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Template-Assembled Synthetic Proteins Designed To Adopt a Globular, Four-Helix Bundle Conformation Form Ionic Channels in Lipid Bilayers

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Abstract: Template-assembled synthetic proteins (TASPs) designed to adopt globular, four-helix bundle structures form ion channels in lipid bilayers. The rationale behind this work is that a bundle of amphiphilic α-helices may constitute a functional pore-forming motif in a lipid environment. TASPs designated $T_4(4\alpha_{11})$, $T_4(4\alpha_{15})$, and $T_4(4\alpha_{18})$ contain four identical, amphiphilic, helical modules of 11, 15, or 18 residues, respectively; secondary structure modules are attached to the lysine ϵ-amino groups of a cyclic template, Ac-CysLysAlaLysProGlyLysAlaLysCys-NH$_2$, which directs the intramolecular folding. $T_4(4\alpha_{15})$ and $T_4(4\alpha_{18})$ form cation selective channels with single channel conductances in 500 mM NaCl of 10 and 50 pS for $T_4(4\alpha_{15})$ and 9 and 25 pS for $T_4(4\alpha_{18})$. In contrast, $T_4(4\alpha_{11})$ does not produce discrete conductance events. Notably, channel activity is observed only for molecules that display well-defined α-helical structure in solution; $K(\alpha_{15})$, which contains a single peptide module attached to the ϵ-amino group of Ac-Lys-NH$_2$, does not elicit single channels and displays low α-helical content. By contrast, $K(\alpha_{18})$ displays spectral features associated with α-helical structure and forms channels with primary conductances of 3 and 9 pS. The occurrence of multiple conductances suggests that molecules aggregate and form heterogeneous conductive oligomers. The minimum length of helical modules required to span a lipid bilayer is established by investigating the channel activity of $T_4(4\alpha_{15})$ in bilayers of increasing width. Taken together, results suggest that the relative orientation of amphiphilic segments depends on the hydrophobicity of surrounding media; accordingly, TASP molecules may form ionic channels through a reorientation of template-assembled helical modules to expose charged residues to a central hydrophilic pore.

Introduction

A key issue in the *de novo* design of proteins involves consideration of how protein structure determines function. Design efforts have focused on the generation of folding motifs composed of modules with specific secondary structure, such as the β-barrel, the helix–loop–helix, and the α-helical bundle. Advances in protein design have led to the realization of such motifs;[1,2] however, correlating structure with function remains a challenge. Here, we address this question using the four-helix bundle motif as a test case. Particularly, is amphiphilicity of peptide modules sufficient to direct the assembly of a functional ion-conducting motif within the hydrophobic environment of a lipid membrane?

Amphiphilic peptides may fold into well-defined secondary structures. Specifically, the formation of α-helical bundles has been demonstrated, in aqueous solution[1–3] as well as in a lipid membrane.[4,5] In a lipid environment, such structures have a functional correlate: the assembly of α-helices such that polar residues line a central pore may generate transmembraneous structures that allow the flux of ions across the membrane. Amphiphilicity of peptide modules may be sufficient to induce this folding motif in a lipid environment and, consequently, to produce functional activity. This notion is addressed by incorporation of template-assembled synthetic proteins (TASPs) in a lipid bilayer. TASP molecules are designed to adopt a globular four-helix bundle conformation, and the sequence of amphiphilic peptide modules is selected exclusively to optimize secondary structure[3] and not for function.

The three-dimensional folded structure of a protein is a consequence of favorable intramolecular interactions and protein–solvent interactions.[6] Accordingly, our goal is to explore whether changes in the polarity of surrounding solvent may induce conformational modifications of designed, non-linear proteins.

TASP molecules were realized by the covalent attachment of amphiphilic peptide modules at the lysine ϵ-amino groups of a carrier template.[3] The lysine side chains that serve as attachment points to the template allow flexibility of tethered peptide modules. It is conceivable, therefore, that polarity of the environment determines the relative orientation of attached peptide segments. This hypothesis is tested by assessing the channel activity in lipid bilayers of the tethered four-helix bundle proteins. Presumably, amphiphilic peptide modules reorient themselves to shield charged or polar residues from the hydrophobic membrane environment,

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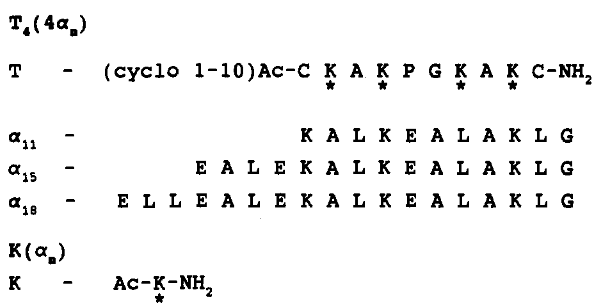

Figure 1. Amino acid sequences of peptide modules, attached to template (T) lysines as indicated (*).

thus creating a structure containing a hydrophilic interior. Four-helix bundles exhibiting a polar interior are anticipated to form ionic channels in lipid bilayers.

