

Design, synthesis and functional characterization of a pentameric channel protein that mimics the presumed pore structure of the nicotinic cholinergic receptor

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Nicotinic cholinergic receptors are membrane proteins composed of five subunits organized around a central aqueous pore. A pentameric channel protein, T₅M2 δ , that emulates the presumed pore-forming structure of this receptor was generated by assembling five helix-forming peptide modules at the lysine ϵ -amino groups of the 11-residue template [K*AK*KK*PGK*EK*G], where * indicates attachment sites. Helical modules represent the sequence of the M2 segment of the *Torpedo californica* acetylcholine receptor (AChR) δ subunit; M2 segments are considered involved in pore-lining. Purified T₅M2 δ migrates in SDS-PAGE with an apparent M_r ~14,000, concordant with a protein of 126 residues. T₅M2 δ forms cation-selective channels when reconstituted in planar lipid bilayers. The single channel conductance in symmetric 0.5 M KCl is 40 pS. This value approximates the 45 pS single channel conductance characteristic of authentic purified *Torpedo* AChR, recorded under otherwise identical conditions. These results, together with conformational energy calculations, support the notion that a bundle of five amphipathic α -helices is a plausible structural motif underlying the inner bundle that forms the pore of the pentameric AChR channel.

Neurotransmitter receptor; Ionic channel; Protein design; Lipid bilayer; Signal transduction

1. INTRODUCTION

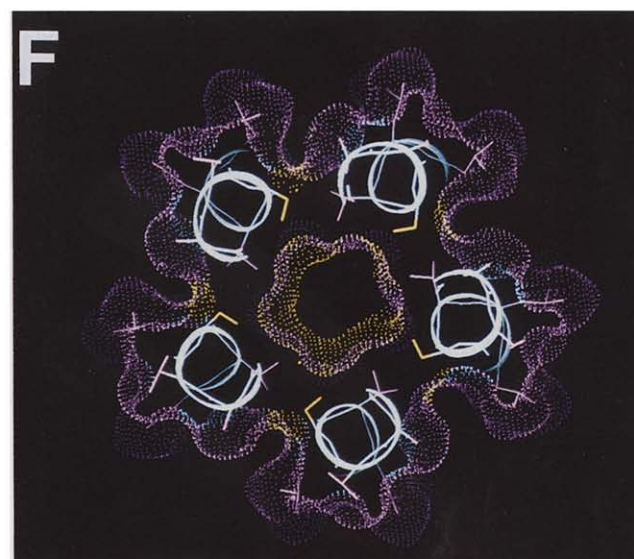
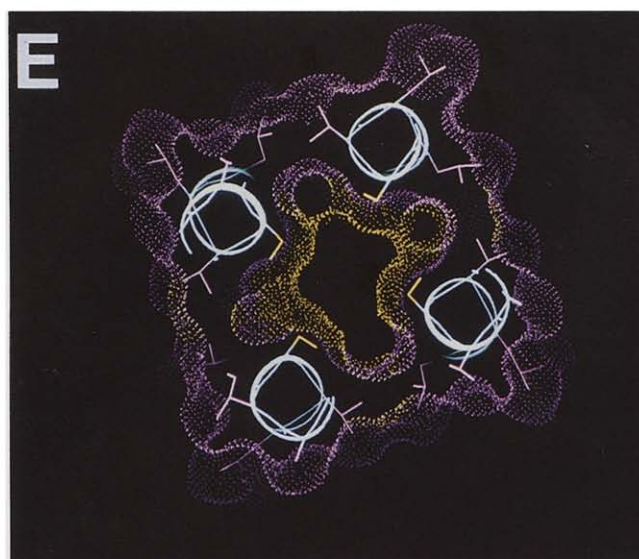
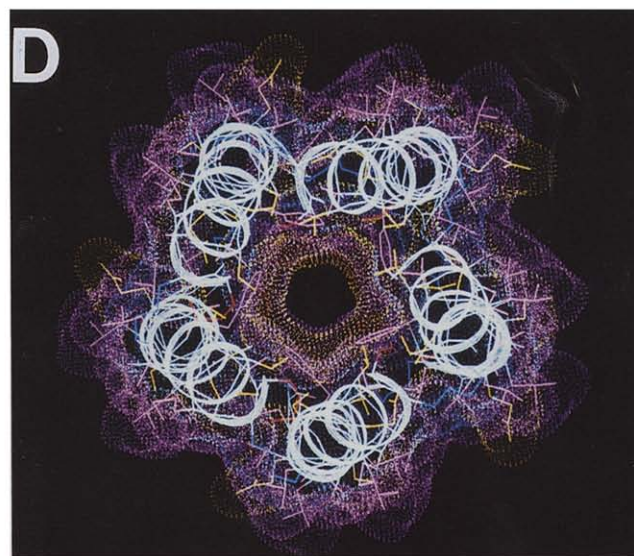
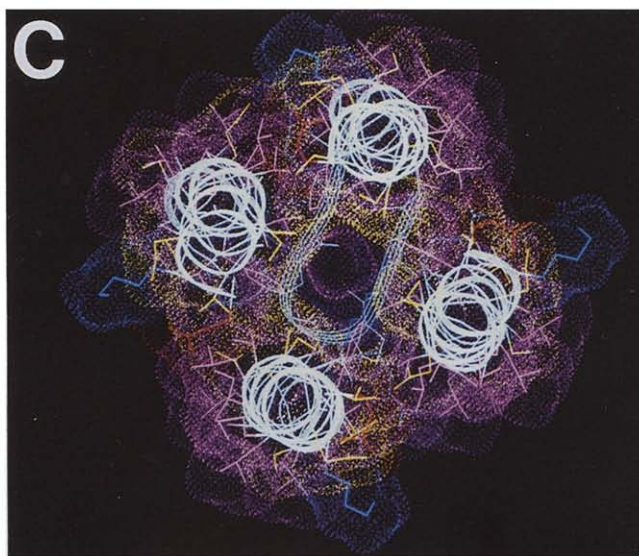
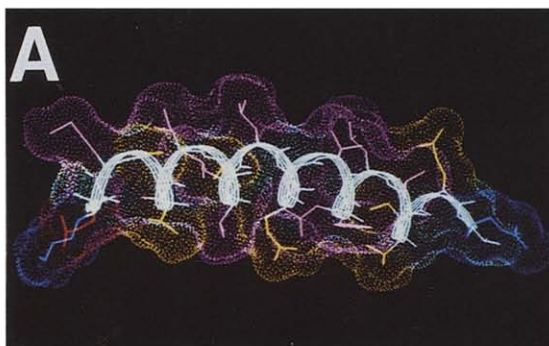
The acetylcholine receptor (AChR) from *Torpedo californica* is a pentameric glycoprotein (M_r ~250 kDa) with a stoichiometry of $\alpha_2\beta\gamma\delta$, assembled around a central ion conducting pore [1–7]. The AChR channel is cation selective, with an effective pore diameter of ~7 Å; [8,9]. Analysis of the primary structure predicts the occurrence of four α -helical transmembrane segments, M1–M4, in each subunit [1–3,6]. M2 is amphipathic, highly conserved among all members of the superfamily of ligand-gated channels, and is thought to be involved in lining the pore [1–3,6,10–24]. A bundle of five M2 α -helices contributed by each of the subunits that cluster together to form a hydrophilic channel has been considered as a plausible structural motif for the pore-forming element of the AChR [1,2,6,10–24].

