Self-Assembling Cyclic β^3-Peptide Nanotubes as Artificial Transmembrane Ion Channels

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Abstract: A new class of self-assembling transmembrane ion channels based on cyclic β^3-peptides is described. Cyclic peptide subunits were designed to adopt flat, ring shaped conformations and stack through extensive backbone-backbone hydrogen bonding to form tubular channel structures. Candidate channel-forming peptides *cyclo*[(-β^3-HTrp)$_4$-] **1**, *cyclo*[(-β^3-HTrp-β-HLeu)$_2$-] **2**, and *cyclo*[(-β^3-HLeu)$_4$-] **3** were examined in liposome-based proton transport assays and single channel conductance experiments. Compounds **1** and **2** exhibited remarkable ion transport activities with single-channel K^+ conductance of 56 pS for peptide **1**, while compound **3** was inactive, possibly due to its poor solubility. Additionally, the putative structure of transmembrane channels formed by peptides **1** and **2** was supported by FT-IR spectroscopy of membrane-peptide preparations. The present system not only complements that of channel-forming cyclic D,L-α-peptides previously reported from this laboratory but also is expected to exhibit novel properties arising from the unnatural β^3-peptide backbone.

Introduction

β-Peptides are an emerging class of unnatural biopolymers with surprising secondary structural propensities.[1,2] Seebach[1b,c] has described helical and extended conformations adopted by short linear oligomers of acyclic β-amino acids, while Gellman[2a,b] has shown that homooligomers of *trans*-2-amino-cyclohexane and cyclopentane carboxylic acids form helical structures. Recently Seebach et al. reported that cyclic tetramers of α-unsubstituted-β-chiral-β-amino acids (β^3-amino acids)[3] can adopt flat ring conformations and stack in the solid state through backbone−backbone hydrogen-bonding.[1a] The resulting hollow tubular ensembles (cyclic β^3-peptide nanotubes) are structurally analogous to those formed by self-assembly of cyclic peptides composed of alternating D- and L-α-amino acids (cyclic D,L-α-peptide nanotubes),[4−6] and these two classes of nanotubular assemblies are expected to complement one another in structure and function. Here we report that cyclic β^3-peptides with appropriately chosen hydrophobic side chains can self-assemble in lipid bilayers to form highly efficient transmembrane ion channels (Figure 1). The present study complements earlier work from this laboratory concerning channel-forming cyclic D, L-α-peptides.[6]

Design Principles

Choice of Backbone Structure. Nanotubes based on cyclic peptides were first proposed in 1972 by Hassal et al. who predicted that cyclic tetrapeptides composed of alternating α- and β-amino acids would stack through backbone-backbone hydrogen-bonding to form hollow cylindrical structures.[7a] Later X-ray crystallographic work partially validated these predictions, although only two of four expected intersubunit hydrogen-bonds were observed.[7b] In 1974 within the context of a theoretical analysis of cylindrical β-helical conformations accessible to linear poly-D,L-α-peptides, De Santis and co-workers recognized

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(3) We have adopted the nomenclature of Seebach in which an α-unsubstituted-β-chiral-β-amino acid is designated β^3-HXaa where Xaa is the three letter abbreviation for the trivial name of the corresponding α-amino acid (e.g., β^3-HTrp). In reporting NMR assignments for β^3-peptides, we have referred to residue atoms using a Greek lettering system analogous to that of standard α-peptides except for obvious changes arising from the extra backbone carbon atom (side chain atom labels shift forward by one letter, e.g., $C^\delta H \rightarrow C^\gamma H$).

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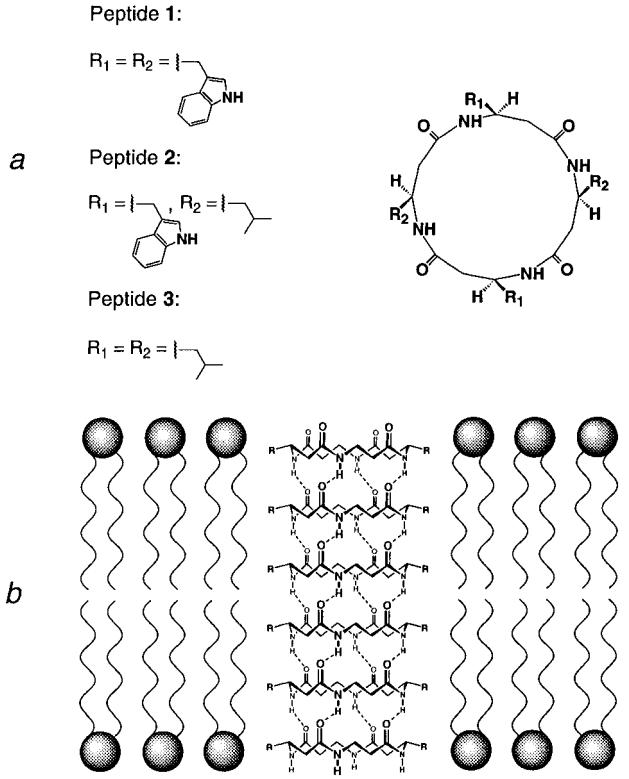

Figure 1. (*a*) Chemical structure of channel-forming cyclic β^3-peptide subunits **1**−**3** represented in a flat ring-shaped conformation. (*b*) Putative structure of self-assembled transmembrane channels formed from cyclic β^3-peptides **1**−**3**. The tubular channel ensemble is represented with expected parallel ring stacking and extensive inter-subunit hydrogen-bonding.[1a] (For clarity most side chains are omitted.)