Design of Template-Assembled Four-Helix Bundle Proteins. Four-helix bundle proteins, $T_4(4\alpha_n)$, composed of four identical amphiphilic α-helical modules of 11, 15, or 18 residues assembled at the ϵ-amino groups of cyclic 10-amino acid template lysines were described;[3] the proteins were designed to adopt globular structures with a hydrophobic core, and amino acid sequences were selected to optimize α-helical conformation (Figure 1). The template moiety is modeled as two antiparallel β-sheets connected by a type II β-turn and a disulfide bridge between terminal cysteines.[7] Four lysine side chains perpendicular to the plane of the template yield a suitable arrangement of attached amphiphilic peptide modules. Spatial constraints are induced by coupling amphiphilic peptides with a propensity for α-helical secondary structure to the template, thereby enhancing intramolecular interactions.[7]

Here, we present a characterization of the channel activity of four-helix bundle proteins $T_4(4\alpha_{11})$, $T_4(4\alpha_{15})$, and $T_4(4\alpha_{18})$ as well as monomeric peptides $K(\alpha_{11})$, $K(\alpha_{15})$, and $K(\alpha_{18})$ incorporated in lipid bilayers (Figure 1). In addition, the length of attached helical modules required for channel formation is established by using $T_4(4\alpha_{15})$: lipid bilayers are formed from synthetic phospholipids containing hydrocarbon chains of increasing length, resulting in increasing width of the hydrophobic core of the bilayer.

Experimental Section

Molecular Modeling. The model of $T_4(4\alpha_{15})$ was generated by using coordinates for a tethered four-helix bundle molecule[4,5] with specific residue replacements. The INSIGHT and DISCOVER program packages (Biosym Technologies, San Diego, CA) were used on a Silicon Graphics 4D/210GTXB supercomputing workstation (Mountain View, CA).

Synthetic Peptides. Syntheses were carried out manually with a methylbenzhydrylamine–1% cross-linked polystyrene (MBHA) resin and solid phase peptide synthesis protocols, using parallel assembly of four helical modules.[3] Peptides were purified extensively by reversed-phase high-performance liquid chromatography and characterized by amino acid analysis, capillary zone electrophoresis, LSI mass spectroscopy, circular dichroism (CD) spectroscopy, and NMR, as described in ref 3.

HPLC. Size exclusion experiments were performed on a Hewlett Packard Model 1090 liquid chromatograph with a BIO-RAD BIO-SIL TSK-125 column (30 × 0.75 cm) at 40 °C. The buffer was 30% acetonitrile/70% 0.02 M triethylammonium phosphate at pH 2.25, applied at a constant flow rate of 1.0 mL/min.

SDS-PAGE. Electrophoresis was carried out with 16% Tricine gels (Novex, San Diego, CA). Molecular weights were determined by using low range markers (Diversified Biotech, Newton Centre, MA).

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were recorded on a JASCO J 600 circular dichrometer with a quartz cell of 0.1 cm path length. Ellipticities are expressed as mean residue ellipticities. All spectra were recorded at 25 °C.

Reconstitution of Protein into Lipid Bilayers. Bilayers were formed by apposition of two monolayers initially formed at the air–water interface, at the tip of patch pipets.[8] Monolayers were formed from solutions of lipid (Avanti Biochemicals, Alabaster, AL): POPE/POPC [1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)], 4:1, 5 mg/mL in hexane (Sigma Chemical Co., St. Louis, MO). Bilayer experiments were performed at 24 ± 2 °C. Aqueous compartments separated by the lipid bilayer contain 500 mM NaCl, 1 mM $BaCl_2$, and 10 mM Hepes (Sigma) at pH 7.3. For experiments involving concentration gradients across the membrane, 100 mM NaCl is substituted in one of the two aqueous phases. Peptide or protein was incorporated into bilayers by extraction with lipid, POPE/POPC, 4:1, 5 mg/mL in hexane, by vortexing 1 min and sonicating for 10 s in a water bath sonicator (Laboratory Supplies Co. Inc., Hicksville, NY) to achieve final protein/lipid ratios in the range of 1:1000. Lipid/protein mixtures were spread into monolayers and bilayers formed as described above. Alternatively, peptide was extracted with 1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine (PC-20), 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (PC-22), or 1,2-dinervonoyl-*sn*-glycero-3-phosphocholine (PC-24), all 2.5 mg/mL in hexane and mixed peptide/lipid bilayers formed as described.

