Three-Dimensional Structure at 0.86 Å of the Uncomplexed Form of the Transmembrane Ion Channel Peptide Gramicidin A

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The crystal structure of the uncomplexed orthorhombic form of gramicidin A has been determined at 120 K and at 0.86 angstrom resolution. The pentadecapeptide crystallizes as a left-handed antiparallel double-stranded helical dimer with 5.6 amino acid residues per turn. The helix has an overall length of 31 angstroms and an average inner channel diameter of 4.80 angstroms. The channel of this crystalline form is void of ions or solvent molecules. The channel diameter varies from a minimum of 3.85 angstroms to a maximum of 5.47 angstroms and contains three pockets where the cross-channel contacts are 5.25 angstroms or greater. The range of variation seen for the ϕ and ψ torsion angles of the backbone of the helix suggests that these potential ion binding sites can be induced to travel the length of the channel in a peristaltic manner by cooperatively varying these angles. The indole rings of the eight tryptophan residues of the dimer are overlapped in three separate regions on the outer surface of the helix when viewed down the barrel of the channel. This arrangement would permit long-chained lipid molecules to nest parallel to the outer channel surface between these protruding tryptophan regions and act like molecular splines to constrain helical twist deformations of the channel.

THE STUDY OF ION CHANNELS IN CELLULAR MEMBRANES has been intimately linked to an understanding of the regulation and control of such events as excitation-contraction and stimulus-secretion coupling. Electrophysiologic experiments indicate that these channels are not simple static pores that are activated or closed by effector ligands or react passively to the changing membrane potential of the cell. Many of these ion channels exist in a number of discrete gating states that are observed to change with the fluctuation of the membrane potential and the availability of transportable ions and ligands that bind to regulatory sites. The mechanism by which these channels are ion specific and by which they cycle through the various gating states is open to speculation.

Gramicidin A is a linear pentadecapeptide made up of alternating L and D configuration amino acids isolated from *Bacillus brevis* (*1*); it has antibiotic activity primarily against Gram-positive bacteria (*2*). The molecular structure of gramicidin A was proved by peptide degradation and de novo synthesis (*3*) to be HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-(L-Trp-D-Leu)$_3$-L-Trp-NHCH$_2$CH$_2$OH. It is one of the simplest known molecules that forms ion channels through lipid membranes, in particular, the membranes of mitochondria where ion influxes inhibit oxidative phosphorylation. These membrane channels are selective for monovalent cations such as the alkali metals, Ag^+, Tl^+, NH_4^+, and H^+ (*4*). Electrophysiologic measurements reveal that the gramicidin A ion transport rate is 10^3 to 10^4 times greater than that for typical ion carriers like valinomycin, which indicates that gramicidin functions as a channel, rather than a shuttle, carrier (*5*). Two facts suggest that the channel is a dimer of fixed length; the rate of conductance decreases exponentially with the thickness of the membrane (*6*), and the rate of channel formation in lipid bilayers is second order in peptide concentration (*7*). Patch clamp analyses of isolated gramicidin A channels have shown that the ion currents fluctuate with time, with the channels cycling through open and closed states that represent different peptide-channel conformations during the course of measurements (*8*). Various models have been proposed for the dimeric form of the gramicidin A channel in lipid membranes, organic solvents, and the solid state. I now describe the crystal structure determined for the orthorhombic uncomplexed form of the gramicidin dimer and suggest how the peptide could function as a channel in lipid bilayers.

Data collection and processing. Commercial samples of gramicidin D (Sigma Chemical Company) are a mixture of 80 percent gramicidin A, 14 percent gramicidin C, and 6 percent gramicidin B (amino acid residue 11 is Trp, Tyr, or Phe, respectively), and were used without further purification. A single crystal of (0.35 by 0.40 by 0.50 mm) of gramicidin was grown from benzene-ethanol azeotrope. The crystal was taken from solution and immediately coated with a film of silicone grease to prevent loss of crystalline solvent, fixed to a glass fiber on a goniometer head, and positioned in the liquid nitrogen cold stream on a Syntex P3 automated four-circle x-ray diffractometer. Data were recorded at 120 K with the use of Ni-filtered CuKα radiation. Cell constants were determined by a least-squares fit of 25 carefully centered Bragg angles in the $(\sin\theta)/\lambda$ range of 0.30 to 0.35 Å^{-1} (Table 1). Intensity data were recorded with a θ-2θ step-scan procedure and subjected to a profile integration analysis (*9*). A total of 21,454 unique reflections were measured to $(\sin\theta)/\lambda = 0.58$ Å^{-1} during the 18-day period of the experiment. These measurements were corrected for Lorentz and polarization effects, but not for absorption. Five reference reflections were remeasured at 2-hour intervals; time-dependent scaling varied only slightly, from 0.990 to 1.015, over the 370 hours of x-ray exposure. In addition to the 176 measurements of each reference

