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Detection of Protein–Ligand Interaction on the Membranes Using C-Terminus Biotin-Tagged Alamethicin

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Abstract—C-terminal biotin-tagged alamethicin, which has several α-aminoisobutyric acid (Aib) residues in its sequence, was synthesized by the preparation of the protected peptide segment using the 2-chlorotrityl resin, followed by conjugation with biotin hydrazide. Suppression of the channel current of the biotin-tagged alamethicin by the addition of streptavidin to the electrolyte was monitorable in real time using the planar lipid-bilayer method. The system was also applicable to the detection of interaction of the biotin-tagged alamethicin with the anti-biotin antibody.

Introduction

Interaction of streptavidin with biotin is one of the most well-known peptide–ligand interactions. The binding constant is reported to be greater than $10^{13}\,M^{-1}$.[1] Utilization of the system as a model of protein–ligand interaction for the design of artificial ion-channels and sensors has been reported. Recently, Bayley and his coworkers reported a novel way of stochastic sensing.[2] Biotin-tagged poly(ethylene glycol) (PEG) was attached inside the pores of α-hemolysin. Interaction of streptavidin with the tag outside the channel pores influenced the channel current, which concept may be developed as a new type of molecular sensor. There are other reports on the detection of streptavidin–biotin interaction where biotin is attached to a gramicidin C-terminus directly or via a spacer.[3,4] Addition of streptavidin to the electrolyte causes a significant decrease in channel current.

Alamethicin is another class of typical channel-forming peptides. The peptide forms a helical structure in the membrane. It is assumed that several molecules of alamethicin associate in the membrane to form a pore in the center of the assembly and allow ions to pass through.[5] The peptide has been employed as a framework of artificial receptors or ion channels,[6] where channel current was modulated by external effects such as metal ions and pH of the electrolyte. If streptavidin can interact with biotin that is directly attached on the C-terminus of alamethicin, this would influence the channel current by closing the channel pore. Construction of many helix-bundle-based artificial peptide ion channels has been reported.[7] If such a way of channel gating is possible, this will contribute to the design of artificial channels of sophisticated function.

Using C-terminal biotin-tagged alamethicin (Alm-Bio), we have preliminarily shown that the interaction of streptavidin with biotin was reflected in the channel current levels.[4] In this report, the characteristics of the channel current modulation by streptavidin are reported. We also exemplify that the same concept is applicable in a more versatile way to the detection of antigen–antibody interaction using the anti-biotin antibody.

Results

Design and synthesis of biotin-tagged alamethicin

Alamethicin has a characteristic structure bearing (i) α-aminoisobutyric acid (Aib) in its sequence and (ii) phenylalaninol (Phol) on the C-terminus as shown in Figure 1. To attach biotin directly to the C-terminus, we replaced the C-terminus Phol with phenylalanine (Phe), which is conjugated with biotin hydrazide. We employed a strategy as follows: (i) preparation of a protected alamethicin segment by Fmoc-solid-phase peptide synthesis[8] on a highly acid-liable 2-chlorotrityl

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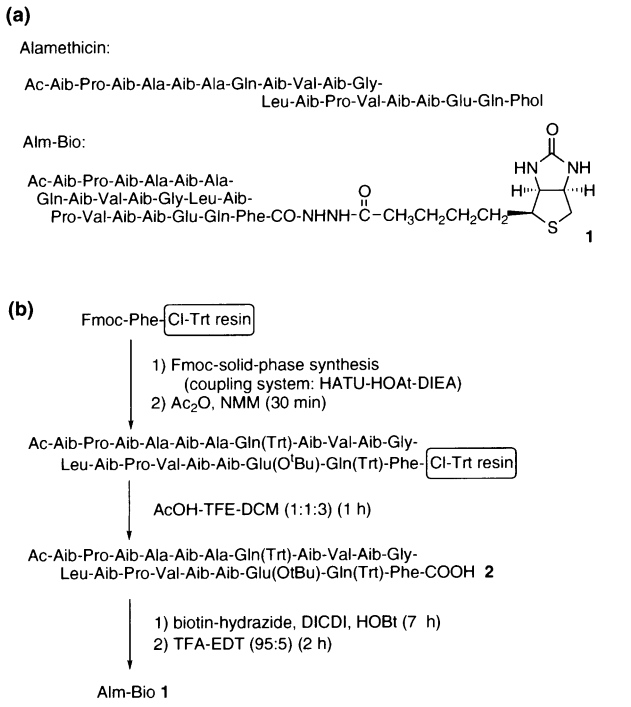