Electrical Recordings and Data Processing. Electrical recordings were carried out with use of a patch clamp system (List L-M EPC-7, Medical Systems Corp., NY). The signal output was stored on videocassette using a VCR (Sony Betamax) equipped with a modified digital audioprocessor (Sony PCM 501ES, Unitrade, Philadelphia). Records were filtered at 2 kHz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized at 100 μs per point using an Axon TL-1 DMA Interface and the Axon pClamp 5.5 program package (Axon Instruments, Burlingame, CA). Conductance values were calculated from current histograms best fitted by the sum of two Gaussian distributions. Open channel probability was determined from integrations of Gaussian distributions. Channel mean open (τ_o) times were determined from exponential fits to probability density distributions of dwell times in the open state.[8] Each conductance and lifetime value was calculated from experiments with ≥200 openings in a continuous recording. Values are reported as mean ± SEM; n denotes the number of experiments.

Results

Structural Organization of Tethered Molecules. Conformational energy calculations indicate that tethered four-helix bundle molecules may adopt a conformation consistent with the formation of an ionic pore. A molecular model of the helical structure of $T_4(4\alpha_{15})$ is shown in Figure 2; the side view illustrates the cyclized, 10-residue template organized with four lysine side chains perpendicular to the plane of the template. Attached helical modules are parallel and feature all hydrophobic residues (leucine 3, 7, 11, 14) facing the exterior of the bundle. Four glutamic acid residues at the untethered N-termini of helical modules are oriented toward the pore lumen. Accordingly, this organization of $T_4(4\alpha_{15})$ is compatible with a membrane-spanning structure featuring a hydrophilic pathway for ionic diffusion.

Template-Assembled Synthetic Proteins. The total synthesis of TASP molecules by solid phase methods and orthogonal protection techniques yielded target molecules of very high purity, obtained following extensive purification by a combination of reversed-phase HPLC and high-performance ion-exchange chromatography.[3] Table I summarizes the apparent molecular weight (MW) of TASP molecules, determined by size exclusion chromatography. The standard curve is based on linear peptides with molecular weights in the range of 1500–7000. Standards are the following, in order of increasing molecular weight: α-melanocyte

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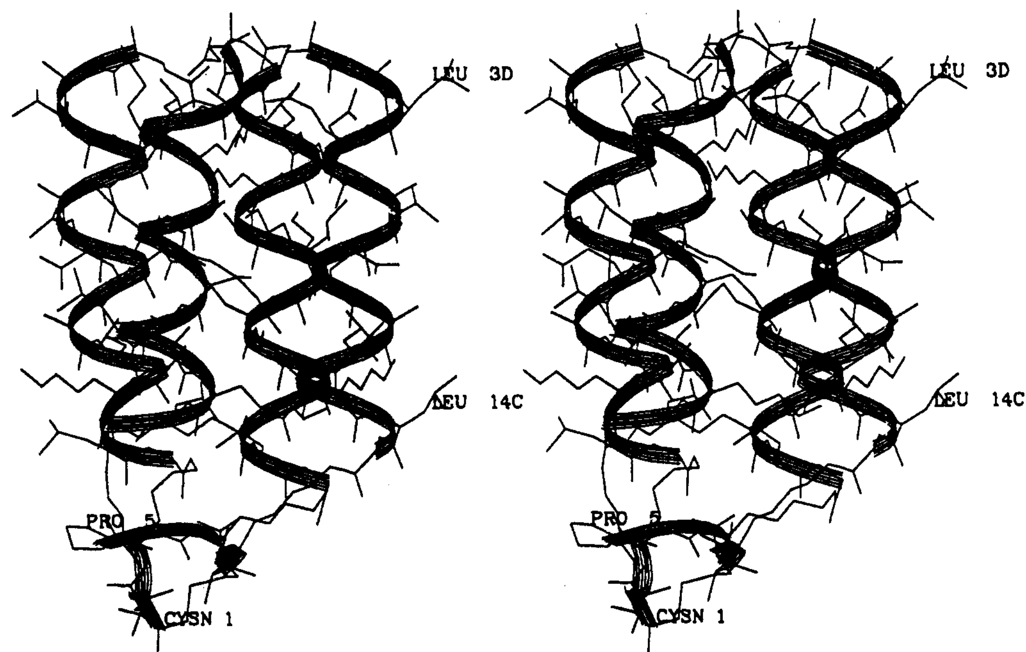

Figure 2. Molecular model of template-assembled synthetic protein $T_4(4\alpha_{15})$. Stereo side view of the parallel tetramer with the cyclized template at the bottom; the N-terminal cysteine of the template (CYSN 1) and proline (PRO 5) of the β-turn region are indicated. The ribbon representation of the α-carbon backbone illustrates the conformation of the bundle. Leucine residues in position 3, 7, 11, and 14 face the exterior of the bundle. The N-terminal glutamic acid residues of helical modules project toward the pore lumen.

