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Synthesis and Ion Conductance Behavior of a Tetrameric Alamethicin Ion Channel

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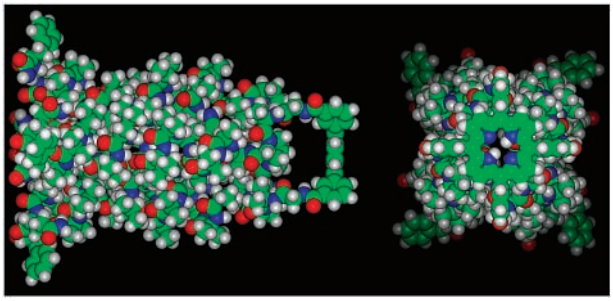
ABSTRACT



A porphyrin-tethered construct, containing four full-length alamethicin monomers, has been synthesized and characterized. The ion conductance data of the assembly in 1 M HCl display long-lived, albeit noisy, channels that appear to be voltage-independent multiples of only one conductance state. The noise in the data is consistent with the molecular modeling studies, which indicate that the side chain of glutamine 7 of alamethicin does not fit well into the narrow pore of a parallel four-helix bundle.

Transmembrane ion channels, which facilitate the transport of ions across the hydrophobic barrier of a cell membrane, are essential to maintaining the critical balance of ion concentrations that forms the basis for such fundamental life processes as intercellular communication and nerve impulse transmission. Alamethicin (Figure 1) is a 20-residue helical peptide that when incorporated in a membraneous environment aggregates to form voltage-gated ion channels, and for this reason it has been studied extensively as a model ion channel peptide.[1,2] However, study of the ion conductance behavior of alamethicin is complicated by its tendency to form channel aggregates of multiple sizes and hence multiple conductance states.[3] The conducting pores are proposed to consist of bundles of parallel helices in which the observed variation in conductance state is attributed to helix bundles of different size. The template assembled synthetic protein (TASP) approach[4] is here employed to isolate an alamethicin channel of a single conductance state by linking an alamethicin analogue to a template molecule, a technique previously applied in modeling other ion channel systems.[3,5−7] The template used in this work is a porphyrin (Figure 1)

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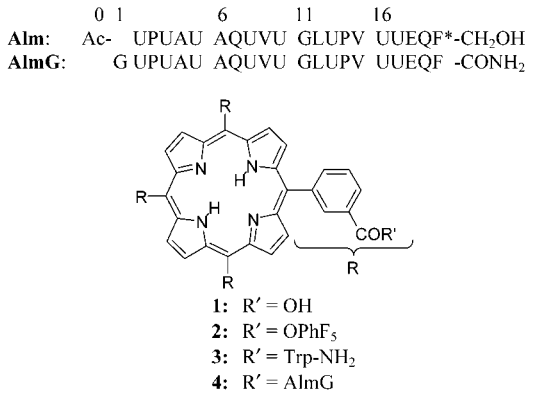

Figure 1. Structures of alamethicin (Alm), alamethicin analogue (AlmG), and the *meso*-tetrakis(*m*-carboxyphenyl)porphyrin template (TmCPP, **1**); Ac = acetyl, F* = phenylalaninol, U = α-amino-isobutyric acid (Aib).

that has previously been used in the synthesis of a tetrameric ion channel model composed of de novo designed peptides.[8]

Templated parallel alamethicin dimers have been made earlier by cross-linking two monomers at their C-terminal ends by a flexible tether.[3] These dimers produce predominantly three conductance states, proposed to correspond to pores consisting of four, five, or six alamethicin units, respectively. Shorter alamethicin derivatives, consisting of up to 17[9] and 18[10] amino acid residues, have previously been attached to linear peptides or cyclic pseudopeptides to produce templated constructs containing up to five alamethicin monomers. In these studies the C-terminal end of alamethicin was attached to lysine residues of the template. This communication describes the first example of a templated alamethicin construct in which the N-terminus of alamethicin has been attached to a scaffold. It also describes the first example of an assembly comprising more than two covalently linked full-length alamethicin monomers. The porphyrin-templated construct is designed to mimic the conductance state of alamethicin that has been attributed by Woolley et al.[3] to correspond to a four-helix bundle. This conductance state is exceedingly short-lived, making a detailed channel characterization difficult. A templated version could extend the open channel lifetimes, making more advanced conductance measurements possible.[8]