Molecular modeling results [10,11,15,17] show that the ion-conducting pore may be mimicked by a parallel

homo-oligomer of five amphipathic α -helices arranged such that polar residues line a central hydrophilic pathway and apolar residues face the hydrophobic bilayer interior [11]. We have demonstrated that a four-helix bundle protein designated as T₄M2 δ , in which four identical helical modules with the sequence of M2 δ were covalently attached to a multifunctional carrier (template) [25,26], forms ionic channels in lipid bilayers. The T₄M2 δ channel is cation-selective and blocked by mM concentrations of the local anesthetic channel blocker QX-22, properties that are characteristic of the AChR channel [25]. However, the single channel conductance (γ) of T₄M2 δ (γ =20 pS), presumably expressing the activity of a conductive tetramer, differs from that of the authentic pentameric AChR channel (γ =45 pS) [25]. Here, we describe the total chemical synthesis of a five-helix bundle protein T₅M2 δ . The purified protein exhibits in SDS-polyacrylamide gels an apparent M_r ~14,000, consistent with a 126-residue protein. The designed molecule forms cation-selective channels after reconstitution in lipid bilayers with a single channel conductance similar to that of the authentic *Torpedo* AChR channel. Results demonstrate that a five helix bundle with the sequence of M2 is sufficient to form the conduction pathway of the authentic AChR and suggest that this motif may represent the inner bundle that lines the pore of the authentic pentameric channel with one M2 segment contributed by each of the five component subunits.

