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Detecting a tag on a channel opening: blockage of the biotinylated channels by streptavidin

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Abstract—C-terminal biotinylated alamethicin and gramicidin A were prepared by a combination of the Fmoc-solid-phase peptide synthesis and the solution method. The ion-channel current of these peptides was dramatically suppressed by the addition of streptavidin, suggesting the potential of this approach for the elucidation of channel protein structure and for the design of artificial sensors and receptors.

Ion-channels and receptors are a class of the most important membrane proteins to transmit extracellular biological signals into cells, which eventually trigger various cellular events to maintain homeostasis. The problem of how these protein molecules transmit outside signals to the inside of the cells is one of the fundamental questions on the mechanisms of these proteins. The information on this issue is not only critical for the understanding of molecular mechanisms in signal transduction, which are often involved with the occurrence of diseases, but also for the design of artificial sensors and receptors. One of the promising approaches to elucidate the questions is to combine the techniques of molecular biology and chemical modification of proteins. For example, the specific amino acids in a putative transmembrane segment of the *Shaker* potassium channel were mutated to cysteine labeled with tetramethylrhodamine–maleimide.[1] By monitoring the fluorescence images, the conformational rearrangement in accordance with membrane depolarization to lead gating of the channel could be monitored in real time. We report here that the ion-channel current of the biotinylated peptide ion-channels was dramatically suppressed by streptavidin, and this method has the potential for elucidating channel protein structure by combining cysteine-mutation and biotin-labeling of the target amino acids.

The systems studied in this report were of two typical peptide ion-channels, alamethicin and gramicidin A. Alamethicin is a fungus-derived peptide antibiotic with a helical structure. The molecules self-assemble in the membrane to form helix bundles that span the membranes perpendicularly.[2] Ions penetrate through the cavity formed in the center of these helices. In contrast, gramicidin A forms a $\beta^{6.3}$-helix dimer structure in the membranes.[3] Two molecules of gramicidin associate with their N-terminal coming together by hydrogen bonding, where ions permeate through the inside of the helices. Based on these peptide channel systems, approaches have been reported not only to study their function as simplified models of natural channel proteins, but also to create channel systems with a sensing function.[4,5]

Design and synthesis of biotin-tagged alamethicin and the channel current suppression by streptavidin

Binding of biotin with streptavidin is known to be one of the strongest ligand–receptor interactions (K_a: 10^{13} M^{-1} or higher) found in nature.[6] When a biotin molecule is placed at the opening, or the entrance, of the channel pore, it is expected that the channel current can be suppressed by covering the channel pore with the attachment of streptavidin. If the biotin is located far from the channel entrance, the current may not be influenced by the attachment of streptavidin. These ideas may be extended to gain information on the topology of specific amino acids in the channel proteins. To ascertain the adaptability of the approach, we designed a biotin-tagged alamethicin **2** (Fig. 1).

To place the biotin moiety close to the channel opening, C-terminal phenylalaninol (Phol) of alamethicin was replaced with phenylalanine, which was directly connected to biotin hydrazide. The peptide chain was

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constructed by Fmoc-solid-phase peptide synthesis[7] on a highly acid-liable 2-chlorotrityl resin.[8] Alamethicin contains α-aminoisobutyric acid (Aib). Because of the steric hindrance at the α-carbon of Aib, peptide-bond formation on the solid support is not easy by ordinal coupling reagents. We then employed the *O*-(7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HATU)–1 - hydroxy - 7 - azabenzotriazole (HOAT)–diisopropylethylamine (DIEA) coupling system, which is reported to be efficient for the synthesis of Aib-containing peptides.[9] After the N-terminal was acetylated by acetic anhydride in the presence of *N*-methylmorpholine (NMM), the peptide resin was treated with acetic acid (AcOH)–trifluoroethanol (TFE)–dichloromethane (DCM) (1:1:3) at room temperature for 1 h to yield a protected peptide **1**. The peptide was then coupled with biotin hydrazide (Sigma) in the presence of diisopropylcarbodiimide (DICDI) and 1-hydroxybenzotriazole (HOBt). The resulting conjugate was treated with trifluoroacetic acid (TFA)–ethanedithiol (EDT) (95:5) at room temperature for 2 h, followed by HPLC purification to yield a pure biotin-tagged alamethicin. The fidelity of the peptide was ascertained by time-of-flight mass spectrometry (TOFMS) [observed, 2219.4 $(M+H)^+$; theoretical, 2219.5].