Figure 1. Design (a) and synthesis (b) of biotin-tagged alamethicin (Alm-Bio) **1**.

resin;[9] (ii) conjugation with biotin hydrazide with the protected alamethicin segment, and (iii) removal of the side-chain-protecting groups (Fig. 1). Because of the steric hindrance at the α-carbon of Aib, peptide-bond formation on the solid support for alamethicin is not easy using ordinary coupling reagents. A method using Fmoc-amino acid fluorides has often been employed for the synthesis of alamethicin derivatives.[10] However, Fmoc-amino acid fluorides are not commercially available, and the applicability to the solid-phase peptide synthesizer has not been well-established. We employed here the *O*-(7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HATU)-1-hydroxy-7-azabenzotriazole (HOAT)–diisopropylethylamine (DIEA) coupling system[11] that has been reported to be efficient for the synthesis of Aib-containing peptides.[12] These reagents are commercially available and fully employable in solid-phase peptide synthesis. Ten equivalents of amino acid were used for incorporation of the Fmoc-amino-acid derivative over the resin by following the standard protocol of Shimadzu PSSM-8 peptide synthesizer. A 10-min preincubation of Fmoc amino acid, HATU, HOAT, and DIEA was employed as reported to increase the efficiency of the introduction of the amino acid.[12] 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 **2**. The peptide was then conjugated with biotin hydrazide in the presence of diisopropylcarbodiimide (DICDI) and 1-hydroxybenzotriazole (HOBt) in dimethylformamide (DMF)–1-methyl-2-pyrrolidone (NMP) (5:1). The resulted conjugate was directly treated with trifluoroacetic acid (TFA)–ethanedithiol (EDT) (95:5) at room temperature for 2 h. Certain amounts of by-products were observed in the HPLC analysis of the deprotected sample. The above by-products were mainly formed by the deletion of sterically hindered amino acids such as Aib and Val. The peptide has a sequence in which these amino acids are continuously aligned, which caused difficulty in the incorporation of amino acid into the peptide chain on the solid-phase resin. However, in this study, a subsequent HPLC purification successfully yielded a pure biotin-tagged alamethicin (Alm-Bio) **1**. The mass of the final product corresponded well with the theoretical value judged by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS).

Ion-channel formed by biotin-tagged alamethicin

We next conducted the characterization of the channel formed by Alm-Bio **1**. Channel current was monitored by the planar-lipid bilayer method[13] using diphytanoylphosphatidylcholine as a lipid. The system has equivalent sensitivity with the patch-clamp system, which enables us to monitor the channel current going through a single channel pore.

The peptide **1** in the absence of streptavidin showed a channel current of multi open states very similar to that of the natural alamethicin (Fig. 2).[4] The frequent switch of the conductance level was attributed to the aggregation number or aggregation state of alamethicin molecules that were continuously interconverted with each