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Abbreviations. AChR, acetylcholine receptor; γ , single channel conductance; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; τ_c , closed times; τ_o , open times



2. MATERIALS AND METHODS

2.1. Conformational energy computations

Molecular modeling, including energy minimization and molecular dynamics simulations, were conducted on a Silicon Graphics IRIS 4D/21 GTX workstation using the INSIGHT and DISCOVER molecular modeling packages of Biosym (San Diego, CA) [11,25,27]. Low-energy arrangements of α -helices and helical bundles were calculated with semi-empirical potential energy functions and optimization routines, and further refined. Constraints were used for symmetry and to maintain regular helical backbone dihedral angles of $\phi = -45$ and $\psi = -60$ [11].

2.2. Protein synthesis, purification and characterization

The designed five-helix bundle protein was synthesized by solid-phase methods using an Applied Biosystems Model 431 peptide synthesizer (ABI, Foster City, CA), according to general principles previously described for the synthesis of four-helix bundle proteins [25–29]. The multifunctional carrier template has the sequence: [K*AK*KK*PGK*EK*G], where * indicates attachment sites for peptide modules at the ϵ -amino groups of lysine. M2 δ modules have the sequence: EKMSTAISVLLAQAVFLLTSQR [6,10,11,25]. Underlined residues are considered to line the pore [11]. Multiple couplings (3–5) were performed to boost coupling efficiencies to >99% per site for the pentameric unit [25,26]. Cleaved proteins were subjected to multiple purification steps by reverse-phase HPLC, and characterized by amino acid analysis, microsequencing and capillary zone electrophoresis [25,26]. SDS-PAGE analysis was according to Laemmli [30] on 10–20% polyacrylamide gradient Tricine gels (Novex, San Diego, CA). Molecular weight was estimated using CNBr-treated myoglobin molecular weight markers [25,26].

2.3. Single channel recordings in lipid bilayers

Lipid bilayers were assembled by apposition of two monolayers, initially formed at the air-water interface, at the tip of patch pipettes [31]. Purified protein was incorporated into lipid bilayers by extraction with lipid, POPE/POPC [1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) (Avanti Biochemicals, Alabaster, AL)], 4:1, in hexane (5 mg/ml), as described [25,26]. The aqueous subphase was composed of 0.5 M KCl, 1 mM CaCl₂ and 10 mM HEPES (pH 7.4). Acquisition and analysis of single channel currents were as described [31–35]. Records were filtered with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized at 0.1 ms per point using an Axon TL-1 interface (Axon Instruments, Burlingame, CA) connected to an Everex Step 386 computer (Everex, Fremont, CA). pClamp 5.5 (Axon Instruments) was used for data processing. Conductance and lifetime values were calculated from continuous recordings lasting 30 s or longer and with ≥ 300 openings; openings with $\tau_o \leq 0.3$ ms were ignored. Values are reported as mean $\pm$ S.E.M., n =number of experiments. Bilayer experiments were performed at $24 \pm 2^\circ\text{C}$.

3. RESULTS AND DISCUSSION

3.1. Bundles of amphipathic α -helices as a structural motif for an ionic pore

Conformational energy calculations suggest that a

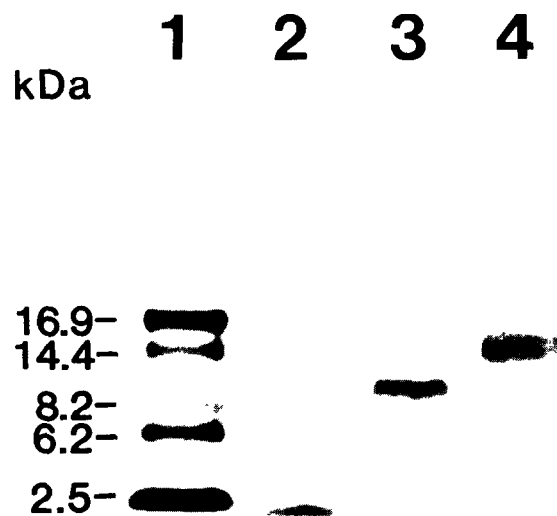


Fig. 2. SDS-PAGE analysis of M2 δ channel-forming peptides and oligomeric proteins. HPLC-purified synthetic M2 δ peptide, tetrameric T₄M2 δ and pentameric T₅M2 δ were subjected to gel electrophoresis on 10–20% polyacrylamide gels. Protein bands were visualized with silver stain. Lanes: 1, molecular weight markers; 2, monomeric M2 δ peptide, 3, tetrameric T₄M2 δ protein, 4, pentameric T₅M2 δ protein. Apparent M_r are 2,000, 11,000 and 14,000, respectively.