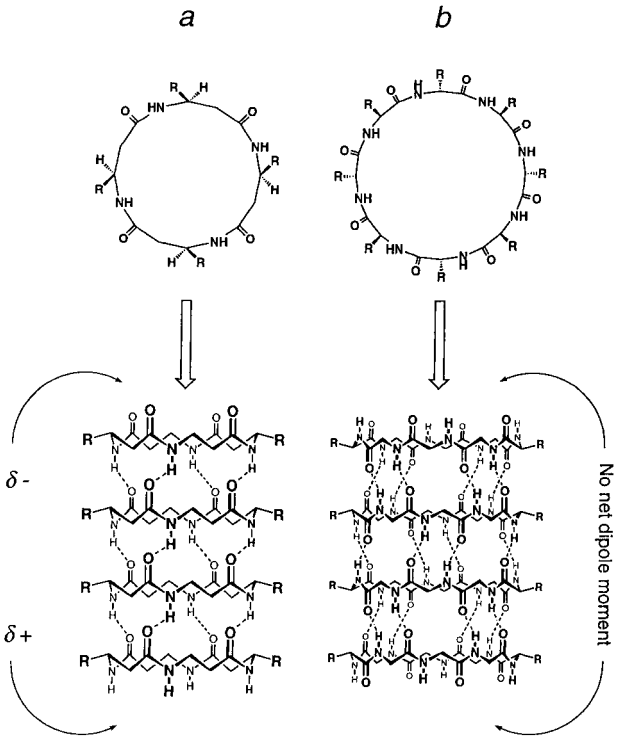

Figure 2. Comparison of anticipated structural attributes of cyclic peptide nanotubes composed of: (*a*) cyclic β^3-tetrapeptides, and (*b*) cyclic D,L-α-octapeptides. Due to unidirectional arrangement of polar backbone amide functionalities, cyclic β^3-tetrapeptide nanotube (*a*) is expected to possess a macrodipole moment reminiscent of an α-helix, while cyclic D,L-α-octapeptide nanotube (*b*) should display no net dipole. (For clarity most side chains are omitted.)

the possibility of forming analogous cylindrical structures by ring-stacking of *cyclic* D,L-α-peptides.[8] Early attempts to experimentally verify this prediction met with limited success, partially due to extreme insolubility of the peptides examined.[5c,d] In 1993, electron microscopy, electron diffraction, FT-IR, and molecular modeling studies reported from this laboratory provided the first compelling evidence for formation of hollow tubular structures by self-assembly of cyclic D,L-α-peptides.[4c] Subsequent investigations have addressed structural and thermodynamic underpinnings of this self-assembly process as well as its scope and limitations.[4,5a,b] Additionally, recent reports from this laboratory have shown that cyclic D,L-α-peptides bearing suitable hydrophobic side chains can self-assemble in lipid bilayers to form highly efficient transmembrane ion and small molecule channels.[6] Such channel-forming cyclic peptides are currently being investigated in design and preparation of novel ion sensing devices and targeted cytotoxic agents.

In order to further explore scope and limitations of ion channel formation by ring-stacking of cyclic peptides, we decided to investigate peptides subunits with alternative backbone structures. Molecular modeling suggested that, like their cyclic D,L-α-peptide counterparts, cyclic peptide subunits composed of homochiral β^3-amino acids could adopt flat, disklike conformations with amino acid side chains occupying equatorial positions on the exterior of the peptide ring, while axial and interior positions remain unobstructed (Figure 2). Due to the extra carbon atom in each β^3-amino acid residue, the register of atoms in the cyclic β^3-peptide backbone was expected to orient amide moieties in a unidirectional manner, with NH and carbonyl groups lying on opposite faces of the peptide ring. These anticipated structural features would allow peptide subunits to stack in a parallel fashion through backbone−backbone hydrogen-bonding, giving rise to hollow peptide nanotubes (Figure 2). While this work was in progress, these design principles were supported by Seebach and co-workers who reported solid-state structures of short linear[1b] and cyclic[1a] homochiral β^3-peptides and indicated that the latter form tubular arrays with expected structural features. Additionally, Seebach et al. indicated that *heterochiral* cyclic β^3-tetrapeptides (*R*,*R*,*S*,*S* and *R*,*S*,*R*,*S*) also give rise to hollow cylindrical structures in the solid state,[1a] further evincing conformational versatility of cyclic β^3-peptides.

Despite similarities to their cyclic D,L-α-peptide counterparts, cyclic β^3-peptide nanotubes were expected to display unique features arising from their unnatural backbone structure (Figure 2). For example, the unidirectional arrangement of polar backbone amide functionalities in cyclic β^3-peptide subunits should endow the tubular ensemble with a macrodipole moment reminiscent of an α-helix. In the context of a transmembrane ion channel, such a macrodipole is expected to influence conductance through effects such as voltage gating and current rectification.[9] Additionally, as a consequence of parallel ring stacking, amino acid side chains of cyclic β^3-tetrapeptide subunits should be oriented in a parallel fashion along the length of the tube. Thus dipole moments arising from side chain polar groups (e.g., the indole moiety of β^3-HTrp) are expected to reinforce one another and further augment voltage gating and current rectification behavior.[10] In contrast, cyclic D,L-α-

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(9) For a study of backbone dipole effects on channel conductance in a simple α-helical model system see: Kienker, P. K.; DeGrado, W. F.; Lear, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4859−4863.

