplet interface bilayers have been introduced to allow easier formation of lipid bilayers. In these methods, aqueous droplets are submerged in an oil/lipid mixture.[60] When the droplets join together, the lipid monolayers surrounding them combine at the interface to form a lipid bilayer. This approach also allows analysis using much smaller volumes than with the traditional planar-lipid-bilayer methods and has potential for high throughput screening of channel peptides/proteins using micro- to nanoliter-range buffer volumes.[61] This system is also beneficial for reducing the electrical noise that typically perturbs the analysis of very fine currents in the nano- to picoampere range. Current studies of artificial-ligand-gated ion channels introduced using this method are in the proof-of-concept stage, but practical application of these techniques in artificial sensors may be achieved in the future.

7. Conclusion

Using our Alm-based channels as examples, we showed that the simple disposition of a structurally switchable extramembrane segment effectively influenced the assembly conditions of the transmembrane Alm segment. This system can serve as an artificial ligand-sensitive receptor channel and has potential uses in the development of artificial sensors, especially in combination with small-scale sensing systems, including the droplet interface bilayer method. Simple conformational alterations effectively transmit assembly states to the transmembrane segment. Various conformationally switchable protein motifs and other molecules, including DNA, RNA, sugars, and synthetic polymers, can be used as extramembrane gating segments to create artificial receptors and channels. It has been suggested that hydrophobic transmembrane segments may form assembled structures in membranes even without functional moieties that interact with each other. We have demonstrated the transmittance of alterations in extramembrane structures into the assembled states of Alm in membranes; this concept may also be used to control the assembly of other membrane proteins. Assuming that transmembrane segments have a tendency to assemble in membranes, membrane proteins composed of more than one protein unit may form higher-ordered structures in membranes even in the absence of strong interactions among extramembrane segments, but extramembrane segments can prevent excess assembly of the subunits in the membrane, as shown in the case of Alm-LeuZ channels. The method involved in the structural formation of membrane proteins is likely to be more complicated than that of soluble proteins because correct folding and packing of the transmembrane segments in the membranes without interfering with each other or the extramembrane segments in aqueous environments are necessary. However, the movement of membrane proteins is two-dimensionally restricted, and their structures can become stabilized, presenting their extramembrane segments on both sides. Therefore, it might be easier to control the structure and assembly of membrane proteins than proteins in solution.

We have reported control of the assembly of transmembrane segment Alm by introducing appropriate extramembrane segments. In addition, we recently developed an alternative approach to the assembly control of the epidermal growth factor receptor using dimerization linkers based on leucine zipper recognition.[62] Extramembrane engineering is a promising area for assembly control of membrane proteins and the eventual control of cell functions.

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BIOGRAPHICAL INFORMATION

Shiroh Futaki obtained his Ph.D. in 1989 from Kyoto University. Following his appointment as a Research Associate and an Associate Professor at the University of Tokushima, he moved to Kyoto University in 1997. Meanwhile, he spent 16 months (1989−1991) in the United States as a Postdoctoral Associate in the Department of Biochemistry, Rockefeller University. He has been a Professor of Biochemistry at the Institute of Chemical Research, Kyoto University, since 2005. His research interests include design of bioactive peptides having unique functions (ion-channel formation, cell penetration, DNA-binding, and so on).

Daisuke Noshiro graduated from Faculty of Pharmaceutical Sciences, Kyoto University in 2007. He obtained his Ph.D. in 2012 from Kyoto University under the supervision of Professor Shiroh Futaki. After obtaining his Ph.D., he joined the research group of Professor Hagan Bayley, Department of Chemistry, the University of Oxford as a postdoctoral fellow. His main research focus is in the field of membrane protein engineering for sensing applications. He is a recipient of the Naito Foundation Subsidy for Inter-Institute Researches.

Tatsuto Kiwada was involved in the development of Alm-[Ida]Fos channels and obtained his Ph.D. in 2005 from Kyoto University. After four years' postdoctoral training at the laboratory of Professor Shigetada Nakanishi, Osaka Bioscience Institute,

he was appointed as an Assistant Professor at Clinical and Analytical Sciences, Kanazawa University in 2009. His current research interest is development of novel platinum anticancer drug.

Koji Asami is an Associate Professor at the Institute for Chemical Research, Kyoto University since 1986. After he obtained his Ph.D. in 1978 from Kyoto University, he was a Research Associate in Physiology at Kochi Medical School from 1979 to 1986 and was a visiting Associate Professor of the Department of Bioengineering at the University of Pennsylvania from 1987 to 1989. His research interests focus on the electric and dielectric properties of artificial and biological membranes.