homopentameric bundle of α -helices is a plausible model for the pore-structure of the cholinergic receptor. Fig. 1 illustrates optimized helical structures for the M2 δ monomer (A, side view; B, end view), the homotetramer T₄M2 δ (C, end view) and the homopentamer T₅M2 δ (D, end view). The template portion of the bundles may be discerned in Fig. 1C as a β -hairpin at the C-terminal of the helical units [25,27]. The N-terminus is in front and corresponds to the untethered end of helical modules. Glu-1, which corresponds to E255 in the protein sequence, is postulated to face the intracellular end of the receptor channel which is considered to be negatively charged [1,2,6,11,18,23]. Attached α -helical modules are parallel. Most of the hydrophobic and hydrophilic residues occur on opposite faces of the helices (A–D). The lumen of the pore in both tetrameric and pentameric bundles is lined with polar/neutral residues. Ser-4 and Ser-8, which correspond to S258 and S262 in the actual protein sequence, face the aqueous pore. These residues are considered to be exposed to the pore lumen and to participate in ionic conduction through the pore [1,2,11,14,17,20–24]. Fig. 1E and F

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Fig. 1. A bundle of five amphipathic α -helices is a plausible structure for the pentameric channel protein T₅M2 δ . Side view (A) and end view (B) of an energy-optimized molecular model of the helix with the sequence of M2 δ . The N-terminus is on the left (A) and at the top (B). The van der Waals envelope (dotted surface) shows the solvent-accessible surface with ribbon representation of the α -carbon backbone. (C and D) End views of the parallel tetramer T₄M2 δ (C) and pentamer T₅M2 δ (D) with the N-terminus in front. The central pore is lined by polar/neutral residues (yellow) and the location of lipophilic residues (purple) is at the exterior of the bundle. The length of the bundle is sufficient to span the bilayer. (E and F) End views of a cross-section at the level of S8 showing approximately two turns of helix, for T₄M2 δ (E) and T₅M2 δ (F). This region corresponds to the section of the pore that features the ring of serines thought to be involved in ionic conduction, with pore dimensions of 4 Å; by 5 Å; for the tetramer (E) and a pore diameter of 7 Å; for the pentamer (F). Color code: light blue, α -carbon backbone; red, acidic, yellow, polar-neutral; white, proline and glycine; and purple, lipophilic residues.

show transverse sections across the bundles depicting approximately one turn of the helix at the location of exposed serines; the pore cross-section, measured at the boundaries of the solvent accessible surface (dotted surface) is, for the tetramer, irregular, with dimensions of 4×5 Å, and for the pentamer ~ 7 Å. The narrowest section of the pore (tetramer, 4 Å, and pentamer, 4.5 Å) is formed by a ring of phenylalanines at position 16, corresponding to F270 in the protein sequence [6,11]. Thus, tetrameric and pentameric bundles of M2 helices satisfy structural and energetic requirements for the inner bundle that forms the pore of the nicotinic AChR

channel [11,14,15], and the pentamer fulfills the constraints imposed by the pentameric subunit structure.

3.2. Chemical characterization of $T_5M2\delta$

We have previously described the strategy and procedures to synthesize a homotetrameric protein $T_4M2\delta$ by the covalent attachment of four M2 δ modules to a carrier molecule [25,26]. Similar protocols were applied for the synthesis, purification and characterization of $T_5M2\delta$. The salient novelty is the modification of the carrier template to generate five attachment sites. $T_5M2\delta$ was synthesized by a two-step procedure: the