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Table 1. Crystal data.

Cell constants	
a (Å)	31.595(4)
b (Å)	32.369(5)
c (Å)	24.219(3)
α,β,γ(°)	90.0
Formula	$(C_{99}H_{140}N_{20}O_{17})_2 \cdot 15C_2H_5OH$
Density (obs)	1.146 g/cm^3 (298 K)
Density (calc)	1.195 g/cm^3 (120 K)
Space group	$P2_12_12_1$
Z	4

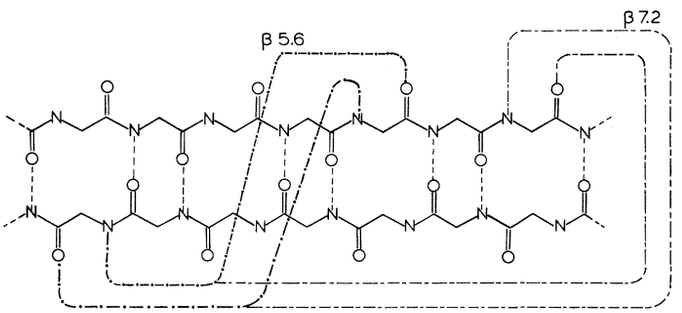

Fig. 1. Schematic diagram illustrating the hydrogen-bonding patterns for the uncomplexed $\beta^{5.6}$ (dot-dashed lines) and cesium-complexed $\beta^{7.2}$ (dashed lines) molecular models of the gramicidin A channel helices. The uncomplexed form is reported here, and the cesium complex structure is from (*26*).

reflection, 1200 reflections were measured in duplicate. Averaging the equivalent data gave a value of 0.043 for $R_w = [\Sigma_i\, w_i(F_i^2 - \langle F^2 \rangle)^2 / \Sigma_i\, w_i(F^2{}_i)^2]^{1/2}$, $w = 1/\sigma^2(F^2{}_i)$. These data were further processed by a Bayesian analysis (*10*) to obtain improved estimates for the weaker intensities, after which 20,268 of the 21,454 data had $|F| > 2\sigma(|F|)$. A Patterson origin integration technique (*11*) was used to determine the absolute scale and overall anisotropic temperature parameters for the data set, and these were used to derive the normalized structure factors for the structure determination.

Structure determination. Crystal structure determination proceeded both through molecular replacement (*12*) and direct methods incorporating estimated three-phase structure invariants (*13*), and both traditional tangent formula (*14*) and random start (*15*) phasing procedures. Molecular replacement methods that used various idealized model helices (*16*) did not provide an unambiguous phase solution or a preferred choice among the models tested. Rotation functions of each of these models readily identified the *b* axis of the unit cell as the approximate direction of the helical axis of the dimers, but were insensitive to the orientation of the dimer about this axis. A similar observation had been noted previously in applications involving α-helices in proteins (*17*). The direct phasing methods also failed to produce a clear-cut solution, although many trial phase sets were generated with different starting points and tentative symbolic phase assignments. Many of the phase sets produced E maps that contained peptide-like fragments, but none of these were large enough to be useful to phase the structure by standard methods. These fragments, however, did give a clue to the possible orientation of the dimer about the helical axis. A model of an antiparallel $\beta^{5.6}$ dimer (*16*) consisting of 60 atoms was fit to a peptide fragment found in one of the maps, which indicated that the helix was tilted 5 degrees from the unit cell *b* axis. Standard tangent refinement methods starting with this partial structure did not appear to produce a phase solution. Phase refinement incorporating ten randomly assigned phase sets in addition to phase values determined by the partial structure produced two equivalent phase sets with negative NQEST figures of merit (*18*). A subsequent E map did not return the complete input model of 60 atoms in two contiguous strands, but rather seven unconnected peptide-like fragments comprising 109 atoms in aggregate, many of which corresponded to an extension of the original helical model. Two cycles of fragment-based phase refinement were sufficient to trace the entire peptide backbone of both strands. Fourier maps disclosed the positions of the amino acid side chains and solvent ethanol molecules in the structure. Full-matrix constrained least-squares refinement (*19*) of the parameters for 334 non-hydrogen atoms, including full and partial occupancy sites, converged to a weighted residual of 0.071 for 19,684 data used in the refinement.

