Synthetic Peptides as Models for Ion Channel Proteins

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We have recently adopted a "minimalist" approach to protein design.[1] As applied to the analysis of natural proteins, this approach involves two steps: first, the structural basis for a given function is predicted; then, a highly simplified peptide or protein sequence is designed that embodies the predicted structural features, while lacking much of the complexity of natural proteins. Features predicted to be superfluous for structure or function are either eliminated or replaced with sequences of minimal complexity. The activities and properties of the minimal designs serve to test the guiding structural hypotheses. This approach has been applied to the design of four-helix bundle proteins,[1] calmodulin-binding peptides,[2] and DNA-binding peptides.[3] This Account will discuss the application of this and related approaches toward understanding some of the structural features underlying the function of ion channel proteins.

Ion channel proteins[4] facilitate the diffusion of ions across cell membranes. These proteins are critical for cell regulation and intercellular communication, and they mediate all electrical excitability of the nervous and muscle systems. Defects associated with ion channel proteins can have severe pathological consequences. For example, a defect in a putative chloride channel is implicated in cystic fibrosis, the most common lethal inherited disease among Caucasians.[5,6] Another example is myasthenia gravis, an autoimmune disorder in which antibodies are produced against the muscle nicotinic acetylcholine receptor.[7] Despite their central role in biology, a structural understanding of the mechanisms of action of ion channel proteins has only recently begun to be elucidated as a result of improved molecular biology and single channel analysis techniques.

The ion channel proteins of the nervous system are proposed to consist of four or five homologous subunits radially arranged around a central hydrophilic pore. The membrane-spanning portions are predicted from the amino acid sequences to be predominantly α-helical[8] (Figure 1). A four-subunit structure appears to form the voltage-gated Na^+, K^+, and Ca^{2+} channels, while the channels of the ligand-gated acetylcholine receptor and other homologous receptors are five-subunit structures.

The acetylcholine receptor is one of the most studied ion channel proteins. This protein forms ligand-gated channels that are selective for cations smaller than approximately 7 Å in diameter. A 5-fold pseudosymmetric channel model has evolved as a result of intensive photoaffinity labeling,[9,10] site-directed mutagenesis,[11] and electron microscopy studies[12–14] and by comparison of amino acid sequences.[15] In the proposed model the channel is composed of five homologous α-helices which contain several conserved serine residues lining the pore. The hydroxyl group of this residue might serve to solvate permeant cations. In addition, the model suggests the presence of several negatively charged residues near the entrance of the channel which may serve to increase the local concentration of cations, thereby increasing their conductance relative to anions. Indeed, mutagenesis of these residues causes a decrease in the conductance rate.[16]

Unfortunately, there are no crystal structures available for channel proteins from electrically excitable tissue, although the structure of porin isolated from

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(1) DeGrado, W. F.; Wasserman, Z. R.; Lear, J. D. *Science* **1989**, *243*, 622–628.
(2) O'Neil, K. T.; DeGrado, W. F. *Trends Biochem. Sci.* **1990**, *15*, 59–64.
(3) O'Neil, K. T.; Hoess, R. H.; DeGrado, W. F. *Science* **1990**, *249*, 774–778.
(4) For recent comprehensive reviews on ligand-gated and voltage-gated ion channel proteins, see: (a) Betz, H. *Biochemistry* **1990**, *29*, 3591–3599. (b) Numa, S. *A molecular view of neutrotransmitter receptors and ionic channels*; Alan R. Liss, Inc.: New York, 1989; Vol. Series 83, pp 121–166. (c) Jan, L. Y.; Jan, Y. N. *Cell* **1989**, *56*, 13–25. (d) Catterall, W. A. *Science* **1988**, *242*, 50–61. For a more detailed account of the factors implicated in structure–function relationships of ion channels, see: (e) Montal, M. *FASEB J.* **1990**, *4*, 2623–2635. (f) Unwin, N. *Neuron* **1989**, *3*, 665–676. (g) Eisenman, G.; Dani, J. A. *Annu. Rev. Biophys. Biophys. Chem.* **1987**, *16*, 205–226. (h) Miller, C. In *Neuromodulation: the Biochemical Control of Neuronal Excitability*; Kaczmarek, L. K., Levitan, I. B., Eds.; Oxford University Press: Oxford, 1986; pp 39–63. (i) Reference 52.
(5) Boat, T. F.; Welsh, M. J.; Beaudet, A. L. In *The Metabolic Basis of Inherited Disease*, 6th ed.; Scriver, C. L., Beaudet, A. L., Sly, W. S., Valle, D., Eds.; McGraw-Hill: New York, 1989; pp 2649–2680.
(6) Anderson, M. P.; Gregory, R. J.; Thompson, S.; Souza, D. W.; Paul, S.; Mulligan, R. C.; Smith, A. E.; Welsh, M. J. *Science* **1991**, *253*, 202–205.
(7) Christadoss, P. *Crit. Rev. Immunol.* **1989**, *9*, 247–278.
(8) It has been proposed that the potassium channel-lining segment might consist of a β-hairpin structure: (a) Hartmann, H. A.; Kirsch, G. E.; Drewe, J. A.; Taglialatela, M.; Joho, R. H.; Brown, A. M. *Science* **1991**, *251*, 942–944. (b) Yellen, G.; Jurman, M. E.; Abramson, T.; MacKinnon, R. *Science* **1991**, *251*, 939–942. (c) Yool, A. J.; Schwartz, T. L. *Nature* **1991**, *349*, 700–704.
(9) Changeux, J. *Trends Biochem. Sci.* **1990**, *11*, 485–492.
(10) Revah, F.; Galzi, J.-L.; Giraudat, J.; Haumont, P.-Y.; Lederer, F.; Changeux, J.-P. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 4675–4679.
(11) Leonard, R. J.; Labarca, C. G.; Charnet, P.; Davidson, N.; Lester, H. A. *Science* **1988**, *242*, 1578–1581.
(12) Unwin, P. N. T.; Toyoshima, C.; Kubalek, E. *J. Cell Biol.* **1988**, *107*, 1123–1138.
(13) Kubalek, E.; Ralston, S.; Lindstrom, J.; Unwin, N. *J. Cell Biol.* **1987**, *105*, 9–18.
(14) Brisson, A.; Unwin, P. N. T. *Nature* **1985**, *315*, 474–477.
(15) Hartman, D.; Claudio, T. *Nature* **1990**, *343*, 372–375.
(16) (a) Imoto, K.; Busch, C.; Sakmann, B.; Mishina, M.; Konno, T.; Nakai, J.; Bujo, H.; More, Y.; Fukuda, K.; Numa, S. *Nature* **1988**, *335*, 645–648. (b) Konno, T.; Busch, C.; von Kitzing, E.; Imoto, K.; Wang, F.; Nakai, J.; Mishina, M.; Numa, S.; Sakmann, B. *Proc. R. Soc. London, B* **1991**, *244*, 69–80.