stimulating hormone (MSH), porcine β-MSH, human atrionatriuretic factor, β-endorphin, rat growth hormone releasing factor, and an analog of cholecystokinin (CCK) [$Tyr^{52}Nle^{32,53,56}$-Nal^{55}]-CCK-58. The standards used are not considered to adopt well-defined secondary structures in aqueous solution. Compared to the calculated MW, TASP molecules $T_4(4\alpha_{15})$ and $T_4(4\alpha_{18})$ display a conspicuously low apparent MW, whereas $T_4(4\alpha_{11})$ features a higher MW. In addition, $T_4(4\alpha_{15}$-Ac) with the side chain helix N-termini acetylated exhibits an even lower apparent MW than $T_4(4\alpha_{15})$. Removal of N-terminal charge by acetylation correlates with α-helix stability.[9] Size-exclusion chromatography measures molecular shape rather than molecular mass. Accordingly, the observed lower apparent MW of nonlinear molecules is consistent with increasing helical content. The apparent MW of TASP molecules, determined from SDS-PAGE, is shown in Table I. Under denaturing conditions a progression of apparent MW is observed, yet the apparent MW is approximately 40% lower than the calculated value.

Helical Content of TASP Molecules. Conformational analysis by CD and NMR spectroscopy established the helix stabilizing effect of attaching peptide segments to the cyclic template moiety.[3] The significance of the template in stabilizing α-helical conformation depends on the length of attached peptide segments: structural properties of $T_4(4\alpha_n)$ were compared to those of $K(\alpha_n)$, in which the peptide module is attached to the ϵ-amino group of an α-acetylated lysine amide, thereby mimicking the attachment of peptide to template lysines.

$T_4(4\alpha_{11})$ and monomeric $K(\alpha_{11})$ adopt a random conformation in aqueous solution as determined by CD spectroscopy; CD spectra of both peptides exhibit a strong negative Cotton effect at 198 nm indicating predominantly unordered conformation.[3] Conversely, spectral features of both $T_4(4\alpha_{18})$ and $K(\alpha_{18})$ are consistent with α-helical structure in both aqueous solution and TFE.[3] Accordingly, the primary role of the template in $T_4(4\alpha_{18})$ may be to organize the designed four-helix bundle conformation rather than to induce secondary structure.

By contrast, α-helical content of $T_4(4\alpha_{15})$ is higher than that of the corresponding monomeric peptide. CD spectra of $K(\alpha_{15})$

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Table I. Apparent MW of TASP Molecules Determined by Size Exclusion Chromatography or by SDS-PAGE

TASP	retention time (s)	apparent MW HPLC	apparent MW SDS-PAGE	calcd MW
$T_4(4\alpha_{11})$	388	6850	3500	5567
$T_4(4\alpha_{15})$	393	6460	4200	7336
$T_4(4\alpha_{15}$-Ac)	403	5740	nd[a]	7505
$T_4(4\alpha_{18})$	392	6540	5700	8757

[a] nd, not determined.

in aqueous phosphate buffer indicate only partial helical content (Θ_{222} = –6400 deg cm² dmol⁻¹)[3] whereas $T_4(4\alpha_{15})$ show significantly higher helical content (Θ_{222} = –22 000 deg cm² dmol⁻¹) under indentical conditions.[3] Thus, conformational studies by CD spectroscopy indicate that the template stabilizes secondary structure, in accord with the precept of the design strategy.[3]

The helix-promoting solvent TFE mimics a more hydrophobic environment. Accordingly, conformational properties of tethered molecules were studied in neat TFE. As shown in Figure 3, the CD spectra of $T_4(4\alpha_{11})$ in TFE exhibits two negative Cotton effects, at 222 (Θ = –8400 deg cm² mol⁻¹; n–π^* transition) and 208 nm (Θ = –14 000 deg cm² mol⁻¹; π–π^* transition), characteristic for α-helical structure. The Θ_{222} value is consistent with a helical content of about 25%.

The CD curve for $T_4(4\alpha_{15})$ (—) displays the typical features of peptides in predominantly α-helical conformation: strong negative Cotton effects at 222 (Θ = –12 500 deg cm² mol⁻¹) and 208 nm, a zero-crossover at 199 nm, and a strong positive Cotton effect at ≈193 nm. Thus, analysis of $T_4(4\alpha_n)$ in both aqueous buffer[3] and TFE indicates that the transition from a random-coil to a helical conformation occurs in the range of $11 < n < 15$.

Similar to $T_4(4\alpha_{15})$, $T_4(4\alpha_{18})$ adopts a well-developed helical conformation (characteristic double minima at 222 and 208 nm), with Θ_{222} = –16 800 deg cm² mol⁻¹ corresponding to an estimated helix content of >50%. Thus, helical content of TASP molecules in TFE, compatible with the formation of a four-helix bundle in a hydrophobic environment, increases with the length of the attached peptides.