The structure of the dimer. Numerous models have been proposed for the dimer structure of gramicidin A in various solvents, lipid bilayers, and the solid state based on nuclear magnetic resonance (NMR), circular dichroism, infrared, and Raman studies (*20*). These include the single-stranded, head-to-head $\beta^{6.3}$ dimer proposed by Urry (*21*) and the double-stranded antiparallel $\beta^{5.6}$ dimer favored by Veatch *et al.* (*22*). Although diffraction studies of the crystalline state of gramicidin were initiated nearly 40 years ago (*23*), the structure has resisted solution in the intervening years. Crystals of a CsSCN complex of gramicidin A obtained by Koeppe and co-workers (*24*) provided a preliminary description of the structure at 2.9 Å resolution as a cylindrical channel 5 Å in diameter and 32 Å long. Data were subsequently recorded to 2.5 Å resolution, and the channel was reported to contain two Cs^+ ions; however, the details of the peptide backbone and side chains were obscure. A 5.0 Å model of a deuterium-hydrogen–difference neutron diffraction structure of the uncomplexed form of the dimer was equally lacking in detail. Wallace and Hendrickson (*25*), using single wavelength anomalous dispersion data, determined the structure of an isomorphous CsCl complex at 1.8 Å resolution which revealed that the earlier space group assignment for the CsSCN complex was incorrect. The channel was originally reported to be a left-handed, double-stranded antiparallel helix that was 4.4 Å in diameter with 5.6 residues per turn. Later (*26*), the number of residues per turn was revised to 6.3, and at the same time a hydrogen-bonding pattern (Fig. 1) corresponding to the double-stranded antiparallel $\beta^{7.2}$ helix described by Lotz and co-workers (*16*) was outlined. The channel was found to contain two cesium and three chloride ions, all separated at nonbonded distances. The refinement of the peptide channel has been hampered by the low resolution of the data, pseudosymmetry, and the dominant influence of the Cs^+ ions in the diffraction amplitudes. Although evidence suggests that multiple double-stranded forms of the dimer exist in the solid state and in organic solvent solutions, the exact structure of the dimeric form of gramicidin in phospholipid bilayers still remains a subject of controversy. Results from studies in which ^{13}C and ^{19}F NMR and lanthanum shift reagents (*27*) were used favor a dimer model that has carbonyl termini at the surface of the membrane and amino termini embedded in the lipid bilayer, consistent with a single-stranded head-to-head dimer. Circular dichroism spectra have been interpreted as showing that gramicidin initially binds to membranes as a double-helical dimer, then slowly converts to the single-stranded head-to-head form (*28*). It is difficult to imagine how such a transition, which would require the breaking and reforming of about 30 hydrogen bonds, would occur in a nonpolar environment.

We have determined the crystal structure of the uncomplexed, orthorhombic form of gramicidin A at 0.86 Å resolution by direct phasing procedures. The structural model has thus far been refined to a constrained least-squares weighted residual of 0.071, the precision of the structure being comparable to that routinely obtained for small peptide molecules. The conformation of the gramicidin A dimer is that of a left-handed, antiparallel, double-