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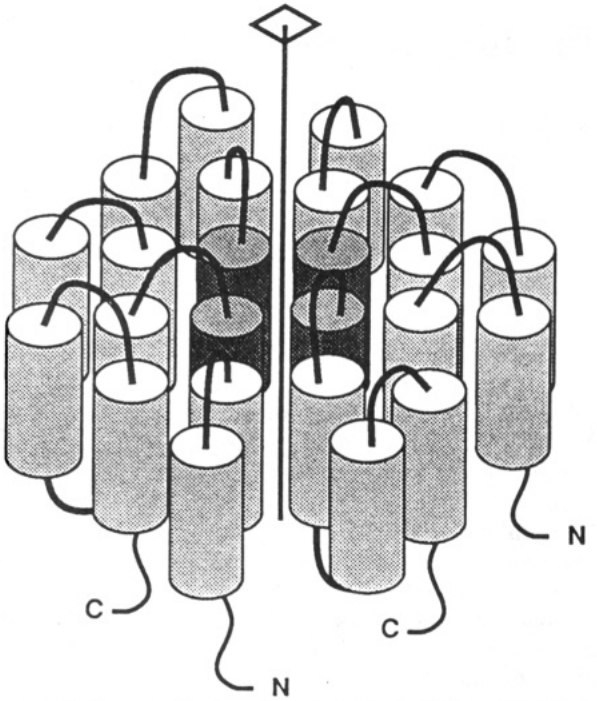

Figure 1. Proposed general model of a 4-fold symmetric ion channel protein. The cylinders represent membrane-spanning α-helices connected by intervening nonhelical peptide sequences (drawn as loops). The four pore-lining α-helices are depicted as dark cylinders. Reprinted with permission from ref 53. Copyright 1988 American Association for the Advancement of Science.

Rhodobacter capsulatus has been determined.[17] Unlike the above-mentioned channel proteins, porin allows the diffusion of a variety of molecules with molecular weights smaller than 600 daltons (Da) and is composed of a 3-fold symmetric array of β-barrel subunits.

Conductance Measurements

Recent technological developments have made it possible to examine the channel properties of a single molecule. In the patch clamp technique[18,19] a micropipet is attached to a patch of cell membrane. Electrodes placed on either side of the patch permit investigation of the electrical properties of the channel protein. An electrical potential is applied across the membrane, and the ion flux (current) is monitored with time. Single channels can be detected electrically because they have a very high turnover rate (10^8 ions/s, giving rise to a current of approximately 20 pA). Ion-conducting molecules can also be studied in artificial planar bilayers formed across a 150-μm-diameter hole in a Teflon film.[20,21] Using this method, the voltage and the peptide or protein concentration in the bilayer can be adjusted to allow the measurement of single ion channels.

Model Peptide Ion Channels

The large size and complexity of natural ion channel proteins has made it attractive to study smaller natural or designed peptides to serve as model systems.

Natural Peptides. The classic example of a naturally occurring ion channel-forming peptide is gramicidin A, a pentadecapeptide consisting of an alternating sequence of D- and L-amino acids.[22-25] The conducting pore, which is cation selective, is formed by a highly unusual "head-to-head" β-helical dimer structure that places all the amino acid side chains on the outside of the helix. The ions pass through the center of the helix and are solvated by the peptide amide carbonyls (Figure 2).

Many natural channel-forming peptides adopt amphiphilic α-helices which form assemblies more similar in structure to channel proteins than the gramicidin β-helix. Several microbial channel-forming peptides (e.g., alamethicins,[26] suzukacillins,[27] trichotoxins,[28] and zervamicins[29]) contain α-aminoisobutyric acid (α,α-dimethylglycine, abbreviated Aib, or B) residues which strongly promote helix formation.[30] One of the earliest studied Aib-containing peptides is alamethicin, a 20-residue peptide which forms discrete ion channels of multiple conductance states thought to consist of bundles of helices.[31] Other natural peptides that interact with membranes as α-helices include melittin,[32] cecropins,[33] magainins,[34] and pardaxin.[35]

Synthetic Peptides: Putative Pore-Lining Sequences. Peptide segments corresponding to the postulated pore-lining sequences of the sodium channel,[36] the acetylcholine receptor δ-subunit,[37] and the glycine receptor[38] have been synthesized and characterized in planar bilayers. This approach assumes that, in the absence of the rest of the protein, these segments will self-associate to produce a channel resembling the pore of the native protein. The putative pore-lining sequences of the acetylcholine receptor δ-subunit, the sodium channel, and the glycine receptor form discrete ion channels with some similarities to their cognate proteins. However, it should be appreciated that many amphiphilic peptide sequences can form channels in a bilayer. For example, a synthetic peptide corresponding to the putative membrane-spanning voltage sensor segment of the sodium channel forms ion-conducting channels.[39] Another concern is that in the absence of the rest of the protein the pore-lining peptides may