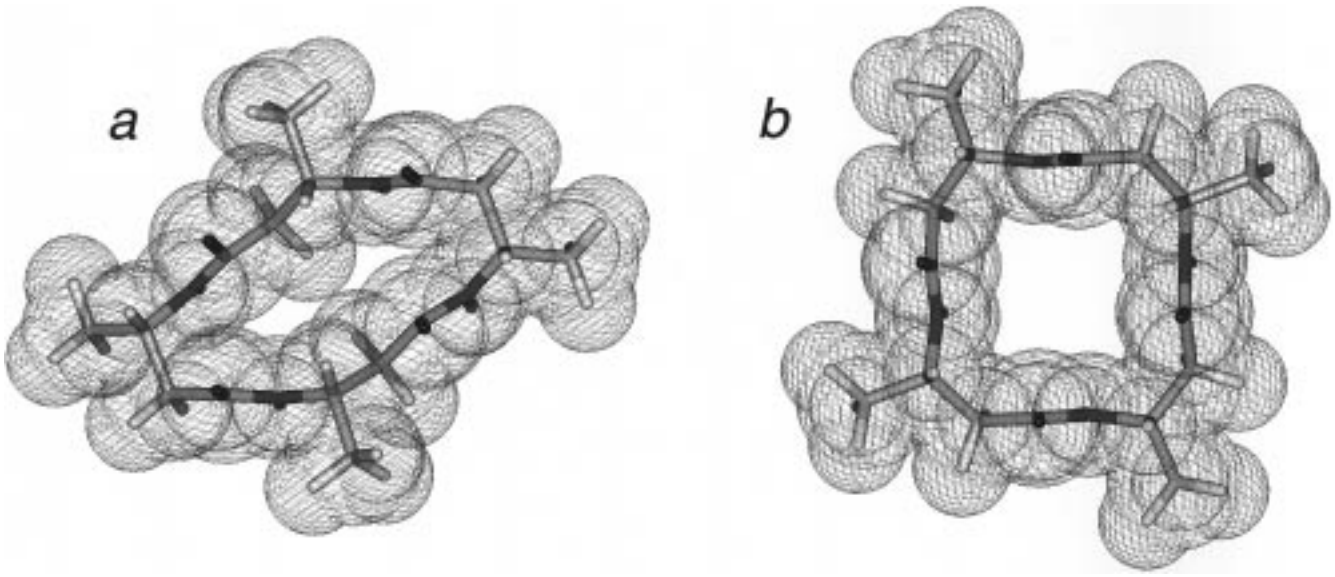

Figure 3. Stick and van der Waals representations of a homochiral cyclic β^3-tetrapeptide shown (*a*) in a flat-ring C_2 symmetrical conformation as recently described by Seebach[1a] and (*b*) in an alternative flat-ring C_4 symmetrical conformation. In (*b*) the molecule displays a central hole 2.6−2.7 Å in diameter, large enough to allow passage of water and small ions.[12] (For clarity side chains are displayed as methyl groups.)

peptides favor antiparallel ring stacking due to greater inherent stability of antiparallel versus parallel β-sheets;[4e] therefore, side chain dipole moments in these nanotubes should cancel. Finally, linear β^2- and β^3-peptides have been shown to resist protease digestion due to their unnatural backbone structure;[1c,11] thus cyclic β^3-peptides possessing cytotoxic or antimicrobial activity would be attractive candidates for development as therapeutic agents.

Cyclic Peptide Ring Size. Cyclic β^3-tetrapeptides were chosen for investigation as potential channel-forming subunits on the basis of molecular modeling. These studies suggested that cyclic tetramers would exhibit greater conformational rigidity and a higher degree of preorganization for self-assembly compared with larger cyclic β^3-penta- or hexapeptides. Seebach et al. have studied the solid-state structure of *cyclo*[(-β^3-HAla)$_4$-] using X-ray powder diffraction and molecular modeling and reported that the molecule exists in a C_2 symmetrical conformation in which the central hole of the peptide ring is collapsed (Figure 3a).[1a] However, van der Waals surface calculations carried out in this laboratory on a cyclic β^3-tetrapeptide model indicate that in an alternative flat-ring C_4 symmetrical conformation such molecules possess an internal diameter of 2.6−2.7 Å, large enough to accommodate water and small ions (Figure 3b).[12] We speculated that in a lipid membrane environment cyclic β^3-tetrapeptide subunits would sample this C_4 symmetrical conformation, thus allowing passage of water and small ions through the central pore of the expected tranmembrane tubular structure.

Choice of Amino Acid Side Chains. Due to their strong propensity to aggregate, nanotube-forming cyclic peptides are often highly insoluble.[1a,4,5] Previous work with channel-forming cyclic D,L-α-peptides had indicated that the indole side chain of Trp is important for imparting solubility in polar organic solvents such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) yet is sufficiently hydrophobic to allow efficient partitioning of tryptophan-rich cyclic peptides into apolar membrane environments.[6] Trp has also been implicated in natural membrane peptides and proteins as being important for anchoring polypeptide chains to lipid bilayers through interaction of side chain NH groups with the hydrophilic water-membrane interface.[13] Thus β^3-HTrp was selected for incorporation into candidate channel-forming cyclic β^3-tetrapeptides. Additionally, β^3-HLeu and β^3-HTrp-β^3-HLeu combinations were also examined.