Channel Formation. The amphiphilic nature of designed peptides $K(\alpha_n)$ and four-helix bundles $T_4(4\alpha_n)$ indicates that the

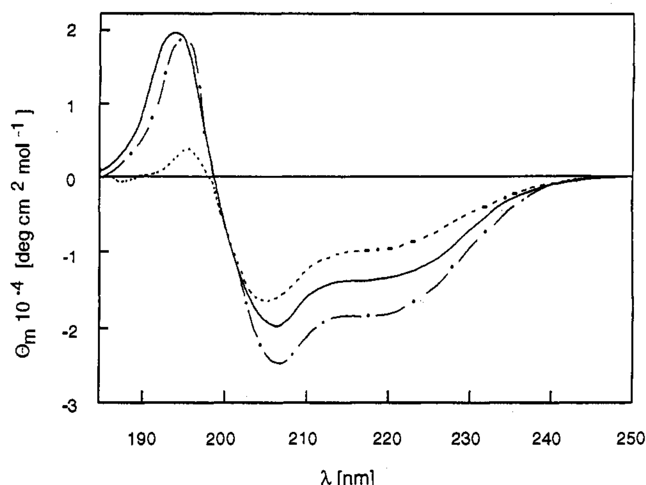

Figure 3. CD spectra of TASP molecules with different length side chain peptides. Spectra were recorded in TFE at a peptide concentration of 1 mg/mL. $T_4(4\alpha_{11})$ (- - -), $T_4(4\alpha_{15})$ (—), $T_4(4\alpha_{18})$ (- • -).

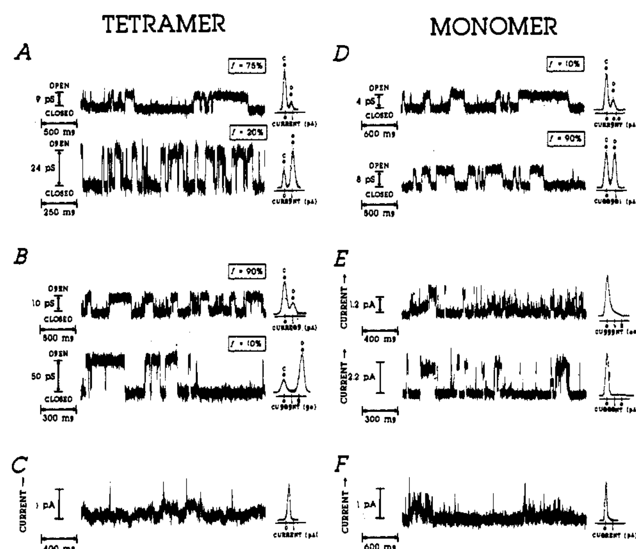

Figure 4. Single channel currents from lipid bilayers containing four-helix bundles (A–C) or corresponding monomeric peptides (D–F). The frequency of occurrence, f, of each individual conductance relative to all observed events is indicated along with the corresponding current histograms: C, closed; O, open. For $T_4(4\alpha_{18})$, channels with $\gamma = 17$ pS occur with a frequency of $f \leq 10\%$. $T_4(4\alpha_{18})$ recorded at 100 mV (9 pS) or 50 mV (24 pS), $T_4(4\alpha_{15})$ at 100 mV (10 pS) or 50 mV (50 pS), $T_4(4\alpha_{11})$ at 100 mV, $K(\alpha_{18})$ at 120 mV, $K(\alpha_{15})$ at 120 mV (1.2 pA) or 100 mV (2.2 pA), and $K(\alpha_{11})$ at 50 mV. Recorded in 500 mM NaCl, 1 mM $BaCl_2$, and 10 mM Hepes at pH 7.3 from POPE/POPC bilayers. Records were filtered at 500 Hz, except $T_4(4\alpha_{15})$ (50 pS, 2 kHz), $K(\alpha_{15})$ (1 kHz), and $K(\alpha_{18})$ (300 Hz).

peptide modules would preferentially shield charged residues from the hydrophobic environment if placed in the nonpolar surroundings of a bilayer interior. Amphiphilic peptides of sufficient length to span the hydrocarbon core of the bilayer self-assemble into conductive oligomers.[5,10] Accordingly, four-helix bundle proteins $T_4(4\alpha_{11})$, $T_4(4\alpha_{15})$, and $T_4(4\alpha_{18})$ and their monomeric analogs were reconstituted in POPE/POPC bilayers and their ability to form ion channels was examined.

Figure 4 illustrates single channel recordings obtained in symmetric 500 mM NaCl. $T_4(4\alpha_{18})$ (Figure 4A) elicits discrete, square events that display transitions between two current levels corresponding to the closed and open states. Single channel conductance values of $\gamma = 8.7 \pm 0.7$ and 24.8 ± 0.4 pS ($n = 7$) are calculated from the corresponding current histograms, reflecting the occurrence of distinct open (O) and closed (C) states. The relative frequency of individual conductances is indicated next to corresponding current histograms. For $T_4(4\alpha_{18})$, channels with $\gamma = 17.3 \pm 0.5$ pS ($n = 4$) are observed with a frequency of $\approx 5\%$.