(17) Weiss, M. S.; Kreusch, A.; Schlitz, E.; Nestel, U.; Welte, W.; Weckesser, J.; Schultz, G. E. *FEBS Lett.* **1991**, *280*, 379–382.
(18) Sakmann, B.; Neher, E. *Annu. Rev. Physiol.* **1984**, *46*, 455–472.
(19) Hamill, O. P.; Marty, A.; Neher, E.; Sakmann, B.; Sigworth, F. J. *Pfluegers Arch.* **1981**, *391*, 85–100.
(20) Mueller, P.; Rudin, D. O.; Tien, H. T.; Wescott, W. C. *Nature* **1962**, *194*, 979–980.
(21) Montal, M.; Mueller, P. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3561–3566.
(22) Urry, D. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 1610–1614.
(23) Wallace, B. A. *Biopolymers* **1983**, *22*, 397–402.
(24) Andersen, O. S. *Annu. Rev. Physiol.* **1984**, *46*, 531–548.
(25) Stankovic, C. J.; Schreiber, S. L. *Chemtracts: Org. Chem.* **1991**, *4*, 1–20.
(26) Hall, J. E.; Vodyanov, I.; Balasubramanian, T. M.; Marshall, G. R. *Biophys. J.* **1984**, *45*, 233–247.
(27) Katz, E.; M. A.; Lucht, N.; Konig, W. A.; Ooka, T.; Jung, G. *Liebigs Ann. Chem.* **1985**, 1041–1062.
(28) Przybylski, M.; Dietrich, I.; Manz, I.; Brückner, H. *Biomed. Mass Spectrom.* **1984**, *11*, 569–582.
(29) Karle, I. L.; Flippen-Anderson, J. L.; Agarwalla, S.; Balaram, P. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5307–5311.
(30) Prasad, B. V.; Balaram, P. *Crit. Rev. Biochem.* **1984**, *16*, 307–348.
(31) Fox, R. O., Jr.; Richards, F. M. *Nature* **1982**, *300*, 325–330.
(32) Tosteson, M. T.; Levy, J. J.; Caporale, L. H.; Rosenblatt, M.; Tosteson, D. *Biochemistry* **1987**, *26*, 6627–6631.
(33) Christensen, B. F. J.; Merrifield, R. B.; Mauzerall, D. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5072–5076.
(34) Westerhoff, H. V.; Juretic, D.; Hendler, R. W.; Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6597–6601.
(35) Shai, Y.; Bach, D.; Yanivsky, A. *J. Biol. Chem.* **1990**, *265*, 20202–20209.
(36) Oiki, S.; Danho, W.; Montal, M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 2393–2397.
(37) Oiki, S.; Danho, W.; Madison, V.; Montal, M. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 8703–8707.
(38) Langosch, D.; Hartung, K.; Grell, E.; Bamberg, E.; Betz, H. *Biochim. Biophys. Acta* **1991**, *1063*, 36–44.
(39) Tosteson, M. T.; Auld, D. S.; Tosteson, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 707–710.

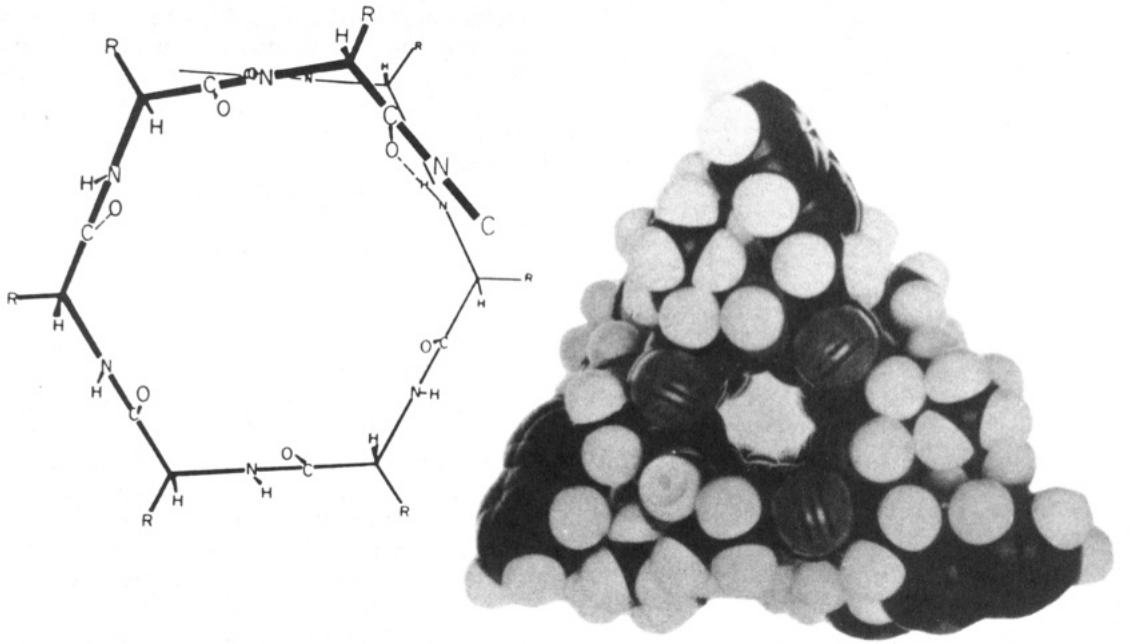

Figure 2. The ion-conducting structure of gramicidin A viewed down the axis of the pore. Due to the alternating D- and L-amino acid sequence, all of the side chains are located on the outside of the β-helix. The hydrogen-bonded peptide backbone lines the channel, which is approximately 4 Å in diameter. Reprinted with permission from ref 22. Copyright 1972 National Academy of Sciences.

form multiple oligomerization states rather than a unique channel structure.

Tethered Peptide Assemblies. To address the problem of multiple aggregation states, Montal and co-workers utilized Mutter's TASP (template-assembled synthetic proteins) technology[40,41] in which secondary structure-forming peptides are attached to a template, promoting their association in a specific tertiary fold. Four copies of the putative pore-lining residues of the acetylcholine receptor δ-subunit[42] or the calcium channel[43] were attached to one face of a β-hairpin peptide template, constraining the four α-helices to form a bundle of parallel helices. The conductance and pharmacological properties of the assembly built to mimic the calcium channel were remarkably similar to those of the native channel protein. In contrast, assemblies built by attaching other fully hydrophobic peptide sequences of the native channel proteins to the template did not result in the formation of channels.