Results

Tetrapeptides *cyclo*[(-β^3-HTrp)$_4$-] **1**, *cyclo*[(-β^3-HTrp-β-HLeu)$_2$-] **2**, and *cyclo*[(-β^3-HLeu)$_4$-] **3** (Figure 1a) were synthesized via established protocols and screened for bulk proton transport activity using a liposome-based assay.[14] Large unilamellar vesicles (LUVs)[15] prepared at pH 6.5 were diluted into pH 5.5 buffer. Collapse of the resulting transmembrane proton gradient upon addition of peptide solution[16] was followed by monitoring the fluorescence of the entrapped pH sensitive dye 5(6)-carboxyfluorescein (CF). Apparent proton transport activities[17] of β^3-HTrp-containing peptides **1** and **2** are similar to those of both gramicidin D and channel-forming cyclic D,L-α-peptides[6c] (Figure 4). However, compound **3** exhibited no activity in this assay (data not shown), possibly due to rapid precipitation of the peptide upon addition to the aqueous liposome suspension. Control experiments[18] monitoring the release of 5(6)-carboxyfluorescein indicated that collapse of the

(10) Tryptophan residues of the Gramicidin A channel have been shown to significantly influence channel conductance. For leading references, see: (a) Hu, W.; Lazo, N. D.; Cross, T. A. *Biochemistry* **1995**, *34*, 14138−14146. (b) Hu, W.; Crosss, T. A. *Biochemisty* **1995**, *34*, 14147−14155. (c) Ketchem, R. R.; Hu, W.; Cross, T. A. *Science* **1993**, *261*, 1457−1460.

(11) Hintermann, T.; Seebach, D. *Chimia* **1997**, *50*, 244−247.

(12) The minimum effective radius of the Gramicidin A channel was recently reported as 1.28 Å. Smart, O. S.; Breed, J.; Smith, G. R.; Sanson, M. S. P. *Biophys. J.* **1997**, *72*, 1109−1126.

(13) Gramicidin A is one example. See refs 10a−c and work cited therein.

(14) Carmichael, V. E.; Dutton, P. J.; Fyles, T. M.; James, T. D.; Swan, J. A.; Zojaji, M. *J. Am. Chem. Soc.* **1989**, *111*, 769−770.

(15) (a) Szoka, F.; Papahadjopoulos, D. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 4194−4198. (b) Olson, F.; Hunt, C. A.; Szoka, F. C.; Vil, W. J.; Papahadjopoulos, D. *Biochim. Biophys. Acta* **1979**, *557*, 9−23.

(16) DMSO solutions (1 mM) of peptides **1** and **2** were used in this assay. However, peptide **3** proved insoluble under these conditions and was first dissolved in HFIP (∼7 mM) followed by solvent exchange via gel filtration on an LH-20 column preequilibarated with MeOH. Peptide containing fractions were identified by UV spectroscopy. The resulting MeOH solution of **3** (∼2 mM) was stable at least 24 h at room temperature with no visible signs of precipitation and was either used directly in this assay or first diluted 1:1 v/v with DMSO (∼1 mM final concentration).

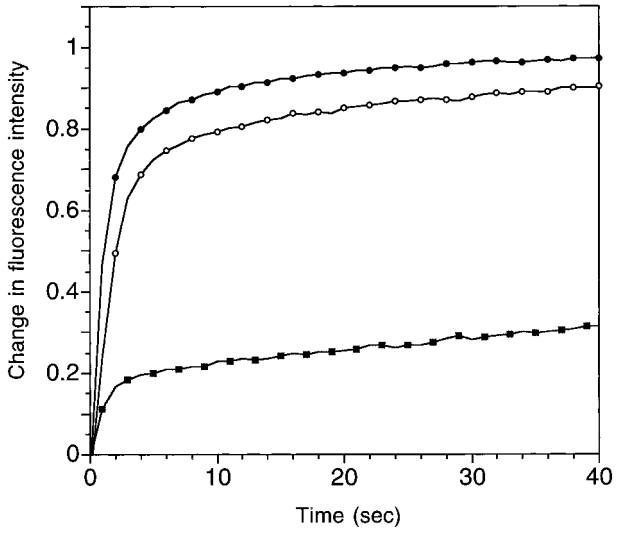

Figure 4. Proton efflux following addition of channel forming peptides, (●) gramicidin D, (○) peptide **1**, and (■) peptide **2**, expressed as fractional change in fluorescence intensity of vesicle entrapped CF versus time (1-s sampling intervals). Peptide **3** displayed no activity in this assay (data not shown). Equimolar amounts of each peptide were used in each experiment,[16] and data were normalized to allow qualitative comparison of initial proton transport activities.[17]

pH gradient upon addition of compounds **1** and **2** was caused neither by rupture of liposomes nor by the presence of small amounts of organic solvents (<5% dimethyl sulfoxide (DMSO)) employed (see Experimental Section).