The effect of streptavidin on the biotin-tagged alamethicin **2** was assessed by channel current measurement using the planar lipid-bilayer method.[10] The biotin-tagged alamethicin in the absence of streptavidin showed a channel current very similar to that of the natural alamethicin. Fig. 1C is a channel current recording of **2** where two or three channels are deduced to be simultaneously open in the membrane. By the addition of streptavidin (0.20 μM) ([streptavidin]/[peptide] = 3.2), the channel current was dramatically reduced, and no channel current could be observed after 3 min. By adding biotin hydrazide (13 μM), the channel current level was recovered. What is interesting is that the channel current was blocked even when the molar ratio of streptavidin to the peptide was 0.66, although a longer incubation time (30 min) was necessary to stop the channel current. Alamethicin was reported to form an assembly comprising 4–12 molecules.[2] This would be sufficient to block the channel current if streptavidin binds one of these alamethicin molecules forming a pore. This fact suggested that, even when a channel is composed of the assembly of multiple subunits, a single biotin label around the channel pore would be detectable with this system. No suppression of the channel current was observed when streptavidin was added to a channel of natural alamethicin that did not have the biotin tag. Thus, streptavidin molecules effectively detected the tag on the membrane to block the opening of the channel.

Design and synthesis of biotin-tagged gramicidin A and the channel current suppression by streptavidin

We next applied this strategy to another class of channel peptide, gramicidin A. The synthetic scheme is shown in Fig. 2A. Here the C-terminal ethanolamine of gramicidin A was replaced with β-alanine for the introduction of biotin hydrazide. The peptide chain was constructed using Fmoc-solid-phase synthesis on the 2-chlorotrityl resin. There is no Aib residue in the sequence; thus the DICDI–HOBt coupling system was employed. N-terminal formylation was conducted using 2,4,5-trichlorophenyl formate (Novabiochem) in the presence of NMM. After the peptide resin was treated with AcOH–TFE–DCM (1:1:3), the peptide **3** was conjugated with biotin hydrazide using DICDI–HOBt as a coupling system. HPLC-purification of the sample gave a pure biotin-tagged gramicidin A **4** [TOFMS: observed, 2173.5 $(M+Na)^+$; theoretical, 2174.1].

The biotin-tagged peptide **4** in the absence of streptavidin showed a single channel conductance (20 pS in 1 M KCl at +180 mV) comparable with that of natural