A potential problem with the TASP method is that the peptides are simultaneously built up on the template by stepwise solid-phase synthesis, giving rise to heterogeneous mixtures of closely related peptides. Mutter and co-workers[44] have shown that the purification to homogeneity of an assembly of four 16-residue water-soluble helices required especially stringent chromatography procedures. Such purification protocols have not yet been applied to the larger and more hydrophobic ion channel assemblies prepared by Montal and co-workers.

De Novo Designed Peptide Models.[45] We have taken an additional step toward the design of peptide models for ion channel proteins. Our method is to test current hypotheses by building the simplest system capable of addressing the following questions: (1) Can ion channels be formed by the association of uncharged amphiphilic α-helical peptides? (2) What factors are important for ion selectivity? In particular, are charged residues necessary to effect anion versus cation specificity? (3) Is it possible to design ion channel-forming peptides that favor the formation of a given aggregation state?

Design Considerations. At the time we began this work the acetylcholine receptor was the best-characterized ion channel protein. The membrane-spanning portions of this protein are predicted from the amino acid sequence to be predominantly α-helical;[46] hence, we chose an α-helix as the basic structural module. An α-helical peptide should be at least 20 amino acid residues long to span a 30-Å lipid bilayer, and it should be sufficiently hydrophobic to be able to insert into the bilayer in a transmembrane orientation. Furthermore, the individual peptides should be able to pack together to produce a well-defined three-dimensional channel structure. We therefore chose hydrophobic and neutral side chains and a 7-residue repeat which would stabilize a coiled-coil structural motif[47] such as that found in the two-stranded GCN4,[48] the three-stranded hemagglutinin,[49] and the four-stranded ColE1 Rop.[50] The unique

(40) Mutter, M.; Altmann, K.-H.; Tuchscherer, G.; Vuilleumier, S. *Tetrahedron* **1988**, *44*, 771–785.
(41) Mutter, M. *Trends Biochem. Sci.* **1988**, *13*, 260–265.
(42) Montal, M.; Montal, M. S.; Tomich, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 6929–6933.
(43) Grove, A.; Tomich, J. M.; Montal, M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6418–6422.
(44) Mutter, M.; Tuchscherer, G. G.; Miller, C.; Altmann, K.-H.; Carey, R. L.; Wyss, D. F.; Labhardt, A. M.; Rivier, J. E. *J. Am. Chem. Soc.* **1992**, *114*, 1463–1470.
(45) There are several interesting examples of de novo designed nonpeptidic ion channels, e.g., crown ether–lipid assemblies, that will not be covered by this Account; see, for example: (a) Voyer, N. *J. Am. Chem. Soc.* **1991**, *113*, 1818–1821. (b) Carmichael, V. E.; Dutton, P. J.; Fyles, T. M.; James, T. D.; Swan, J. A.; Zojaji, M. *J. Am. Chem. Soc.* **1989**, *111*, 767–769. (c) Jullien, L.; Lehn, J.-M. *Tetrahedron Lett.* **1988**, *29*, 3803–3806. (d) Neevel, J. G.; Nolte, R. J. M. *Tetrahedron Lett.* **1984**, *25*, 2263–2266.
(46) Guy, R. H.; Hucho, F. *Trends Neurosci.* **1987**, *10*, 318–321.
(47) Cohen, C.; Parry, D. A. D. *Trends Biochem. Sci.* **1986**, *11*, 245–248.
(48) O'Shea, E. K.; Klemm, J. D.; Kim, P. S.; Alber, T. *Science* **1991**, *254*, 539–544.

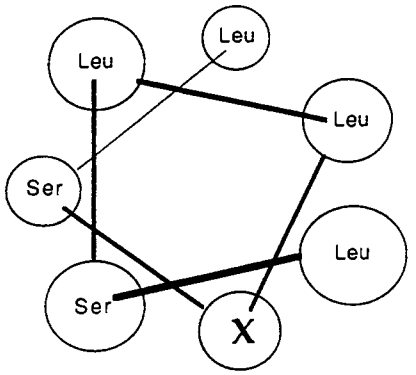

Figure 3. Helical wheel projections of (A) $(LSSLLSL)_3$ (X = S) and (B) $(LSLLLSL)_3$ (X = L) heptad repeats. (Modified from ref 53.)

characteristic of the coiled coil is a repeating heptad sequence pattern containing residues that pack well in a "knobs-into-holes" fashion. The supercoiled helix arrangement is energetically favorable because it maintains maximum interactions along the entire length of the helices, which would otherwise diverge.

In choosing the channel-lining residues it is instructive to consider the energy profile of an ion going through a channel. For fast ion transport, the initial rate-determining step should be diffusion into and out of the channel. The channel interior should provide a solvation structure that is as similar as possible to what the ion experiences in bulk water: too weak an interaction between the entering ion and the channel interior provides a high energy barrier for the incoming ion, whereas too tight binding would make the ion fall into an energy well and get trapped.

On the basis of the above considerations the peptide H_2N-(LeuSerSerLeuLeuSerLeu)$_3$-$CONH_2$, $(LSSLLSL)_3$, was designed (Figure 3A). The repeating heptad sequence should encourage the formation of coiled coils. Serine was chosen as the pore-lining residue because its hydrophilic side chain should serve to solvate mobile ions. Leucine was selected for the remaining positions since it is hydrophobic and packs very well in a "knobs-into-holes" fashion.[48,50]

$(LSSLLSL)_3$ was synthesized by a polymer-supported segment condensation of protected heptamer units prepared on the *p*-nitrobenzophenone oxime resin.[51] The peptide has also been synthesized by standard stepwise solid-phase methodology. The crude peptide $(LSSLLSL)_3$ was purified by high-performance liquid chromatography employing styrene-based reversed phase supports.