stranded $\beta^{5,6}$ helix (Fig. 2). The helix is approximately 31 Å long and 4.80 Å in diameter and has a different hydrogen-bonding pattern from that reported for the cesium complex (Fig. 1). The channel dimer is held together by 28 intermolecular hydrogen bonds involving 14 of the 15 amino acids in each peptide strand. Only the Gly^2 residues in both strands are not involved in holding the dimer together. The channel is devoid of solvent molecules, but does contain three pockets where the diameter exceeds 5.25 Å. These pockets are spanned by four amino acid residues on each of the opposing chains and are bound by channel constrictions that are 4.5 Å or less. A hypothetical potassium ion placed in the center of one of these pockets (Fig. 3) was found to make four keto oxygen and four peptide nitrogen contacts averaging 2.73 Å, with no oxygen-nitrogen contact less than 2.66 Å. These distances, although reasonable for an eight-coordinate potassium, would be quite unusual if the hydrogen-bonding pattern of the helix were not disrupted, as the bond angles at the metal-binding donor atoms would be near 90 degrees. Each of these four oxygens is hydrogen bonded pairwise to the four peptide nitrogens involved in this hypothetical potassium complexation. These four hydrogen bonds would weaken if these carbonyl oxygens were bound to a potassium ion, which would allow the peptide channel to flex and twist the nitrogen atoms out of the way as the cation passes. The cesium complex would require a larger binding pocket, with bond lengths ~3.2 Å, which is consistent with the larger pore diameter afforded by the double-stranded antiparallel $\beta^{7,2}$ helical model (*16*) determined for that structure.

The averaged ϕ,ψ torsion angles (± SEM) of the peptide backbone of the uncomplexed channel form are -152 ± 9, 110 ± 22 degrees for the L residues and 101 ± 20, -142 ± 8 degrees for the D residues. These values, compared with -141, 116 and 102, -159 degrees for the idealized $\beta^{5,6}$ helix, indicate the extent to which the pitch of the helix varies from one residue to the next. The idealized helix has a twofold axis normal to the helical axis at the center of the dimer that relates the A and B peptide strands to one another. This diad axis is only approximate in the crystal structure dimer because of the asymmetry in the crystalline environment of these molecules. The average pairwise difference between the ϕ and ψ torsion angles of residues related by this axis is only 8 degrees. Moreover, the largest discrepancies ($\Delta\psi$ Val^7 = 24 degrees; $\Delta\phi$ Val^8 = -30 degrees) involve adjacent torsion angles that compensate for one another to help preserve the pseudo twofold symmetry. The root-mean-square displacement between the main chain and β-carbon atoms related by this pseudo twofold axis is 0.55 Å.

The gramicidin channels pack in a hexagonal array when viewed down the *b* axis of the crystal (Fig. 4). Nearly all of the tryptophan rings associate in pairs with the planes of the rings orthogonal to one another; only two pairs of tryptophan rings form a parallel stacking arrangement between neighboring channels. The eight tryptophan indole rings are clustered in three distinct regions around the periphery of the channel, as seen in projection in Fig. 4. The Trp^{13} indoles of the A and B chains form a cluster of two projected on the pseudo twofold axis. The remaining six tryptophans form two clusters, each containing indoles Trp^9 and Trp^{15} from one chain together with Trp^{11} of the companion strand. The clustering of indoles is also a feature in the single-stranded $\beta^{6,3}$ head-to-head dimer proposed by Urry (*21*), except that the tryptophan residues are concentrated at the two ends of that model and are not distributed along the entire length as is observed here. It is reasonable to assume that a channel with this double-helical structure could readily insert into a membrane bilayer with the long fatty acid side chains of the lipid keyed tightly into the vertical hydrophobic contours of this channel, nested between the protruding surfaces defined by the three tryptophan clusters as seen in projection. Under these circumstances the lipid molecules could act as splines, preventing the channel from helically twisting and altering its pitch, and constraining the channel in the conformation necessary for ion transport. In this regard, molecular dynamical calculations of the lipid-free dimer would not be appropriate for modeling this behavior.