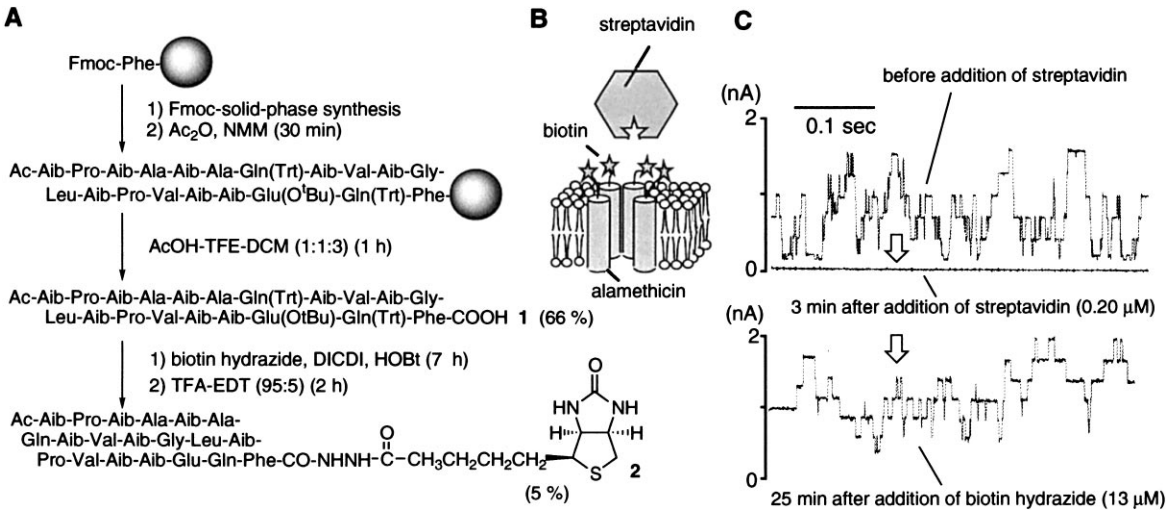

Figure 1. Synthesis of the biotin-tagged alamethicin **2** (A), schematic representation of a channel formed by **2** (B), and channel current records of **2** (C). Lipid: diphytanoylphosphatidylcholine; Solution: 1 M KCl. The peptide (63 nM) and streptavidin were added to the electrolyte at one side of the membrane. The applied voltage (+190 mV) was defined as the voltage of the side where the peptide was added with respect to the compartment of the other side.

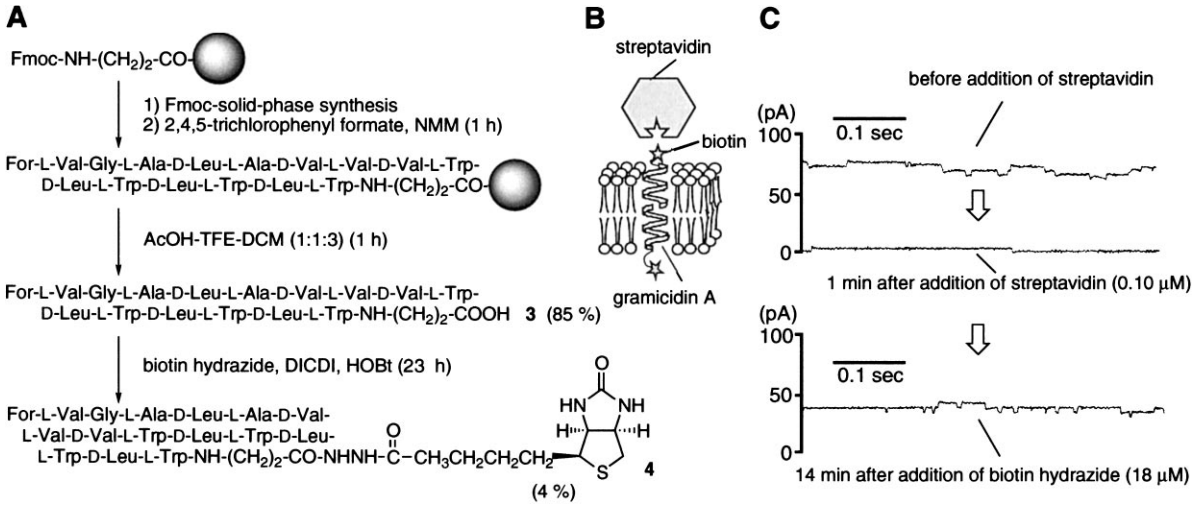

Figure 2. Synthesis of the biotin-tagged gramicidin A **4** (A), schematic representation of a channel formed by **4** (B), and channel current records of **4** (C). Lipid: diphytanoylphosphatidylcholine; Solution: 1 M KCl; Applied voltage; +180 mV. The peptide was mixed with the lipid to achieve a final peptide/streptavidin molar ratio of 1/2000. Streptavidin was added to the electrolytes at both side of the membrane.

gramicidin A (21 pS under the same condition). The peptide **4** was also recognized by streptavidin. Fig. 2C is a channel record, which corresponds to the state of about 12–20 channels in the membrane simultaneously open. Adding 0.10 μM of streptavidin to a solution of the biotin-tagged gramicidin effectively reduced the channel current to less than 10% in 1 min compared with the current levels before the addition of streptavidin. Addition of biotin hydrazide (18 μM) also recovered the channel current levels. No effect on the membrane current was observed when streptavidin was added to the channel formed by the natural gramicidin A.

The present data suggested the applicability of this approach for detecting a biotin tag around the channel pore for understanding the topology and the conformational switch of specific amino acids in channel proteins. This system would also be useful as a working model for designing artificial functional ion-channels and receptors where the ligand–receptor interaction on the membranes can be monitored in real time as a membrane current. Details of the above studies will be reported elsewhere.

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