Determination of $(LSSLLSL)_3$ Ion Channel Properties. When $(LSSLLSL)_3$ is incorporated into diphytanoylphosphatidylcholine planar bilayers at peptide lipid molar ratios in the range of 1/100 to 1/1000, single channels are observed that resemble those of ion channel proteins. The peptide channel produces one predominant conductance state (around 70 pS at −100 mV in 0.5 M KCl, corresponding to a turnover rate of 4.4×10^7 ions/s) with millisecond open lifetimes. The peptide length requirement was tested using the 14-residue peptide $(LSSLLSL)_2$ which contains two heptad repeats and is thus not long enough to span a lipid bilayer in an α-helical conformation. This peptide elicited erratic conductance changes in lipid bilayers rather than discrete channel formation.

The $(LSSLLSL)_3$-peptide produces cation-selective channels similarly to the acetylcholine receptor. The ion selectivity was determined by measuring the conductances of various-sized cations as their hydrochloride salts. These experiments showed that the channels are cation selective with an effective diameter of approximately 8 Å, which is very similar to that observed for the acetylcholine receptor.[52]

Single channels are formed far more frequently when the $(LSSLLSL)_3$-peptide is added to the negative side of the bilayer, implying that it is the negative C-terminal end of the peptide that inserts toward the opposite, positively charged side of the bilayer. The asymmetric incorporation of the peptide in the bilayer provides evidence that the model peptides form parallel bundles in the membrane.[26,53] If the formal charge of the protonated N-terminus of the peptide is neutralized by N-acetylation, the channel lifetime is prolonged, consistent with a reduction of charge repulsions between peptides of parallel ordering (unpublished results).

Modeling the Structure of $(LSSLLSL)_3$. Computer models of the $(LSSLLSL)_3$-peptide in trimeric, tetrameric, pentameric, and hexameric aggregation states were generated by an iterative process of interactive computer graphics and energy minimization. To achieve good interhelical side chain packing, each bundle of parallel helices, in which the polar side chains face the interior, was initially given a left-handed twist as in natural protein coiled coils. C_n symmetry (n = the aggregation number) was maintained both to restrict the conformational space to be sampled and to maximize the pore diameter for any aggregation state. The trimer and the tetramer are well-packed with the side chains interdigitated in a "knobs-into-holes" fashion, and the pentamer and hexamer are somewhat less well-packed. However, in the tetrameric model one serine residue is located in the hydrophobic region between helices rather than in the pore (Figure 4A), whereas in the hexamer all of the serine side chains face the interior of the channel (Figure 4B). Furthermore the hexameric model has a pore diameter of about 8 Å, consistent with the ion permeation studies described above.

The energy-minimized models of $(LSSLLSL)_3$ provide a rationale for how uncharged amino acid residues might make a channel cation selective. In an α-helix the proton of a serine hydroxyl side chain i generally engages in hydrogen bonding to the backbone carbonyl at position $i-4$.[54] This arrangement projects the lone pairs of the serine hydroxyl oxygen atoms toward the pore, where they are accessible for solvation of cations.

Design of $(LSLLLSL)_3$, a Proton Channel Peptide. The initial stages of our modeling study of $(LSSLLSL)_3$ were focused on tetrameric bundles. We were struck by the observation that in all of the well-packed models the hydrophilic serine residue of the third position of the heptad repeat was located in the

(49) Wilson, I. A.; Skehel, J. J.; Wiley, D. C. *Nature* **1981**, *289*, 366–373.
(50) Banner, D. W.; Kokkinidis, M.; Tsernoglou, D. *J. Mol. Biol.* **1987**, *196*, 657–675.
(51) (a) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1982**, *47*, 3258–3261. (b) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, *45*, 1295–1300.
(52) Hille, B. *Ion Channels of excitable membranes*, 2nd ed.; Sinauer Associates Inc.: Sunderland, MA, 1992; p 607.
(53) Lear, J. D.; Wasserman, Z. R.; DeGrado, W. F. *Science* **1988**, *240*, 1177–1181.
(54) Baker, E. N.; Hubbard, R. E. *Prog. Biophys. Mol. Biol.* **1984**, *44*, 97–179.

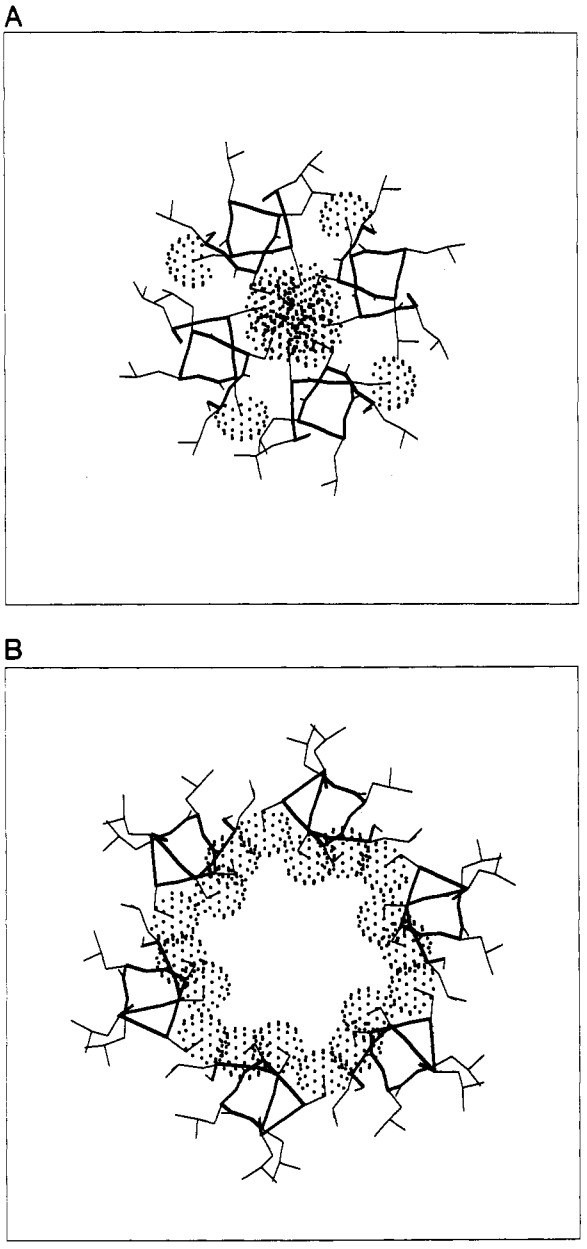

Figure 4. Minimum-energy models of $(LSSLLSL)_3$ in (A) tetrameric and (B) hexameric aggregation states. For each model a single heptad is shown; heavy lines indicate the helix backbone, and dots indicate the van der Waals surfaces of the hydroxyl oxygen atoms of the serine side chains. In the tetrameric model, one serine hydroxyl per heptad is located in the hydrophobic region between the helices, whereas all the hydroxyls face the channel interior in the hexamer.