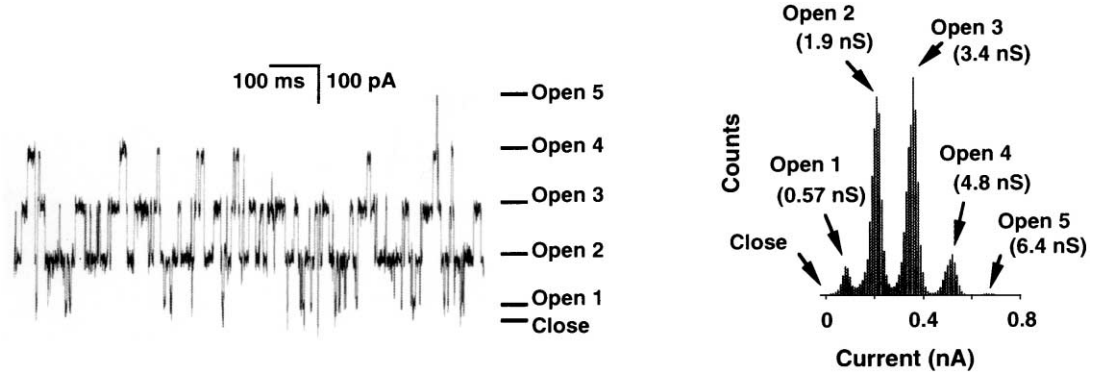

Figure 2. A single channel recording (left) and the histogram (right) of Alm-Bio **1**. Voltage, +100 mV; peptide, 63 nM; electrolyte, 1 M KCl; lipid, diphytanoylphosphatidylcholine.

other. The observed conductance levels corresponded well with that for natural alamethicin.[4] Therefore, C-terminal biotinylation seem to have little effect on the channel characteristics. Here the peptide was added from one side of the membrane (designated the *cis* side). A significant channel current was observed only when the voltage on the *cis* side was positive, suggesting that **1** was incorporated predominantly at the N-terminus.

Detection of streptavidin–biotin interaction

We next examined whether the streptavidin–biotin interaction was detectable as changes in the channel current levels. Figure 3 shows a channel current recording of **1** where two or three channels were simultaneously open in the membrane. By the addition of streptavidin (final concentration: 0.24 μM), the channel current was dramatically reduced, and no channel current could be observed after 3 min. Further addition of biotin hydrazide (final concentration: 13 μM) recovered the channel current level. No suppression of the channel current was observed when streptavidin was added to a channel formed by natural alamethicin, which does not have the biotin tag. No significant suppression of the channel current was observed when streptavidin (0.24 μM) was added from the other side of the membrane (designated as the *trans* side), which suggested that alamethicin was incorporated in the membrane predominantly from the N-terminus of the molecules and that streptavidin on the *trans* side was not able to pass through the channel pore to interact with biotin on the *cis* side. Likewise, after alamethicin current was suppressed by adding streptavidin (0.24 μM), significant recovery of the channel current was not observed on the addition of biotin hydrazide (13 μM) to the *trans* side, which was assumed to correspond to the N-terminus side of the alamethicin in the membrane. Thus streptavidin molecules effectively detected the tag on the membrane to block the opening of the channel.