The structure of the alamethicin analogue used in this study (AlmG) included an additional *N*-terminal Gly residue to allow flexibility of the peptide upon attachment to the template molecule, a modification suggested by preliminary molecular modeling of the proposed model channel. The free N-terminal amine of AlmG was necessary for coupling to the carboxylic acid groups of the template. For synthetic convenience, the C-terminal residue was changed from phenylalaninol to phenylalanine, which was then protected as an amide to prevent cross reactions among AlmG monomers.

AlmG was synthesized using solid-phase techniques with Fmoc chemistry. As for all Alm analogues, the synthesis of AlmG was complicated by the presence of many Aib residues. Although these strongly helix-promoting residues predispose AlmG to a helical structure, they are also sterically hindered and couple with difficulty to other sterically hindered residues (e.g., Pro, Val). For this reason, double couplings with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) were used in all coupling steps. Purification of the AlmG peptide was achieved by reversed-phase high performance liquid chromatography (HPLC, 23% recovery) employing a diphenyl column, and its identity was confirmed by electrospray ionization mass spectrometry (ESI MS).

Linkage of AlmG to the TmCPP template was complicated considerably by the Glu residue near the C-terminus. The presence of this additional carboxylic acid led to problematic cross-linking reactions among AlmG monomers under conditions designed to couple the peptide to TmCPP. Preactivation of the carboxylic acid moieties of TmCPP with the coupling reagent HBTU prior to reaction with AlmG failed to reduce the extent of side reactions among AlmG monomers and resulted in an unresolvable mixture of products.

Formation of the tetrameric channel construct was achieved via preactivation of the TmCPP template as the tetrakis(pentafluorophenyl) ester, **2**. This intermediate was synthesized by coupling pentafluorophenol (10.0 equiv) to **1** in *N*,*N*-dimethylformamide (DMF) using the coupling reagent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 4.4 equiv). Ester **2** was easily isolated by precipitation with H_2O. Although HPLC revealed two minor impurities in the crude product mixture, coupling of Trp-NH_2 to a sample of crude **2** demonstrated that side products resulting from these impurities could be readily separated from the desired product **3** by HPLC. Ester **2** was amenable to storage without perceptible degradation.

Reaction of **2** with purified AlmG produced the tetrameric alamethicin channel **4**. Unlike many ion channel peptides, which have limited solubility in conventional organic solvents, compound **4** is readily soluble in acetonitrile. This unusual property allowed purification of the tetrameric channel by HPLC using a reversed-phase analytical C4 column and a standard water−acetonitrile gradient. The identity of the purified product was confirmed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Both AlmG and templated AlmG were incorporated into solvent-free diphytanoyl phosphatidylcholine planar bilayers, prepared as previously described.[7] Single ion channel activity was measured at −40 to −60 mV in symmetrical 1 M HCl electrolyte or at −90 mV in 1 M KCl and 10 mM MOPS buffer, pH 7. Peptides incorporated spontaneously after the addition of a few microliters of a ca. 0.1 mg/mL peptide in methanol solution to the electrolyte. In both HCl and KCl,