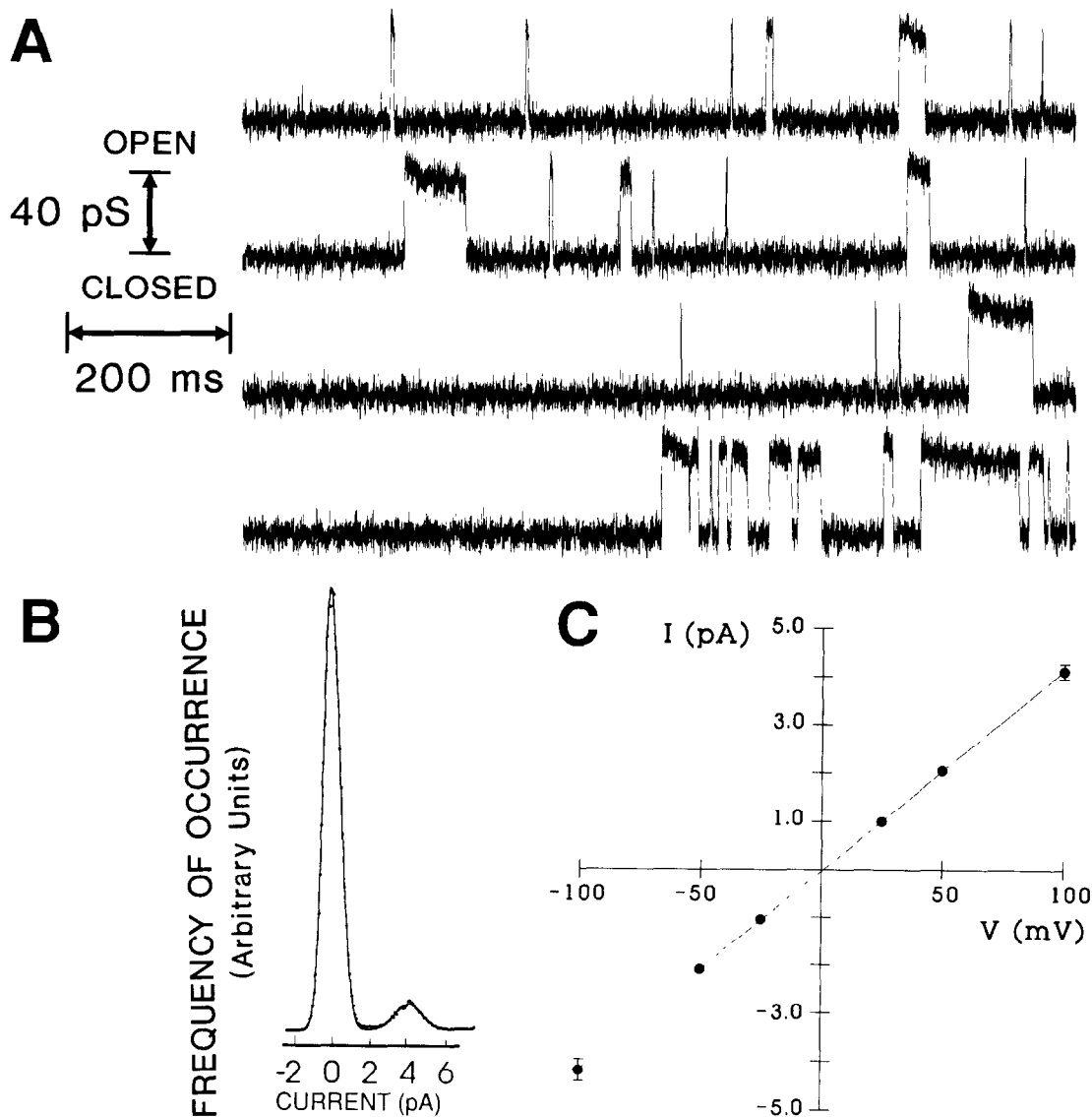


Fig. 3. Single channel currents from POPE/POPC bilayers containing the synthetic pentameric protein $T_5M2\delta$. Currents were recorded at $V=100$ mV in symmetric 0.5 M KCl, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4. (A) Segments of a continuous record displayed at a time resolution sufficient to illustrate the occurrence of discrete openings of homogeneous current amplitudes that exhibit short and long residence times in the open and closed states. Records filtered at 2 kHz. (B) Current histogram generated from segments of records lasting several minutes. Data points were fitted by the sum of two Gaussian distributions (smooth curve). The prominent fitted distribution centered at zero current corresponds to the channel closed state and that centered at 4 pA to the open state. The single channel current, calculated as the difference between the peaks associated with the closed and open states, is 4 pA; the corresponding value for single channel conductance is 40 pS. (C) Current-voltage ($I-V$) characteristics of single channel events of $T_5M2\delta$ in symmetric 0.5 M KCl. The single channel $I-V$ is ohmic with a slope conductance of 40 pS. Currents reported as mean values $\pm$ S.D.; $n=5$.

carrier molecule was synthesized using orthogonal lysine side-chain protection followed by the simultaneous assembly of M2 δ peptide modules [25,26]. Amino acid composition, sequence, and purity of the protein were confirmed by amino acid analysis (hydrolysis with 6 M HCl at 165°C), microsequencing (automated Edman degradation), and analytical HPLC. T₅M2 δ migrates in SDS-polyacrylamide gels as a single band with M_r ~14,000, as illustrated in Fig. 2 (lane 4). This molecular weight is consistent with a protein of 126 residues. In contrast, the tetrameric protein T₄M2 δ (lane 3) appears as a band with M_r ~11,000, compatible with a protein of 101 residues [25,26], whereas the monomer form of M2 δ (lane 2) migrates with M_r ~2,000 [10,25,26].