FOOTNOTES

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The authors declare no competing financial interest.

REFERENCES

1 Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of the Cell*, 5th ed.; Garland Science: New York, 2007.

2 Hille, B. *Ion Channels of Excitable Membranes*, 3rd ed.; Sinauer Associates Inc.: Sunderland, MA, 2001.

3 Numa, S. Molecular basis for the function of ionic channels. *Biochem. Soc. Symp.* **1986**, *52*, 119–143.

4 Miyazawa, A.; Fujiyoshi, Y.; Unwin, N. Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **2003**, *423*, 949–955.

5 Matile, S.; Vargas Jentzsch, A.; Montenegro, J.; Fin, A. Recent synthetic transport systems. *Chem. Soc. Rev.* **2011**, *40*, 2453–2474.

6 Sakai, N.; Mareda, J.; Matile, S. Rigid-rod molecules in biomembrane models: from hydrogen-bonded chains to synthetic multifunctional pores. *Acc. Chem. Res.* **2005**, *38*, 79–87.

7 Bayley, H. Designed membrane channels and pores. *Curr. Opin. Biotechnol.* **1999**, *10*, 94–103.

8 Astier, Y.; Bayley, H.; Howorka, S. Protein components for nanodevices. *Curr. Opin. Chem. Biol.* **2005**, *9*, 576–584.

9 Fyles, T. M. Synthetic ion channels in bilayer membranes. *Chem. Soc. Rev.* **2007**, *36*, 335–347.

10 Futaki, S. Peptide ion channels: design and creation of function. *Biopolymers* **1998**, *47*, 75–81.

11 Jung, Y.; Bayley, H.; Movileanu, L. Temperature-responsive protein pores. *J. Am. Chem. Soc.* **2006**, *128*, 15332–15340.

12 Gorteau, V.; Bollot, G.; Mareda, J.; Pasini, D.; Tran, D. H.; Lazar, A. N.; Coleman, A. W.; Sakai, N.; Matile, S. Synthetic multifunctional pores that open and close in response to chemical stimulation. *Bioorg. Med. Chem.* **2005**, *13*, 5171–5180.

13 Sakai, N.; Sordé, N.; Das, G.; Perrottet, P.; Gerard, D.; Matile, S. Synthetic multifunctional pores: deletion and inversion of anion/cation selectivity using pM and pH. *Org. Biomol. Chem.* **2003**, *1*, 1226–1231.

14 Jog, P. V.; Gin, M. S. A light-gated synthetic ion channel. *Org. Lett.* **2008**, *10*, 3693–3696.

15 Tian, Y.; Hou, X.; Wen, L.; Guo, W.; Song, Y.; Sun, H.; Wang, Y.; Jiang, L.; Zhu, D. A biomimetic zinc activated ion channel. *Chem. Commun.* **2010**, *46*, 1682–1684.

16 Xia, F.; Guo, W.; Mao, Y.; Hou, X.; Xue, J.; Xia, H.; Wang, L.; Song, Y.; Ji, H.; Ouyang, Q.; Wang, Y.; Jiang, L. Gating of single synthetic nanopores by proton-driven DNA molecular motors. *J. Am. Chem. Soc.* **2008**, *130*, 8345–8350.

17 Hou, X.; Guo, W.; Xia, F.; Nie, F. Q.; Dong, H.; Tian, Y.; Wen, L.; Wang, L.; Cao, L.; Yang, Y.; Xue, J.; Song, Y.; Wang, Y.; Liu, D.; Jiang, L. A biomimetic potassium responsive nanochannel: G-quadruplex DNA conformational switching in a synthetic nanopore. *J. Am. Chem. Soc.* **2009**, *131*, 7800–7805.

18 *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*; Chan, W. C., White, P. D., Eds.; Oxford University Press: Oxford, 2000.

19 Montal, M. Reconstitution of channel proteins from excitable cells in planar lipid bilayer membranes. *J. Membr. Biol.* **1987**, *98*, 101–115.

20 Neher, E.; Sakmann, B. Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* **1976**, *260*, 799–802.

21 Sansom, M. S. The biophysics of peptide models of ion channels. *Prog. Biophys. Mol. Biol.* **1991**, *55*, 139–235.

22 Woolley, G. A.; Wallace, B. A. Model ion channels: gramicidin and alamethicin. *J. Membr. Biol.* **1992**, *129*, 109–136.

23 Gordon, L. G.; Haydon, D. A. The unit conductance channel of alamethicin. *Biochim. Biophys. Acta* **1972**, *255*, 1014–1018.

24 Oiki, S.; Danho, W.; Madison, V.; Montal, M. M2 delta, a candidate for the structure lining the ionic channel of the nicotinic cholinergic receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8703–8707.