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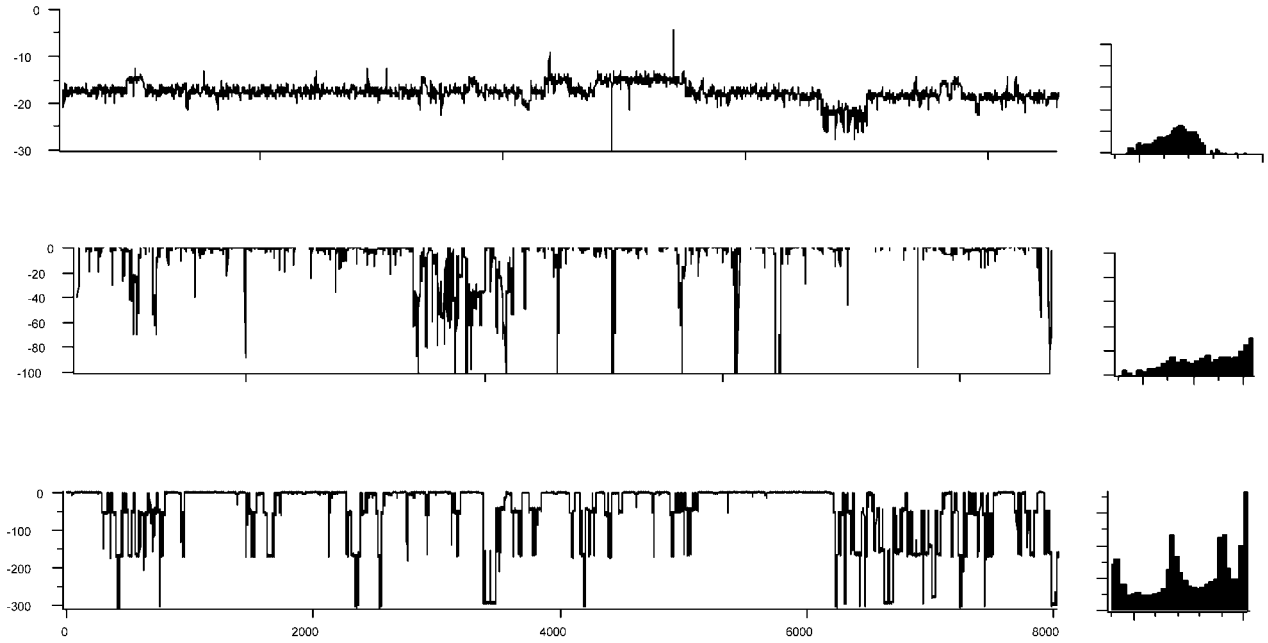

Figure 2. Current (pA) versus time (ms) traces for templated AlmG (top), AlmG measured in 1 M HCl (middle), and AlmG measured in 1 M KCl (bottom). Each trace is 8 s long and accompanied by a histogram showing the square root of the open probability (ordinate) versus current level (abcissa) in bins spanning the current range shown in the respective current−time traces.

AlmG produces channels with short lifetimes and multiple conductance states. These states have voltage-dependent occurrence frequencies that are qualitatively similar to those observed for native alamethicin, Alm. AlmG forms channels of slightly shorter lifetimes in HCl compared to in KCl, but both electrolytes produce similar mixtures of varying conductance states. In contrast, templated AlmG **4** produces longer lived, but noisy current levels in 1 M HCl that appear to be voltage-independent multiples of only one conductance state. In 1 M HCl, AlmG has an average open lifetime of 1 ms or less, which is extended to ca. 5000 ms for templated AlmG. The sample data traces in Figure 2 exemplify these differences. The much smaller and more uniform conductance produced by the templated peptide channels is also consistent with the expected aggregate size of a tetramer, which has a narrow pore. When the trans chamber was perfused with 1 M KCl, channel activity disappeared; however, more extensive, systematic experiments, beyond the scope of this communication, will be required to reliably establish channel selectivity.

The untethered peptide AlmG forms channels whose occurrence frequency and conductance distribution depends on the applied voltage across the membrane. In contrast, the lack of a strong voltage dependence of channel formation for templated AlmG (data not shown) is indicative of a channel assembly that maintains its transmembrane orientation in the absence of an applied field. The same channel behavior was previously found for templated (LSLULSL)[3,7] for which CD spectroscopy experiments in oriented lipid bilayers verified that the assembly was inserted into the membrane with the helix axis perpendicular to the membrane surface.[11]

Consistent with previous studies,[12,13] molecular modeling indicates that the glutamine residue in position 7 of AlmG does not pack well into the narrow pore of a parallel four-helix bundle. Interference of this residue with the channel conductance pathway might account for the relatively noisy character of the open channels. We are currently synthesizing larger templated alamethicin assemblies, consisting of more than four helices. We are also synthesizing an alamethicin derivative containing an asparagine[14] in place of the glutamine residue to test whether this modified peptide will form more stable channels when attached to the porphyrin template.

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Supporting Information Available: Experimental procedures for the synthesis, isolation, and characterization of AlmG-TmCCP **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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