3.3. Channel formation by T₅M2 δ

T₅M2 δ forms channels in lipid bilayers, as shown in Fig. 3. Conductance events are homogeneous in amplitude (Fig. 3A) with single channel conductance γ =40 $\pm$ 3 pS (n =8) in symmetric 0.5 M KCl (Fig. 3B). Channel openings with γ =80 $\pm$ 5 pS were detected at significantly lower frequency of occurrence (<10%). The channel is ohmic (Fig. 3C) and cation selective; the cation transference number, determined from reversal potential measurements under single KCl concentration gradients, is 0.90 $\pm$ 0.05 (n =3). Channel openings tend to occur in bursts of openings followed by very long salient periods with little or no activity. The probability of the channel being open, P_o , calculated from the area under the fitted Gaussian curve (Fig. 3B), is 0.078 $\pm$ 0.013 (n =3). Analysis of channel lifetimes in the open and closed states during the periods of activity shows that channel open (τ_o) and closed (τ_c) times are well fitted by the sum of two exponentials, τ_{o1} =1.5 $\pm$ 0.2 ms and τ_{o2} =78 $\pm$ 5 ms, and τ_{c1} =1.4 $\pm$ 0.2 ms and τ_{c2} =28 $\pm$ 3 ms (n =3). The efficiency of channel formation by T₅M2 δ is considerably lower (~20%) than that observed with the tetrameric T₄M2 δ [25,26] or with monomeric M2 δ [10,25,26]. Channel activity of purified T₅M2 δ is highly sensitive to aging and heat inactivation: activity is lost within 24 h at 4°C or after incubation at 50°C for 5 min.

3.4. Comparison with authentic AChR channels

We have previously shown that synthetic M2 δ peptides (Fig. 1A and B; Fig. 2, lane 2) form channels in lipid bilayers of heterogeneous conductance and lifetimes, resulting from the self-assembly of conductive oligomers of distinct sizes [10]. The design of tethered oligomeric proteins was introduced to generate proteins of defined oligomeric number and predetermined orientation of peptide modules relative to one another [25–27]. This approach has led to the generation of homotetrameric (T₄M2 δ) and homopentameric (T₅M2 δ) proteins that exhibit homogeneous conductances. Accordingly, whereas M2 δ peptide forms channels in POPE/POPC bilayers with primary conductances of 20

pS and 40 pS (0.5 M KCl), T₄M2 δ and T₅M2 δ form homogeneous channels with unitary conductances of 20 pS [25] and 40 pS (Fig. 3), respectively. This suggests that the γ =20 pS and γ =40 pS events recorded with M2 δ peptide arise from non-covalently bonded tetrameric and pentameric arrays, respectively [10].

The pore area calculated for a pentameric cluster of α -helices, 42 Å² [11], is consistent with the observed channel conductance of T₅M2 δ . The 40 pS conductance events recorded with T₅M2 δ approximate the single channel conductance of authentic, purified *Torpedo* AChR reconstituted in POPE/POPC bilayers (γ =45 pS in symmetric 0.5 M KCl [35]). Whereas the *Torpedo* AChR is a heteropentamer, T₅M2 δ is a homopentamer. This feature may explain the observed ~10% difference in single channel conductances. Thus far, we have focused on the design of pore-forming structures without the ligand-binding domain or other modulatory structures of the receptor [14,25–27]. The design of functional homomeric channel proteins sets us now in position to undertake the challenging task of the step-by-step reconstitution of a channel protein from its elementary functional units [14].

3.5. Conclusion

The synthesis of a pentameric protein designed to function as a transmembrane ionic pore was achieved. T₅M2 δ displays several pore properties of the purified *Torpedo* AChR channel reconstituted in lipid bilayers, namely, single channel conductance, cation selectivity and channel open lifetimes in the millisecond time range [7,32]. These results support the notion that a five helix bundle may represent the structural motif underlying the inner bundle that forms the pore of the pentameric AChR channel.

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