hydrophobic region between the helices (Figure 4A). We, therefore, sought to improve the stability of the tetramer structure by substituting leucine for this serine residue, giving the sequence H_2N-(LeuSerLeuLeuLeu-SerLeu)$_3$-$CONH_2$, or $(LSLLLSL)_3$ (Figure 3B). The resulting ion channels did not conduct Li^+ or other cations but were completely selective for protons. This finding could be rationalized if we assumed that $(LSLLLSL)_3$ forms the originally designed tetramer (which in minimized models has a pore diameter of less than 1 Å at the positions of closest approach), and that the main conductance state of $(LSSLLSL)_3$ is the above-mentioned hexamer. Consistent with this suggestion, the major conductance state of $(LSSLLSL)_3$ has a much larger turnover for protons (900 pS, or 5.6×10^8 protons/s) than $(LSLLLSL)_3$ (190 pS, or 1.2×10^8 protons/s) in 1.0 M HCl at −100 mV.

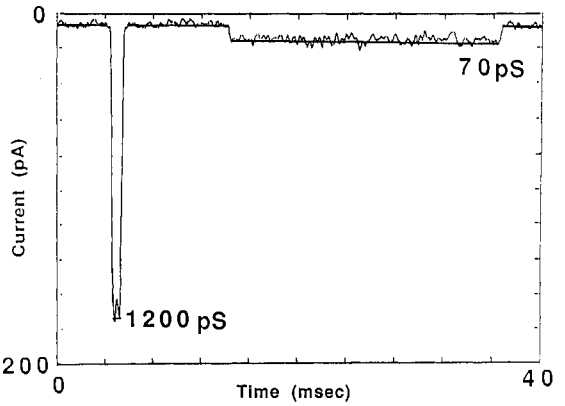

Figure 5. Plot of current versus time for the $(LSSLLSL)_3$-peptide incorporated in a diphytanoylphosphatidylcholine bilayer measured at −140 mV with 1 M HCl as electrolyte. Channel openings are shown as downward current deflections. Two types of channels are observed with conductances of 70 and 1200 pS.

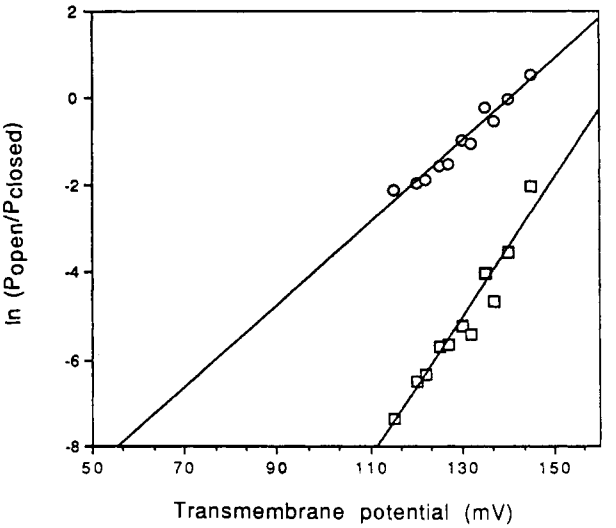

Figure 6. Plot of $\ln(P_{open}/P_{closed})$ versus transmembrane potential, where P_{open} is the probability per unit time that the bilayer is in a conducting state with a single channel open, and P_{closed} is the probability that the bilayer is closed in its resting state with no channels open. The measurements were made under the same conditions as described in Figure 5 in which the simultaneous opening of two channels is very rare. The data were analyzed by an Ono Sokki CFT 360 portable spectrum analyzer, and the lines were obtained by linear regression to provide gating charges (see text) of 3.8 for the large channels (circles) and 2.3 for the small channels (squares).

These findings stimulated us to search for alternate conductance states of $(LSSLLSL)_3$, associated with aggregation states smaller than the postulated hexamer. Indeed, a proton selective channel state has recently been detected with a conductivity of 70 pS at −140 mV in 1.0 M HCl (under these conditions the conductance of the larger channel is 1200 pS) (Figures 5 and 6).

Testing the Helical Model. Circular dichroism spectroscopy studies indicate that $(LSSLLSL)_3$ and $(LSLLLSL)_2$ adopt predominantly α-helical conformations in diphytanoylphosphatidylcholine bilayers. However, it should be realized that the channel-forming state of the peptide is exceedingly rare: approximately 10^8 peptide molecules are present in a typical planar bilayer, yet at moderate voltages the probability of finding a single channel open at any given time is less than 1. How do we know whether the channel-forming

state is not a rarely occurring conformational isomer of the predominant α-helical state? To test this possibility we substituted α-aminoisobutyric acid, a residue which strongly promotes α-helix formation,[30] into $(LSLLLSL)_3$ to give $(LSLBLSL)_3$.[55] If the four-stranded coiled-coil model is correct for $(LSLLLSL)_3$, it would place the Aib residue at exterior positions on the bundle. A mutation of this "outside position" from a leucine to an Aib residue should have a minimal effect on the channel interior while promoting helix formation.