On the basis of their apparent proton transport activities in liposome assays described above, compounds **1** and **2** were selected for further biophysical characterization. FT-IR studies of liposome-peptide complexes provided evidence for the putative structure of transmembrane channels formed by these peptides (Figure 5). Samples were prepared for IR analysis by adding DMSO solutions of **1** or **2** to an aqueous suspension of 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC) LUVs,[15] resulting in rapid partitioning of peptide into lipid bilayers. Following gel filtration and air-drying, the resulting preparations displayed all expected peptide IR signals including amide I, amide II, and N−H bands. Observed amide N−H stretching bands at 3289 cm^{-1} (peptide **1**) and 3297 cm^{-1} (peptide **2**) indicate the existence of tight backbone hydrogen-bonding networks[19,20] and are consistent with solid-state IR data on related cyclic D,L-α-peptide nanotubes.[4,5b,6b,c,e]

Formation of discrete ion channels by compounds **1** and **2** was conclusively established through single channel conductance measurements using planar lipid bilayers.[21] Figure 6 shows a continuous conductance trace of a single channel formed by

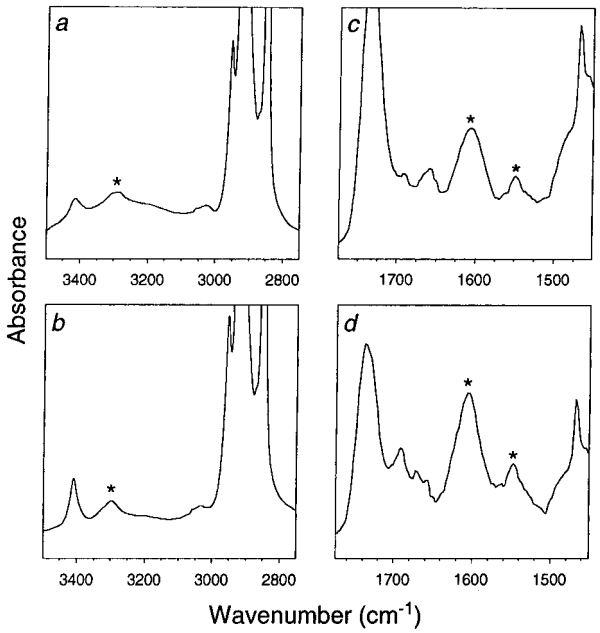

Figure 5. Infrared absorbance spectra (at 4-cm^{-1} resolution) of channel-forming cyclic β^3-peptides **1** and **2** incorporated into DMPC bilayers: (*a*) and (*b*) N−H stretch region; (*c*) and (*d*) amide I, amide II region for peptides **1** and **2**, respectively. Signals due to peptide channel structures are marked with an asterisk (*) and appear at the following wavenumbers (cm^{-1}; peptide **1** and **2**, respectively): 3289, 3297 (N−H stretch); 1607, 1605 (amide I); 1549, 1548 (amide II).[20]

cyclic peptide **1**. The observed conductance in 500 mM KCl with peptide concentrations of ∼30 μM in the subphase is 56 pS. Thus the rate of channel mediated K^+ transport is 1.9×10^7 ions s^{-1} for compound **1**, greater than that of gramicidin A under similar conditions.[22] Compound **2** also displays similar single channel activity (data not shown). Both single channel opening and closing events as well as multiple conductance levels of individual channels were often observed within the same experiment. Channel gating most likely arises from peptide nanotube assembly−disassembly processes as well as ring opening and collapse (see Figure 3), while multiple conductance levels may reflect fluctuations in the number of cyclic peptide subunits per channel structure.[6d]

Summary and Discussion

In summary, we have presented a new class of highly efficient artificial transmembrane ion channels based on cyclic β^3-peptides. Single channel conductance measurements establish K^+ conductance of 56 pS for compound **1**, similar to that of previously reported channel-forming peptide *cyclo*[-(L-Trp-D-Leu)$_3$-L-Gln-D-Leu-][6c] and greater than that of Gramicidin A under similar conditions.[22] Peptides **1** and **2** also displayed bulk proton transport activity in a liposome-based assay and were investigated by FT-IR within the context of lipid membranes and found to exist in a tightly hydrogen-bonded network,[19,20] consistent with the expected β-sheetlike tubular structure (Figure 1).[4,5b,6b,c,e] Lack of activity displayed by *cyclo*[(-β-HLeu)$_4$-] **3** in liposome-based proton transport assays suggests that β^3-HTrp residues of **1** and **2** play an important role in solubilizing these peptides and allowing their incorporation into lipid membranes (vide supra). Additionally, the indole NH moiety of β^3-HTrp may help to orient subunits at the lipid−water interface, as has

(17) The initial rapid proton efflux observed in these experiments reflects rate-limiting steps of peptide insertion into the lipid membrane and self-assembly, the latter in turn being influenced by peptide subunit stoichiometry required for channel formation (2 for gramicidin D vs ∼6−12[5d] for peptide **1** or **2**). Thus this assay cannot provide comparative information on the actual rate of channel mediated ion transport, which is faster for peptide **1** than for gramicidin A as judged by single channel conductance measurements (see text).

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(20) For IR data on related linear β^3-peptides, see: ref 1c.

(21) (a) Suarez-Isla, B. A.; Lindstrom, W. K.; Montal, M. *Biochemistry*, **1983**, *22*, 2319−2323. (b) Hille, B. *Ionic Channels of Excitable Membranes* 2nd ed.; Sinauer Associates: Sunderland, 1992.