$T_4(4\alpha_{15})$ produces clearly resolved events of $\gamma = 9.7 \pm 0.4$ ($n = 13$) and 50.8 ± 0.8 pS ($n = 5$) with residence times in the open state in the millisecond time range (Figures 4B and 6, legend). Current histograms clearly disclose the existence of distinct closed and open states. In contrast, $T_4(4\alpha_{11})$ induces erratic current fluctuations of transient duration ($n = 13$) rather than the uniform conductance events characteristic of channel-forming peptides (Figure 4C); the current histogram shows a single broad band rather than two distinct peaks. Presumably, $T_4(4\alpha_{11})$ is not able to span the hydrophobic core of the lipid bilayer whereas $T_4(4\alpha_{15})$ contains amphiphilic peptide modules of sufficient length to form transmembraneous pores.

Monomeric peptide $K(\alpha_{18})$ forms discrete channels in POPE/POPC bilayers exhibiting clearly resolved transitions between the closed and open states (Figure 4D). The primary events have single channel conductance values of $\gamma = 3.3 \pm 0.1$ ($n = 3$) and 9.1 ± 0.4 pS ($n = 7$). By contrast, $K(\alpha_{15})$ elicits only very fast transitions between low and high conductance levels (Figure 4E; $n = 6$). The residence time in the higher conducting state is very brief and activity is frequently characterized by erratic current fluctuations. $K(\alpha_{11})$ only induces irregular current fluctuations of varying amplitude (Figure 4F; $n = 14$). Evidently, well-defined single channel activity is elicited only by the 18-mer peptide.

When incorporated into the hydrophobic environment of a lipid bilayer, the amphiphilic peptides may self-associate into conductive species of different oligomeric number.[5,10] The occurrence of a 9-pS channel, elicited by both monomer $K(\alpha_{18})$ (Figure 4D) and tetramer $T_4(4\alpha_{18})$ (Figure 4A), is consistent with a tetrameric, parallel arrangement of peptide modules. An antiparallel—or trimeric—arrangement of monomeric peptides may give rise to a conductance of smaller amplitude, consistent with the absence of channels with conductances ≤ 8 pS for the tethered tetramer, $T_4(4\alpha_{18})$. Analogously, larger (>4) oligomeric arrangements may not be sufficiently stable to reproducibly occur with $K(\alpha_{18})$; attachment of peptides to template may stabilize such oligomeric clusters, resulting from the self-assembly of tetrameric molecules, yielding the observed conductances of 17–25 pS (Figure 4A).

Notably, a distinct difference in channel activity is apparent between $K(\alpha_{15})$ and the corresponding tethered bundle, $T_4(4\alpha_{15})$. The single channel activity elicited by $T_4(4\alpha_{15})$ may be the functional correlate of the template-induced secondary structure determined in solution.[3] Accordingly, $T_4(4\alpha_{15})$ is selected for further examination of single channel properties.

Ionic Selectivity. Current–voltage relationships in symmetric 500 mM NaCl or under a single salt concentration gradient were recorded for $T_4(4\alpha_{18})$ and $T_4(4\alpha_{15})$ (Figure 5). Channels are ohmic in both conditions. Ionic selectivity is determined from the reversal potential, V_{rev}, measured under a 5-fold concentration gradient for the most frequent conductances. For $T_4(4\alpha_{15})$ ($\gamma = 10$ pS), $V_{rev} = 36.8 \pm 3.2$ mV (Figure 5). For $T_4(4\alpha_{18})$ ($\gamma = 9$ pS), $V_{rev} = 32.3 \pm 3.7$ mV ($n = 3$). The selectivity for cations, calculated from reversal potentials, is 95% and 89%.

Bilayer Thickness. The occurrence of discrete conductance events, observed with $T_4(4\alpha_{15})$, indicates the formation of membrane-spanning hydrophilic pores. To explore the peptide chain length required for channel formation, $T_4(4\alpha_{15})$ was incorporated into bilayers composed of phospholipids containing the 20-carbon fatty acid 11-*cis*-eicosenoic (PC-20), the 22-carbon 13-*cis*-docosenoic acid (PC-22), or the 24-carbon 15-*cis*-tetracosenoic acid (PC-24).

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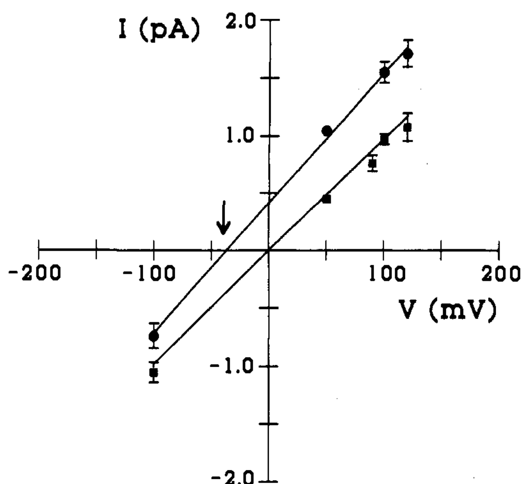

Figure 5. Current-voltage (*I–V*) relationship in symmetric 500 mM NaCl (■) and under an imposed 5-fold concentration gradient (●). Linear fits have regression coefficients of 0.9969 and 0.9989. Slope conductance is 11 pS (■). Reversal potential (voltage at which $I = 0$) is 36.8 mV, indicated by an arrow (●). Each point represents the mean of ≥3 different experiments.