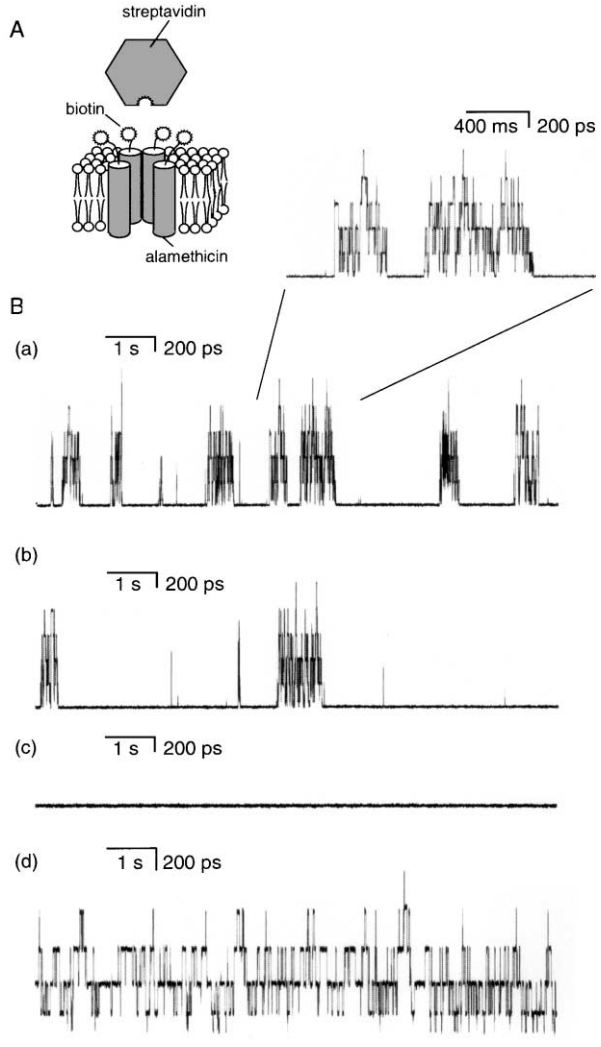

Figure 3. A schematic representation of a channel formed by Alm-Bio **1** (A) and the effect of streptavidin on the Alm-Bio channel current (B). (a) Before addition of streptavidin; (b) 1 min and (c) 3 min after addition of streptavidin (0.24 μM); (d) recovery of the channel current by biotin hydrazide (13 μM) (20 min after addition of biotin hydrazide). Peptide concentration, 63 nM; electrolyte, 1 M KCl; applied voltage, +191 mV.

A dose-dependent manner of channel current suppression by streptavidin was observed (Fig. 4). With the addition of streptavidin (0.24 μM), complete suppression was obtained in 3 min. The smaller the concentration of the streptavidin became, the less efficient the suppression became. At a streptavidin concentration of 0.006 μM, complete suppression was not attained in 60 min. Interestingly, the channel current was blocked even at a streptavidin concentration of 0.03 μM (molar ratio of streptavidin to Alm-Bio **1** = ~0.5), although a longer incubation time (30 min) was necessary to stop the channel current. Alamethicin was reported to form an assembly comprising several molecules.[4] It would be sufficient to block the channel current if streptavidin binds one of these alamethicin molecules forming a channel. Another idea to explain this result may be the

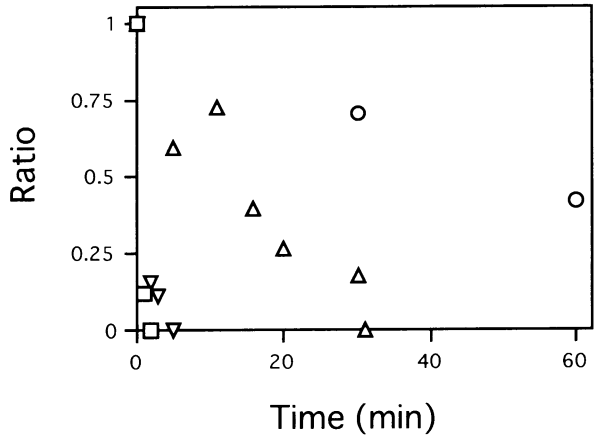

Figure 4. Dose-dependent manner of the channel current suppression of Alm-Bio **1** by streptavidin. The ordinate: the ratios of the total electric charges going through the membrane in 30 s beginning at the indicated time over those just before addition of streptavidin. Abcissa: time after addition of streptavidin. Streptavidin concentration: 0.24 μM (open square); 0.12 μM (open downward triangle); 0.03 μM (open upward triangle); 0.006 μM (open circle). Because exact control of the number of channels formed by alamethicin in the membrane is difficult using this measuring system, the applied voltage in each experiment (+191, +143, +165, and +140 mV, respectively) was adjusted so that the channel current did not exceed the maximum range of detection (~2 nA). Other conditions were the same as in Figure 3.

involvement of steric hindrance among the streptavidins attaching to the biotin tags, as was observed in the alamethicin bearing a helical extramembrane segment.[6h] The eventual prevention of the alamethicin molecules from assembling in the membrane may result in the decrease in the channel current.