The $(LSLBLSL)_3$-peptide produces channels that have properties similar to those of $(LSLLLSL)_3$. Although the peptide makes proton-selective channels with significantly shorter lifetimes (<0.1 ms), the conductance rates (200 pS at −100 mV) are similar to those of $(LSLLLSL)_3$. Furthermore, the circular dichroism spectra of $(LSLLLSL)_3$ and $(LSLBLSL)_3$ incorporated into phospholipid vesicles are identical. This finding suggests that the helical content of $(LSLLLSL)_3$ is as high as that of the Aib analog and that the peptides in the active conducting state are α-helical. By inference, the $(LSSLLSL)_3$-peptide may be assumed to form helical bundles in the conducting state as well since circular dichroism spectroscopy shows that the peptide adopts a helical conformation in phospholipid vesicles.

Gating. The frequency of channel formation depends strongly on voltage (Figure 6) and peptide concentration,[56] suggesting that the peptide helix dipole undergoes a major reorientation between the open and closed channel states. The lifetime of the open channel state is relatively independent of voltage and peptide concentration whereas the time in between the open states depends on these variables.[56] We hypothesized that, in the predominant closed state of the channel, the peptide, P, is oriented with its α-helix parallel to the bilayer surface ($P_=$), Scheme I. This orientation allows dehydration of the leucine residues and exposure of the serine residues to bulk water. Also present in low concentration are vertically inserted closed states of the peptide ($P_\parallel$). An applied electrical field stabilizes a transmembrane orientation of those α-helices (which contain a large macrodipole) oriented with the negative end of their dipoles toward the positive side of the bilayer. Thus, the concentration of inserted peptides should increase with voltage, increasing the probability of the formation of an open channel ($P_{\parallel n(\text{open})}$). The driving forces for pore formation are presumably the favorable interhelical packing interactions as well as self-association of the inserted peptides to bury their hydrophilic faces from the lipophilic environment:

Scheme I

$$nP_= \leftrightarrow nP_\parallel \leftrightarrow \cdots \leftrightarrow P_{\parallel n(\text{open})}$$

A similar gating model has also been suggested for alamethicin.[57]

The proposed model makes three experimentally testable predictions. First, in the predominant, closed channel state the orientation of the helical peptide is parallel to the lipid bilayer. To test the orientation of

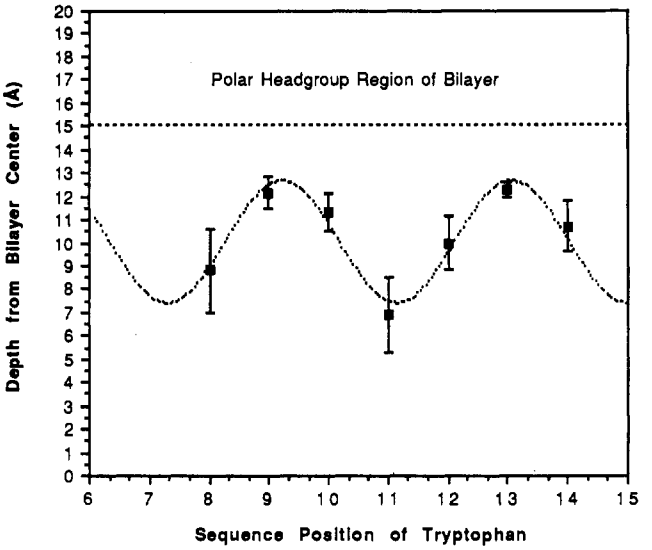

Figure 7. The calculated distance between the tryptophan residue and the center of the lipid bilayer (filled squares) as a function of sequence position, as determined by the parallax method. Note that the distance changes with a periodicity consistent with an α-helical peptide conformation. (From ref 58.)

$(LSSLLSL)_3$ in the closed state,[58] we utilized the sensitivity of the tryptophan fluorescence emission wavelength to its surrounding dielectric environment.[59] Seven peptide analogs were synthesized in which each position of the middle heptad unit of $(LSSLLSL)_3$ was replaced with a tryptophan residue, one per peptide analog. The peptide analogs were studied in diphytanoylphosphatidylcholine phospholipid vesicles at a peptide/lipid ratio of 1/150. The positions of the tryptophan residues with respect to the center of the bilayer were determined using the "parallax method".[60] This technique involves the calculation of the position of a fluorophore relative to two spin-labels (or other quenchers) located at two different positions on the lipid acid side chains of the bilayer. By this method it was determined that, in the predominant closed state of the channel, the peptide is an α-helix parallel to the bilayer with the long helix axis located approximately 10 Å from the bilayer center (Figure 7).

It is important to note that the preferred orientation of our peptides depends on the concentration of the peptide in the bilayer. We conducted the fluorescence experiments at relatively low peptide/lipid ratios similar to those used in our ion channel measurements, at such low peptide/lipid ratios that the monomeric, surface-bound state predominates. If the concentration of the peptide in the lipid bilayer is increased, the fraction of peptide in the self-associated transmembrane α-helical bundle will increase.

Due to the peptide helix dipole, the C- and N-terminal ends carry effective charges of approximately −0.6 and +0.6, respectively.[61] Reorientation of the dipole from a parallel to a perpendicular membrane orientation is, therefore, associated with the translocation of 0.6 charges per peptide or 3.6 (6 × 0.6) for $(LSSLLSL)_3$, assuming that the conducting channel is hexameric. In

(55) DeGrado, W. F.; Lear, J. D. *Biopolymers* **1990**, *29*, 205–213.
(56) Lear, J. D.; Wasserman, Z. R.; DeGrado, W. F. In *Membrane Protein Structural Methods*; White, S. H., Ed.; Oxford University Press: New York, in press.
(57) Baumann, G.; Mueller, P. *J. Supramol. Struct.* **1974**, *2*, 538–557.
(58) Chung, L.; Lear, J.; DeGrado, W. F. *Biochemistry* **1992**, *31*, 6608–6616.
(59) O'Neil, K. T.; Wolfe, J. H. R.; Erickson-Viitanen, S.; DeGrado, W. F. *Science* **1987**, *236*, 1454–1456.
(60) Chattopadhyay, A.; London, E. *Biochemistry* **1987**, *26*, 39–45.
(61) Pethig, R. *Dielectric and Electronic Properties of Biological Material*; John Wiley & Sons: New York, 1979; pp 49–53.

fact, the gating charge, z, was determined to be 3.2 from the voltage dependence of the average time between channel openings, τ, according to the following equation:[56]

$$z = (RT/F)\, \mathrm{d}(\ln \tau)/\mathrm{d}V$$

The third prediction of our gating model is that features that stabilize the oligomeric channel state should increase channel lifetime and/or decrease time between channel openings. As discussed earlier, N-acetylation of $(LSSLLSL)_3$, which removes charge repulsion between the N-terminal ends of the peptides of the helical bundle, increases the lifetime of the channels by approximately 10-fold.