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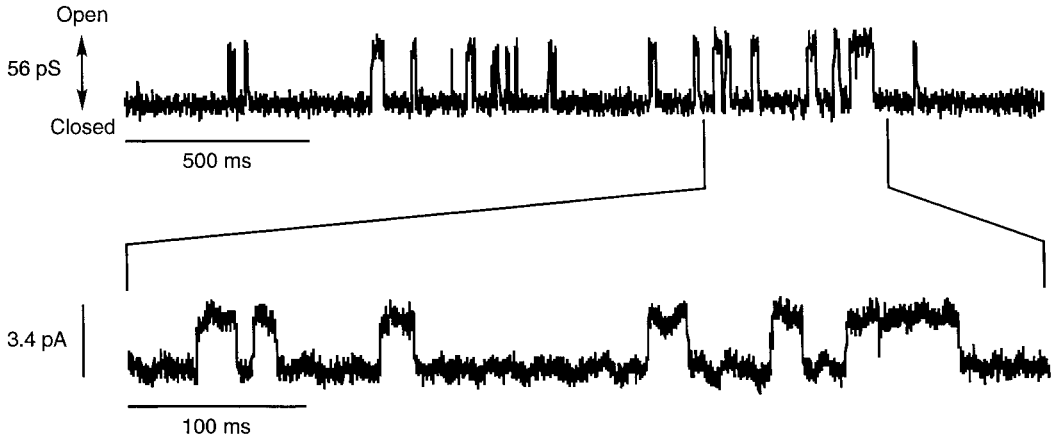

Figure 6. Continuous K^+ single channel conductance trace recorded at +60 mV (peptide **1**, open probability = 0.14). Planar lipid bilayers were formed on micropipet electrodes using Type II−S soybean phosphatidylcholine (Sigma). Sharp transitions in measured current indicate channel opening and closing events and may reflect rapid conformational changes (see Figure 3) or assembly disassembly of the channel structure.[6d]

been demonstrated for many natural membrane peptides and proteins.[13]

The present system not only complements previously described channel-forming cyclic D,L-α-peptides but also is expected to exhibit novel properties arising from the unnatural β^3-peptide backbone. For example, in flat ring conformations of compounds **1** and **2** all backbone amide functionalities are oriented in a unidirectional manner with N−H and carbonyl groups pointing in opposite directions (Figures 1 and 2), endowing the cyclic β^3-peptide subunit with permanent dipole character. These dipole moments should orient themselves in the direction of an applied electric field, favoring channel self-assembly and causing voltage gating behavior.[9] Additionally, the tubular channel ensemble is expected to display a macrodipole similar to that of an α-helix, causing cations and anions to pass more easily through the δ^- and δ^+ ends of the channel, respectively, and giving rise to a non-Ohmic current−voltage relationship known as current rectification.[9] Furthermore, due to parallel ring-stacking, axial components of side chain dipole moments should align in a parallel fashion along the length of the channel, thus augmenting dipole conductance effects.[10] Finally, linear β^2- and β^3-peptides have been shown to resist protease digestion due to their unnatural backbone structure;[1c,11] thus channel-forming cyclic β^3-peptides may prove useful as antimicrobial or cytotoxic agents.

It is important to note that the data here presented cannot unequivocally establish the actual mechanism of channel-mediated ion transport by compounds **1** and **2**. These peptides were designed to partition into lipid membranes and self-assemble into tubular structures capable of passing water and small ions through their central pores. However, alternative possibilities exist, such as formation of tubular bundles with central holes large enough to serve as a conduit for water and ions. We feel, however, that such a scenario is unlikely due to the hydrophobic character of constituent residues in channel-forming peptides **1** and **2**. In any case, the observed high ion throughput in single channel conductance experiments definitively establishes channel-mediated ion transport by these peptides and excludes carrier mechanisms as the primary mode of ion conveyance.[21b] Ongoing efforts to define ion size and charge selectivity as well as voltage gating and current rectification behavior of ion channels formed by compounds **1** and **2** should shed further light on these issues.

Experimental Section

Chemicals. Acetonitrile (optima grade), dichloromethane (ACS grade), and dimethylformamide (DMF, sequencing grade) were purchased from Fisher and used without further purification. Diisopropylethylamine (DIEA, peptide synthesis grade) was purchased from Fisher, distilled first from ninhydrin then from CaH_2,[23a] and stored over KOH before use. Trifluoroacetic acid (TFA, New Jersey Halocarbon), 2-(1*H*-benzotriazoL-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, Richelieu Biotechnologies), *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, Perceptive Biosystems),[23b−d] 4-dimethylaminopyridine (DMAP, 99%, Aldrich), anisole (anhydrous, Aldrich), trifluoroethanol (TFE, 99%, Acros), hexafluoro-2-propanol (HFIP, 99.5%, Acros), hexafluoroacetone trihydrate (HFA·$3H_2O$ 98%, Aldrich), and dimethyl sulfoxide (DMSO, 99%, Aldrich) were used without further purification. *N*−Boc-α-amino acids were used as obtained from Novabiochem or Advanced Chemtech. Deuterated solvents for ^{1}H NMR spectroscopy (DMSO-d_6, 99.9%, Cambridge Isotope Labs; TFE-d_3, 99.5%, Isotech) were used as obtained. All lipids were used as obtained from Avanti, while 5(6)-carboxyfluorescein (CF, Molecular Probes) was recrystallized prior to use.[24]

Synthesis of Amino Acids and Derivatives. Precursor protected β^3-amino acids were synthesized according to published procedures. *N*−Boc-β^3-HTrp(CHO) was prepared from *N*−Boc-L-Trp(CHO) by the methods of Plucinska[25] and Seebach.[1c] *N*−Boc-β^3-HLeu was prepared by the method of Seebach.[1d] *N*−Boc-aminoacyl-4-(oxymethyl)phenylacetamidomethyl-polystyrene resins (*N*−Boc-aminoacyl-OCH_2−Pam resins) were prepared using the preferred method of Kent and co-workers.[26]