25 Lear, J. D.; Wasserman, Z. R.; DeGrado, W. F. Synthetic amphiphilic peptide models for protein ion channels. *Science* **1988**, *240*, 1177–1181.

26 Ghadiri, M. R.; Granja, J. R.; Buehler, L. K. Artificial transmembrane ion channels from self-assembling peptide nanotubes. *Nature* **1994**, *369*, 301–304.

27 Bayley, H. Triggers and switches in a self-assembling pore-forming protein. *J. Cell Biochem.* **1994**, *56*, 177–182.

28 Cafiso, D. S. Alamethicin: a peptide model for voltage gating and protein-membrane interactions. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 141–165.

29 Futaki, S.; Asami, K. Ligand-induced extramembrane conformation switch controlling alamethicin assembly and the channel current. *Chem. Biodiversity* **2007**, *4*, 1313–1322.

30 Kaduk, C.; Wenschuh, H.; Beyermann, M.; Forner, K.; Carpino, L. A.; Bienert, M. Synthesis of Fmoc-amino acid fluorides via DAST, an alternative fluorinating agent. *Lett. Pept. Sci.* **1995**, *2*, 285–288.

31 Futaki, S.; Fukuda, M.; Omote, M.; Yamauchi, K.; Yagami, T.; Niwa, M.; Sugiura, Y. Alamethicin-leucine zipper hybrid peptide: a prototype for the design of artificial receptors and ion channels. *J. Am. Chem. Soc.* **2001**, *123*, 12127–12134.

32 Landschulz, W. H.; Johnson, P. F.; McKnight, S. L. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **1988**, *240*, 1759–1764.

33 O'Shea, E. K.; Rutkowski, R.; Kim, P. S. Evidence that the leucine zipper is a coiled coil. *Science* **1989**, *243*, 538–542.

34 Chugh, J. K.; Wallace, B. A. Peptaibols: models for ion channels. *Biochem. Soc. Trans.* **2001**, *29*, 565–570.

35 Woolley, G. A. Channel-forming activity of alamethicin: effects of covalent tethering. *Chem. Biodiversity* **2007**, *4*, 1323–1337.

36 Matsubara, A.; Asami, K.; Akagi, A.; Nishino, N. Ion-channels of cyclic template-assembled alamethicins that emulate the pore structure predicted by barrel-stave model. *J. Chem. Soc., Chem. Commun.* **1996**, 2069–2070.

37 Duclohier, H.; Kociolek, K.; Stasiak, M.; Leplawy, M. T.; Marshall, G. R. C-terminally shortened alamethicin on templates: influence of the linkers on conductances. *Biochim. Biophys. Acta* **1999**, *1420*, 14–22.

38 Sakoh, M.; Okazaki, T.; Nagaoka, Y.; Asami, K. N-terminal insertion of alamethicin in channel formation studied using its covalent dimer N-terminally linked by disulfide bond. *Biochim. Biophys. Acta* **2003**, *1612*, 117–121.

39 Okazaki, T.; Sakoh, M.; Nagaoka, Y.; Asami, K. Ion channels of alamethicin dimer N-terminally linked by disulfide bond. *Biophys. J.* **2003**, *85*, 267–273.

40 Futaki, S.; Aoki, M.; Fukuda, M.; Kondo, F.; Niwa, M.; Kitagawa, K.; Nakaya, Y. Assembling of the four individual helices corresponding to the transmembrane segments (S4 in repeat I-IV) of the sodium channel. *Tetrahedron Lett.* **1997**, *38*, 7071–7074.

41 Futaki, S.; Aoki, M.; Ishikawa, T.; Kondo, F.; Asahara, T.; Niwa, M.; Nakaya, Y.; Yagami, T.; Kitagawa, K. Chemical ligation to obtain proteins comprising helices with individual amino acid sequences. *Bioorg. Med. Chem.* **1999**, *7*, 187–192.

42 Cornell, B. A.; Braach-Maksvytis, V. L. B.; King, L. G.; Osman, P. D. J.; Raguse, B.; Wieczorek, L.; Pace, R. J. A biosensor that uses ion-channel switches. *Nature* **1997**, *387*, 580–583.

43 Kiwada, T.; Sonomura, K.; Sugiura, Y.; Asami, K.; Futaki, S. Transmission of extramembrane conformational change into current: construction of metal-gated ion channel. *J. Am. Chem. Soc.* **2006**, *128*, 6010–6011.

44 Futaki, S.; Kiwada, T.; Sugiura, Y. Control of peptide structure and recognition by Fe(II)-induced helix destabilization. *J. Am. Chem. Soc.* **2004**, *126*, 15762–15769.