Chemicals of Defined Aggregation State. Although our model studies of $(LSSLLSL)_3$ and $(LSLLLSL)_3$ suggest specific packing arrangements that are in agreement with their channel properties, we would like to eliminate this uncertainty, in particular to be able to study the effect of amino acid substitution on ion selectivity.

To provide assemblies of defined aggregation state we have attached ion channel-forming peptides to a tetraphenylporphine derivative carrier, which is considerably more rigid than the linear or cyclic[44] peptide templates employed by Mutter and co-workers. Tetraphenylporphine is also somewhat more rigid and chemically more stable compared to coproporphyrin, which was introduced to tether a water-soluble four-helix bundle in pioneering work by Sasaki and Kaiser.[62]

To avoid the potential problem of microheterogeneity associated with the buildup of TASP assemblies by the stepwise solid-phase peptide synthesis methodology, the 21-residue $(LSLBLSL)_3$ peptide was synthesized separately and the protecting groups were removed. After purification, the unprotected peptide was coupled to *meso*-tetrakis(3-carboxyphenyl)porphine to furnish tetraphilin 1 (Figure 8).[64]

The four-helix bundle assembly forms discrete ion channels when incorporated into diphytanoylphosphatidylcholine planar bilayers. Like $(LSLLLSL)_3$, the porphyrin–peptide assembly is selective for protons. Further, tetraphilin 1 has a gating charge of 0.5, suggesting that the assembly is vertically inserted in the bilayer in its predominant state. If this is correct, the tether stabilizes the peptide in the inserted transmembrane orientation. We are currently testing this hypothesis by examining the optical properties of the assembly as well as monomeric derivatives of $(LSLBLSL)_3$ in oriented multilayer films.

Discussion

These results indicate that neutral amphiphilic α-helical peptides can form ion channels in lipid bilayers. A surprising result is that neutral bundles of helices can form ion channels that are both large and cation selective. The small neutral peptide gramicidin A forms channels that are selective for alkali metal cations, but in this case the mechanism of selectivity involves very specific solvation of the cations by backbone amide carbonyls. The channels formed by the association of amphiphilic α-helical peptides, such as $(LSSLLSL)_3$, presumably orient neutral polar groups toward the interior of pores of larger diameter compared to gramicidin A, giving a broader size specificity while maintaining cation selectivity.

The use of templates to assemble bundles of α-helices, or other secondary structure building blocks, is a particularly promising technique, because the aggregation state can be defined and varied using different templates (e.g., cyclodextrins). Ultimately, modifications in existing synthetic approaches will allow the introduction of asymmetric arrays of secondary structural units, providing additional control of the channel. This method also has promise for the design of models for membrane-associated proteins other than ion channels.

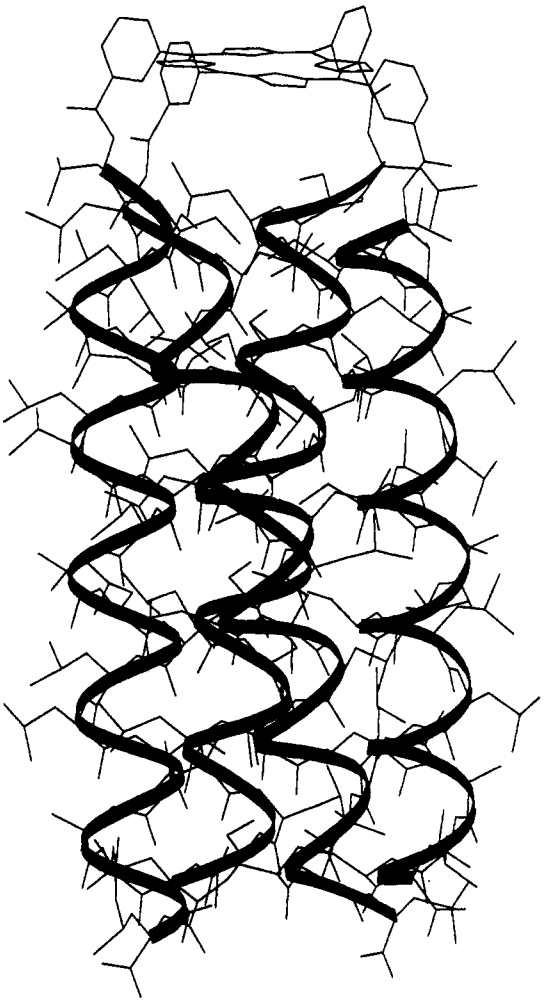

Figure 8. The energy-minimized computer graphics model of tetraphilin 1. Reprinted with permission from ref 64. Copyright 1992 American Chemical Society.

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(62) Sasaki, T.; Kaiser, E. *J. Am. Chem. Soc.* **1989**, *111*, 380–381.
(63) Jameson, G. B.; Molinaro, F. S.; Ibers, J. A.; Collman, J. P.; Brauman, J. I.; Rose, E.; Suslick, K. S. *J. Am. Chem. Soc.* **1980**, *102*, 3224–3237.
(64) Åkerfeldt, K. S.; Kim, R. M.; Camac, D.; Groves, J. T.; Lear, J. D.; DeGrado, W. F. *J. Am. Chem. Soc.* **1992**, *114*, 9656–9657.