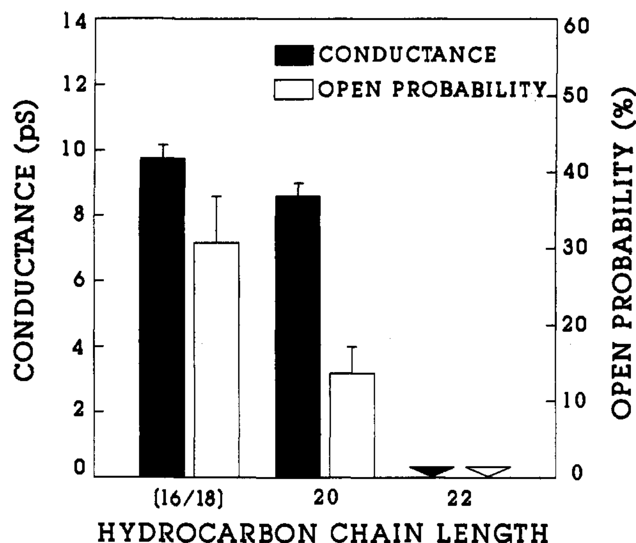

Figure 6. Single channel conductance and open probability of $T_4(4\alpha_{15})$, recorded in 500 mM NaCl from bilayers composed of lipids of increasing hydrocarbon length. No single channel activity was observed in PC-22, indicated by arrowheads. Open probability was calculated for 30-s segments of recording from POPE/POPC (hydrocarbon length 16/18) or PC-20 bilayers. Channel mean open times in POPE/POPC are $\tau_{o1} = 3.3 \pm 0.5$ ms and $\tau_{o2} = 248 \pm 45$ ms ($n = 10$); in PC-20 $\tau_{o1} = 3.8 \pm 0.3$ ms and $\tau_{o2} = 197 \pm 37$ ms ($n = 4$).

$T_4(4\alpha_{15})$ elicits single channel activity in bilayers composed of POPE/POPC, both containing the 16-carbon hexadecanoic acid and the 18-carbon 9-*cis*-octadecenoic acid, with $\gamma = 9.7 \pm 0.4$ pS (Figure 4). Figure 6 summarizes results obtained in bilayers of different composition. In PC-20, $\gamma = 8.6 \pm 0.4$ pS ($n = 4$). No discrete events are discerned in PC-22 ($n = 21$) or PC-24 ($n = 7$; data not shown), only erratic current fluctuations indicating that the protein is embedded in the membrane. Notably, the open channel probability is significantly reduced in PC-20, as channels occur in brief bursts of activity. Apparently, $T_4(4\alpha_{15})$ forms ionic pores in the thicker bilayers, however of transient duration.

Discussion

A major goal in protein design is to decrease flexibility of the folding polypeptide chain through the introduction of conformational constraints. The TASP concept was introduced as a strategy for the construction of novel proteins with predicted structural as well as functional properties.[7] The resulting molecules are nonlinear structures in which the template promotes interactions between covalently attached amphiphilic peptide modules and stabilizes the predetermined three-dimensional conformation; amphiphilicity of peptide modules is a requirement for folding of the protein into the desired conformation. The design of four-helix bundle proteins may be accomplished such that the resulting molecule resembles a globular protein with a hydrophobic core and a hydrophilic surface[3] or such that polar residues line a central pore.[4] Optimization of design includes adjusting the extent of hydrophilic and hydrophobic surfaces of each helical module.[3,4,7]

Four-helix bundle molecules have been realized by solid-phase synthesis methods,[3,4,11] and conformational studies indicate the potential of the template moiety in stabilizing secondary structure.[3] Helix–helix interactions provide the primary driving force for folding. Accordingly, a stable three-dimensional structure reflects interhelical interactions as well as helix–solvent interactions.