Peptide Synthesis and Cyclization. Manual Boc solid-phase synthesis of linear peptides[27] was carried out according to the in situ neutralization protocol of Kent[28] with HBTU activation and employing *N*−Boc-aminoacyl-OCH_2−Pam resins (0.54 mequiv/g loading) as indicated for each peptide. Peptides were cleaved from resin using standard HF procedures (10 mL 9:1 HF/anisole per gram of peptidyl resin; 1 h at 0 °C) and eluted with DMF or, in the case of peptide (-β^3-HLeu)$_4$ **3a**, TFA. Following purification by reverse phase high performance liquid chromatography (RP−HPLC/C_4 column/CH_3CN/ ^{i}PrOH/ H_2O/0.1% TFA), cyclization was effected as follows:

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Linear peptide and 1.2 equiv HATU[23b−d] were dissolved in sufficient DMF such that the final peptide concentration was 1 mM. In the case of linear peptide (-β-HLeu)$_4$ **3a**, DMF containing 20 mM LiCl was required in order to solubilize the compound completely.[29] Cyclization was initiated by addition of 3 equiv DMAP followed by 6 equiv DIEA, and the resulting reaction mixture was stirred 12 h at room temperature. Piperidine was then added to a final concentration of 10% v/v, followed by stirring an additional 5 h. After removal of solvent under reduced pressure, the solid residue was dissolved in 19:1 DMF:H_2O at a peptide concentration of 5 mg/mL or, in the case of peptide **3a**, at 1 mg/mL in 7:2:1 TFE:HFIP:HFA·3H_2O, and purified by RP−HPLC (CH_3CN/^{i}PrOH/H_2O/0.1% TFA).

***cyclo*[(-β^3-HTrp)$_4$-] 1.** Linear peptide (-β^3-HTrp(CHO))$_4$ **1a** was synthesized on *N*−Boc-β^3-HTrp(CHO)-OCH_2−Pam resin and purified by RP−HPLC to give the desired product as its N-terminal TFA salt (IS-MS $[M + H]^+$ calculated = 931.4, found = 931). After cyclization, deprotection, and purification by RP−HPLC, cyclic peptide **1** was obtained in high purity as judged by ^{1}H NMR and mass spectral data. ^{1}H NMR (400 MHz, 293 K, DMSO-d_6): NH (6.83, d, J = 6.5 Hz); C$^\beta$H (3.98, m); C$^\alpha$H$_2$ (2.21, m); C$^\gamma$H$_2$ (2.71, dd, J = 6.5, 12.4 Hz; 2.82, dd, J = 4.3, 12.4); C$^{\epsilon 1}$H (6.51, d, J = 1.6 Hz); N$^{\zeta 1}$H (9.80, d, J = 1.6 Hz); C$^{\zeta 3}$H (6.87, d, J = 7.0 Hz); C$^{\eta 2}$H (6.70, d, J = 7.0 Hz); C$^{\eta 3}$H (6.36, dd); C$^\theta$H (6.46, dd). IS-MS: $[M + H]^+$ calculated = 801.4, found = 801.

***cyclo*[(-β-HTrp^1-β-HLeu^2)$_2$-] 2.** Linear peptide (-β^3-HTrp(CHO)-β-HLeu)$_2$ **2a** was synthesized on *N*−Boc-β^3-HLeu-OCH_2−Pam resin resin and purified by RP−HPLC to give the desired product as its N-terminal TFA salt (IS-MS $[M + H]^+$ calculated = 729.4, found = 729). After cyclization, deprotection, and purification by RP−HPLC, cyclic peptide **2** was obtained in high purity as judged by ^{1}H NMR and mass spectral data. ^{1}H NMR (400 MHz, 293 K, DMSO-d_6): β^3-HTrp^1: NH (6.88, d, J = 7.0 Hz); C$^\beta$H (3.90, m); C$^\alpha$H$_2$ (2.24, m); C$^\gamma$H$_2$ (2.73, dd, J = 7.0, 12.4 Hz; 2.88, dd, J = 4.8, 12.4 Hz); C$^{\epsilon 1}$H (6.52, d, J = 1.6 Hz); N$^{\zeta 1}$H (9.81, d, J = 1.6 Hz); C$^{\zeta 3}$H (6.92, d, J = 7.0 Hz); C$^{\eta 2}$H (6.72, d, J = 7.0 Hz); C$^{\eta 3}$H (6.40, dd); C$^\theta$H (6.48, dd); β^3-HLeu^2: NH (6.84, d, J = 7.0 Hz); C$^\beta$H (3.74, m); C$^\alpha$H$_2$ (2.15, m; 2.32, m); C$^\gamma$H$_2$ (1.31, m; 1.60, m); C$^\delta$H (1.61, m); C$^{\epsilon 1,\epsilon 2}$H$_3$ (1.03, d, J = 4.9 Hz; 1.04, d, J = 5.3 Hz). IS-MS: $[M + H]^+$ calculated = 801.4, found = 801.