Detection of interaction of anti-biotin antibody with biotin

To explore wider applicability of this approach, we then examined whether interaction of anti-biotin antibody with biotin was detectable in the channel current levels. Goat polyclonal antibody (Vector Laboratories SP-3000, affinity purified) was used as the antibody. As shown in Figure 5, the addition of the antibody (0.24 μM) to the *cis* side effectively suppressed the ion channel current of Alm-Bio **1**. The suppression by the antibody seemed to be slightly less effective than that by streptavidin, because a longer time (15 min) was needed to obtain complete suppression compared with the use of the same concentration of streptavidin (3 min). Addition of biotin hydrazide (18 μM) to the electrolyte of the *cis* side led to a recovery of the channel current, which fact confirmed that the suppression of the channel current was due to the binding of antibody to the biotin. These results exemplified that the ligand–receptor interaction was monitorable in real time through this system.

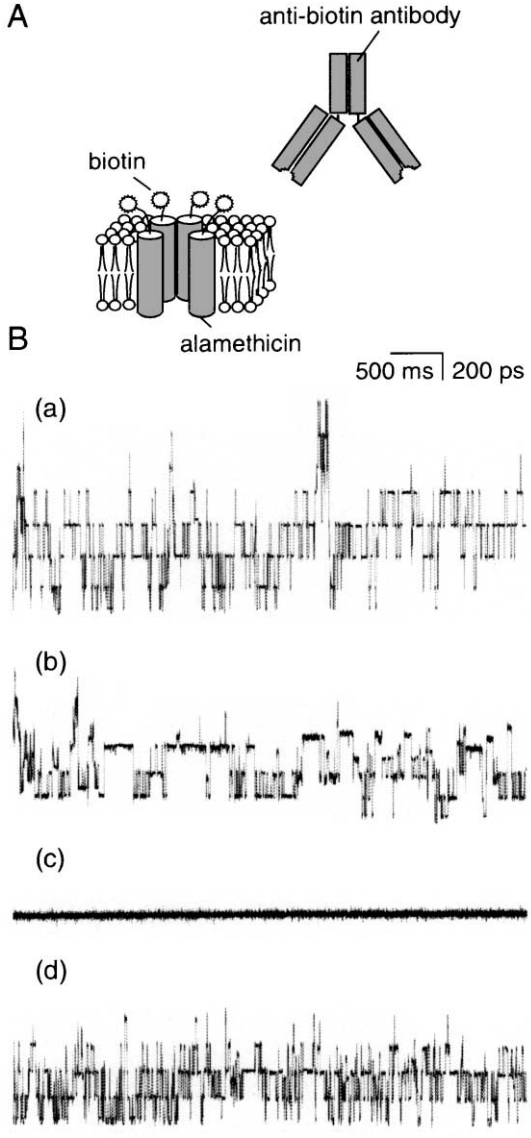

Figure 5. Schematic representation of Alm-Bio **1** with anti-biotin antibody (A), and real-time monitoring of the interaction of biotin with anti-biotin antibody (0.24 μM) (B). Channel current recordings before addition of streptavidin (a), 5 min (b), and 15 min (c) after addition of the antibody were shown, respectively. Further addition of biotin hydrazide (18 μM) recovered the channel current (a channel current record 10 min after addition of biotin hydrazide) (d). Peptide concentration, 63 nM; electrolyte, 1 M KCl; applied voltage, +160 mV.