TASP molecules designed to adopt a globular four-helix bundle conformation form channels in lipid bilayers (Figure 4). The sequence of peptide modules was selected to optimize helical conformation. By contrast, the *de novo* design of functional channel proteins involves the careful selection of segments of specific amino acid sequence from a naturally occurring protein, encompassing features such as amphiphilicity and the presence of residues compatible with observed pore properties.[4,5] This molecular design strategy identified a functional motif for the pore-forming structure of authentic channel proteins as a bundle of amphipathic α-helices, arranged such that polar or charged residues face the central pore. The channel activity of TASP molecules indicates that in a lipid environment, the tethered bundles of amphiphilic peptides mimic this functional motif. Accordingly, amphiphilicity may be sufficient to generate a functional motif for an aqueous pore, yet resulting single channel properties do not imply biological significance. Sequence specificity is necessary to reproduce the unique functional and pharmacological properties that are characteristic of authentic channel proteins.[4,5]

Ionic selectivity of a bundle of amphiphilic peptide modules generating a central aqueous pore is determined by residues lining the pore.[4,5,10] Notably, aspartic acid residues facing the central aqueous pore within a four-helix bundle protein representing the presumed pore-forming structure of voltage-gated calcium channels are considered involved in forming specific sites for interaction with permeant cations.[4] Further, clusters of amphiphilic α-helices have been identified from crystal structures of integral membrane proteins capable of forming hydrophilic pores; α-helical bundles are considered responsible for pore-formation.[12] Bundles of α-helices considered to form the aqueous pore of a channel protein feature polar or charged residues projecting into the pore lumen and nonpolar residues interacting with surrounding hydrophobic parts of the protein and the bilayer core.[4,5,12]

Accordingly, the cation selectivity of channels elicited by TASP molecules (Figure 5) is predicted to arise from negatively charged residues (Figure 1) projecting toward the pore lumen (Figure 2). Such orientation of acidic residues may result from a rearrangement of peptide modules in the nonpolar surroundings of the lipid bilayer. The pore-forming conformation adopted by a TASP molecule embedded in the bilayer would provide a more favorable arrangement of helical modules that exposes charged amino acids

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to the pore lumen and hydrophobic residues to the apolar milieu. Changes in conformation are expected as proteins are dynamic and not static structures. The molecule does not stay in one unique conformation; particularly, changes in hydrophobicity of the environment may induce the protein to explore other conformational states.[6]

The presence of a central hydrophilic pore within $T_4(4\alpha_{15})$ is substantiated by the channel activity recorded from thicker bilayers. The ability of the TASP molecule to span the hydrophobic core of the membrane is a requirement for the generation of a patent pore (Figure 6). The thickness of the hydrocarbon region of lipid bilayers containing fatty acyl chains of 16/18 carbons, determined from X-ray data, is approximately 30 Å. The relative dielectric thickness, estimated from capacitance measurements, is 22 Å.[13] Accordingly, the effective dielectric thickness of the hydrocarbon region is less than its physical dimension, presumably due to limited penetration of water molecules into the hydrophobic core region.[13] For bilayers containing phospholipids with 22-carbon fatty acyl chains, the dielectric thickness was estimated at 33 Å.[14] The dimensions of $T_4(4\alpha_{15})$ are consistent with observed channel activity in POPE/POPC bilayers and predict the observed inability of the molecule to span a bilayer of 22-carbon or 24-carbon fatty acyl chains.

The heterogeneity of conductance events suggests that TASP molecules, like monomeric amphiphilic peptides, may self-assemble in the bilayer, resulting in oligomers of different size.[5,10] Such clusters may involve parallel as well as antiparallel orientation of individual molecules, resulting in further heterogeneity of conductance events. α-Helices bear an intrinsic dipole moment, and it is expected that transmembraneous orientation will be influenced by the strength and polarity of the electric field. Applied voltage across the membrane would offset the unfavorable parallel organization of helices due to the helix dipole, which would otherwise favor an antiparallel organization.

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Conclusion

Template-assembled synthetic proteins designed to adopt a globular four-helix bundle conformation form cation-selective channels in lipid bilayers. Channel activity correlates with secondary structure in solution; monomeric as well as tethered four-helix bundle molecules composed of amphiphilic peptide modules of 18 amino acids form ionic channels in lipid bilayers, and both display spectral features consistent with α-helical secondary structure.[3] By contrast, the covalent attachment of amphiphilic 15-residue peptides to a cyclic template molecule is required for the induction of significant helical content, as well as for channel activity.[3]

Amphiphilicity of attached peptide modules may be sufficient to induce the formation of a functional pore-forming motif in a hydrophobic environment. Ionic selectivity is defined by residues projecting into the pore lumen. Accordingly, the cation selectivity of TASP molecules suggests that glutamic acid residues face the pore lumen, indicating relative flexibility of tethered amphiphilic peptide modules. Conceivably, peptide segments reorient themselves in response to the polarity of the surroundings to shield charged residues from an apolar environment and thereby generate a central hydrophilic pore. The ability of the TASP molecule to span the hydrophobic core of the bilayer is a requirement for the formation of a patent pore, as evidenced by the absence of channel activity in bilayers of increasing thickness.

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Supplementary Material Available: Plot of Θ_m vs λ for $K(\alpha_{18})$ (1 page). Ordering information is given on any current masthead page.