***cyclo*[(-β-HLeu)$_4$-] 3.** Linear peptide (-β-HLeu)$_4$ **3a** was synthesized on *N*−Boc-β-HLeu-OCH_2−Pam resin and purified by RP−HPLC to give the desired product as its N-terminal TFA salt (IS-MS $[M + H]^+$ calculated =527.4, found = 527). After cyclization and purification by RP−HPLC, cyclic peptide **3** was obtained in 70% yield. ^{1}H NMR (400 MHz, 293 K, TFE-d_3): NH (7.36, br s); C$^\beta$H (4.03, m); C$^\alpha$H$_2$ (2.39, m; 2.56, m); C$^\gamma$H$_2$ (1.31, m; 1.60, m); C$^\delta$H (1.60, m); C$^{\epsilon 1,\epsilon 2}$H$_3$ (0.92, d, J = 2.7 Hz; 0.94, d, J = 2.7 Hz). IS-MS $[M + H]^+$ calculated = 509.4, found = 509.

Solution ^{1}H NMR characterization of Cyclic β^3-Tetrapeptides. The ^{1}H NMR spectrum of *cyclo*[(-β-HTrp-β-HLeu)$_2$-] **2** (∼3 mM in DMSO-d_6) was assigned[3] on the basis of its double quantum filtered COSY (2QF−COSY)[30] spectrum and literature precedent for assignment of Trp indole ring protons.[31] The ^{1}H NMR spectra of *cyclo*[(-β^3-HTrp)$_4$-] **1** (∼3 mM in DMSO-d_6) and *cyclo*[(-β-HLeu)$_4$-] **3** (∼3 mM in TFE-d_3) were assigned from the corresponding 1D spectra by analogy to **2**. Due to conformational averaging on the NMR time scale, the ^{1}H NMR spectrum of peptide **2** exhibited C_2 symmetry, while those of peptides **1** and **3** were C_4 symmetrical. All NMR experiments were performed on a Bruker AMX-400 spectrometer using Bruker's standard pulse sequences, and spectra were referenced to residual protic solvent peaks.

Liposome Fluorescence Assays. LUVs[15] were prepared by the reverse evaporation method[22] from 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC, 11 mg), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC, 200 μL of 50 mg/mL in $CHCl_3$), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylserine (POPS, 100 μL of 10 mg/mL in $CHCl_3$), and cholesterol (6 mg) in 1.5 mL PBS pH 6.5 buffer (137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, 1.4 mM K_2HPO_4, pH 6.5) containing 20 μM CF. Following sizing by extrusion (10 × with stacked 0.8, 0.4, and 0.2 μm polycarbonate membranes) unincorporated dye was removed via gel filtration on Sephadex G-25 in PBS pH 6.5 buffer. In each experiment, 1.32 mL PBS pH 5.5 buffer in a 1 cm quartz cuvette was treated with 75 μL of liposome preparation, placed in the fluorometer and stirred gently for 1 min. To the cuvette was added 60 μL of peptide solution (1 mM in DMSO for compounds **1** and **2** or 1−2 mM in MeOH or 1:1 v/v MeOH:DMSO for compound **3**)[16] through an injection port with continuous fluorescence monitoring at 520 nm (excitation at 470 nm). Data were normalized for comparison into fractional change in fluorescence given by $(I_0 - I_t)/(I_0 - I_\infty)$ where I_0 is the initial, I_∞ the final, and I_t the observed fluorescence intensity, respectively.[14]

In control experiments, release of 5(6)-carboxyfluorescein was monitored by quenching its fluorescence with externally added anti-fluorescein rabbit IgG.[18] In a typical experiment, 1.4 mL of PBS pH 6.5 buffer in a 1 cm quartz cuvette was treated with 5 μL CF-containing liposome preparation described above (20 μM CF in PBS pH 6.5), followed by 10 μL anti-fluoresecein rabbit IgG (≥1 mg/mL, Molecular Probes), then placed in the fluorometer, and stirred gently for 1 min. To the cuvette 10 μL of peptide solution (1 mM in DMSO) was added through an injection port with continuous fluorescence monitoring at 520 nm (excitation at 470 nm). A drop in fluorescence upon addition of peptide indicated leakage of CF from liposomes. Peptides **1** and **2** were determined not to cause rupturing of vesicles or leakage of CF, while control experiments with melittin[32] and Triton X-100 showed expected CF leakage.

FT-IR of Membrane-Peptide Complexes. In a typical experiment, 25 μL of peptide solution (∼20 mM in DMSO) was added quickly to a stirred suspension of DMPC liposomes in H_2O (150 μL, 20 mg lipid/mL). After 1 min, the sample was loaded onto a ∼10 mL Sephadex G-25 column and eluted in H_2O. IR samples were prepared by spreading 50 μL vesicle containing fraction onto a CaF_2 plate and air-drying overnight. Spectra were acquired in transmission mode using a Nicolet Magna-IR 550 spectrometer with a step size of 4 cm^{-1}.

Channel Conductance Measurements. Planar lipid bilayers were formed on micropipet electrodes using Type II−S soybean phosphatidylcholine (Sigma). In a typical experiment, a 5 μL aliquot of peptide solution (1mM in DMSO) was added to 150 μL subphase buffer (symmetrical salt conditions; 500 mM KCl, 5 mM $CaCl_2$, 10 mM HEPES, pH 7.5), resulting in spontaneous partitioning of peptide into the lipid bilayer. Channel activity appeared within 2 min of peptide addition in eight out of nine membranes studied. Data acquisition and analysis were performed using the pClamp software package (Axon Instruments).

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