Discussion

Using the biotin-tagged alamethicin **1**, we have shown that real time monitoring of the interaction of streptavidin with biotin, and anti-biotin antibody with biotin, was possible. The concept can be extended to the detection of a variety of biological interactions. Mechanism of suppression may be understandable by the blocking of channel openings by streptavidin or antibody. However, further study is necessary to interpret the precise mechanisms of channel current inhibition. It may be possible that attachment of streptavidin to biotin causes steric hindrance and inhibits the assembling of alamethicin molecules as was suggested in our study using alamethicin equipped with an extramembrane segment.[6h]

The effectiveness of the antibody on the channel current suppression seemed to be slightly less than that of streptavidin. This may be because the antibody used in this report was a polyclonal antibody, and a mixtures of the antibody of various binding titers with biotin might have resulted in a less efficient suppression. Use of a monoclonal antibody would result in more effective suppression. The difference in the molecular weight between streptavidin and the antibody may cause the difference in the effectiveness of their complex formation with biotin. Nevertheless, the result clearly shows that antigen-antibody interaction was monitorable in real time using this system.

The design of artificial ion channels and sensors is one of the challenges of peptide and protein engineering. The results obtained in this study will provide valuable information on the design of these functional molecules.

Experimental

Preparation of biotinylated alamethicin

Peptide synthesis was conducted by Fmoc-solid-phase synthesis as reported. Starting with Fmoc-Phe-2-chlorotrityl resin (0.43 mmol/g) (61 mg, 26 μmol), the peptide chain was constructed using a Shimadzu PSSM-8 synthesizer. For removal of the Fmoc-moiety on the N-terminal, 30% piperidine in DMF (4 min×2) was

used. Ten equivalents of a Fmoc-amino acid derivative were employed for each coupling, which was pre-activated with HATU (95 mg, 10 equiv), HOBt (48 mg, 10 equiv), and DIEA (121 μL, 20 equiv) for 10 min. The activated amino acid was then reacted with the peptide resin for 40 min. By repeating the cycles of N-terminus deprotection and amino acid introduction, the peptide chain was constructed. After N-terminal acetylation using acetic anhydride (12 μL, 5 equiv) and NMM (14 μL, 5 equiv) in DMF at rt for 30 min, the peptide resin was washed successively with DMF, methanol, and diethyl ether and dried to yield a protected peptide resin (110 mg). A part of the peptide-resin (50 mg, 22 μmol) was then treated with DCM–TFE–AcOH (3:1:1) (5 mL) for 1 h. Solvent was removed under a N_2 stream. H_2O was added to produce a precipitate, which was dried by lyophilization to yield **2** (20 mg, 67%). A part of the powder (11 mg, 4.1 μmol) was dissolved in DMF–NMP (5:1) (120 μL) and treated with biotin hydrazide (Sigma) (2.1 mg, 8 μmol), HOBt (1.11 mg, 8 μmol), and DICDI (13 μL, 41 μmol) for 7 h. Progress of the reaction was monitored by analytical HPLC. Solvent was removed in vacuo. H_2O was added to produce a precipitate, which was washed with *n*-hexane twice. The obtained powder (11 mg) was treated with TFA (1 mL) containing EDT (50 μL) at rt for 3 h. After TFA was removed under a N_2 stream, ether was added to produce a precipitate. HPLC purification of the product gave the desired peptide **1** (0.24 mg, 3% from **2**). Purity determined by the HPLC analysis, >95%; retention time in HPLC, 29.8 min [Column, Cosmosil 5C4-AR-300 Å (4.6×150 mm); gradient, 35–55% B in A over 40 min (A = H_2O–0.1% TFA, B = CH_3CN–0.1% TFA); flow, 1 mL/min; detection, 215 nm]. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS), 2219.4 $(M+H)^+$ (theoretical: 2219.5).

Channel activity measurements

Planar lipid bilayers were formed by the painting method.[13] Diphytanoylphosphatidylcholine dissolved in decane (20 mg/mL) was used as a bilayer-forming lipid. Electrolytes were unbuffered 1 M KCl, and all the measurements were done at 22 ± 1 °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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