45 Noshiro, D.; Sonomura, K.; Yu, H. H.; Imanishi, M.; Asami, K.; Futaki, S. Construction of a Ca^{2+}-gated artificial channel by fusing alamethicin with a calmodulin-derived extramembrane segment. *Bioconjugate Chem.* **2013**, *24*, 188–195.

46 Babu, Y. S.; Sack, J. S.; Greenhough, T. J.; Bugg, C. E.; Means, A. R.; Cook, W. J. Three-dimensional structure of calmodulin. *Nature* **1985**, *315*, 37–40.

47 Finn, B. E.; Evenäs, J.; Drakenberg, T.; Waltho, J. P.; Thulin, E.; Forsén, S. Calcium-induced structural changes and domain autonomy in calmodulin. *Nat. Struct. Biol.* **1995**, *2*, 777–783.

48 Barth, A.; Martin, S. R.; Bayley, P. M. Specificity and symmetry in the interaction of calmodulin domains with the skeletal muscle myosin light chain kinase target sequence. *J. Biol. Chem.* **1998**, *273*, 2174–2183.

49 Martin, S. R.; Bayley, P. M. The effects of Ca^{2+} and Cd^{2+} on the secondary and tertiary structure of bovine testis calmodulin. A circular-dichroism study. *Biochem. J.* **1986**, *238*, 485–490.

50 Linse, S.; Helmersson, A.; Forsén, S. Calcium binding to calmodulin and its globular domains. *J. Biol. Chem.* **1991**, *266*, 8050–8059.

51 Xu, M. Q.; Evans, T. C., Jr. Intein-mediated ligation and cyclization of expressed proteins. *Methods* **2001**, *24*, 257–77.

52 Futaki, S.; Sogawa, K.; Maruyama, J.; Asahara, T.; Niwa, M.; Hojo, H. Preparation of peptide thioesters using Fmoc-solid-phase peptide synthesis and its application to the construction of a template-assembled synthetic protein (TASP). *Tetrahedron Lett.* **1997**, *38*, 6237–6240.

53 Noshiro, D.; Asami, K.; Futaki, S. Metal-assisted channel stabilization: disposition of a single histidine on the N-terminus of alamethicin yields channels with extraordinarily long lifetimes. *Biophys. J.* **2010**, *98*, 1801–1808.

54 Noshiro, D.; Asami, K.; Futaki, S. Control of leakage activities of alamethicin analogs by metals: side chain-dependent adverse gating response to Zn^{2+}. *Bioorg. Med. Chem.* **2012**, *20*, 6870–6876.

55 Maglia, G.; Heron, A. J.; Stoddart, D.; Japrung, D.; Bayley, H. Analysis of single nucleic acid molecules with protein nanopores. *Methods Enzymol.* **2010**, *475*, 591–623.

56 Ma, L.; Cockroft, S. L. Biological nanopores for single-molecule biophysics. *ChemBioChem* **2010**, *11*, 25–34.

57 Kawano, R.; Osaki, T.; Sasaki, H.; Takinoue, M.; Yoshizawa, S.; Takeuchi, S. Rapid detection of a cocaine-binding aptamer using biological nanopores on a chip. *J. Am. Chem. Soc.* **2011**, *133*, 8474–8477.

58 Li, W. W.; Claridge, T. D.; Li, Q.; Wormald, M. R.; Davis, B. G.; Bayley, H. Tuning the cavity of cyclodextrins: altered sugar adaptors in protein pores. *J. Am. Chem. Soc.* **2011**, *133*, 1987–2001.

59 Zweifach, A.; Lewis, S. A. Characterization of a partially degraded Na^{+} channel from urinary tract epithelium. *J. Membr. Biol.* **1988**, *101*, 49–56.

60 Bayley, H.; Cronin, B.; Heron, A.; Holden, M. A.; Hwang, W. L.; Syeda, R.; Thompson, J.; Wallace, M. Droplet interface bilayers. *Mol. BioSyst.* **2008**, *4*, 1191–1208.

61 Funakoshi, K.; Suzuki, H.; Takeuchi, S. Lipid bilayer formation by contacting monolayers in a microfluidic device for membrane protein analysis. *Anal. Chem.* **2006**, *78*, 8169–8174.

62 Nakase, I.; Okumura, S.; Tanaka, G.; Osaki, K.; Imanishi, M.; Futaki, S. Signal transduction using an artificial receptor system that undergoes dimerization upon addition of a bivalent leucine-zipper ligand. *Angew. Chem., Int. Ed.* **2012**, *51*, 7464–7467.