The channel helices are required by the unit cell translation along

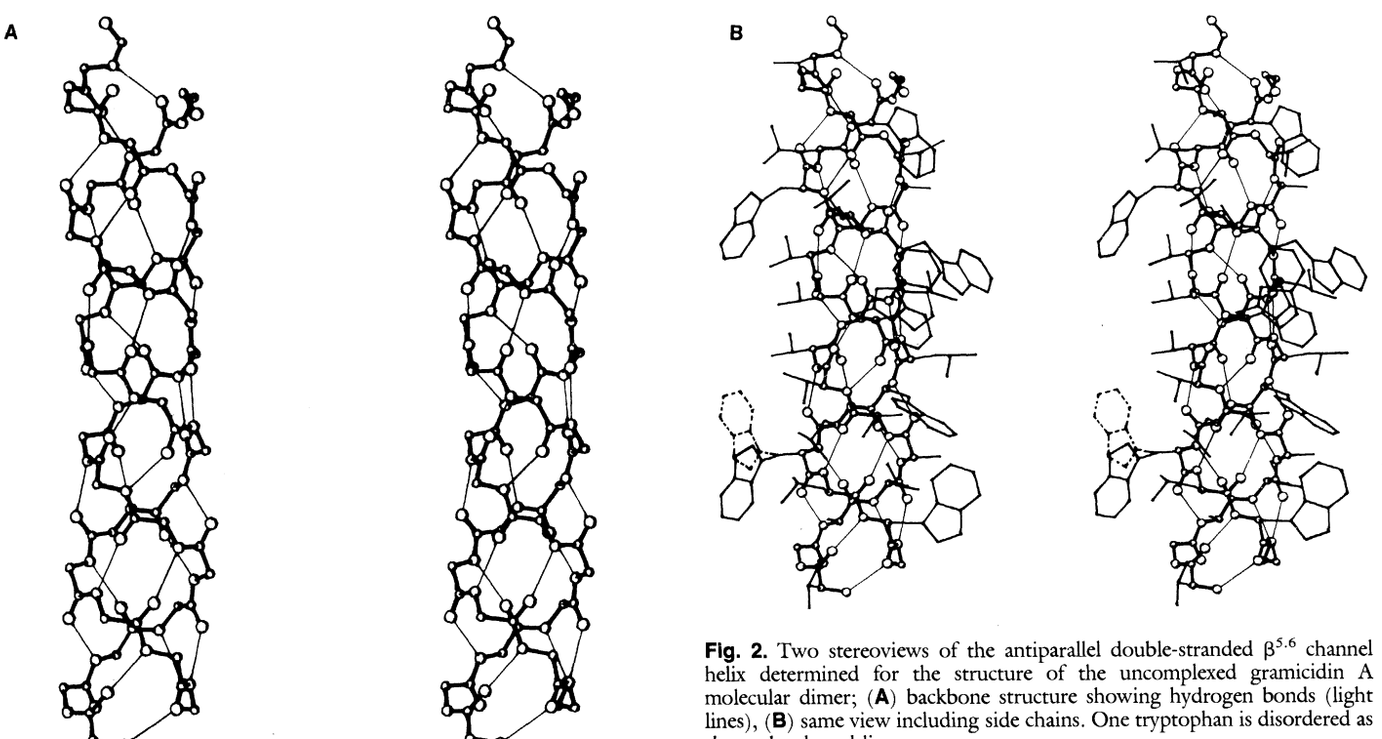

Fig. 2. Two stereoviews of the antiparallel double-stranded $\beta^{5,6}$ channel helix determined for the structure of the uncomplexed gramicidin A molecular dimer; (**A**) backbone structure showing hydrogen bonds (light lines), (**B**) same view including side chains. One tryptophan is disordered as shown by dotted lines.

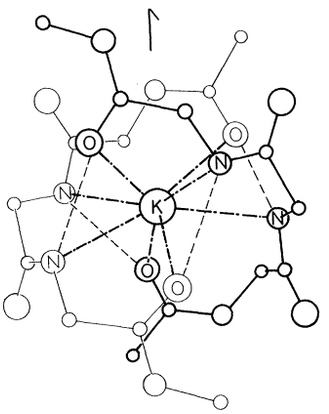

Fig. 3. The hypothetical binding site of a potassium ion within one of the preformed channel pockets observed for the uncomplexed form of the channel dimer. The peptide hydrogen bonds involved at the binding site are indicated by dotted lines. These bonds would be weakened if a cation were in the site, allowing the peptide nitrogen atoms to swing away from the ion so that the helix conformation may flex to permit the ion to pass down the channel. The arrow indicates the direction of the helix.

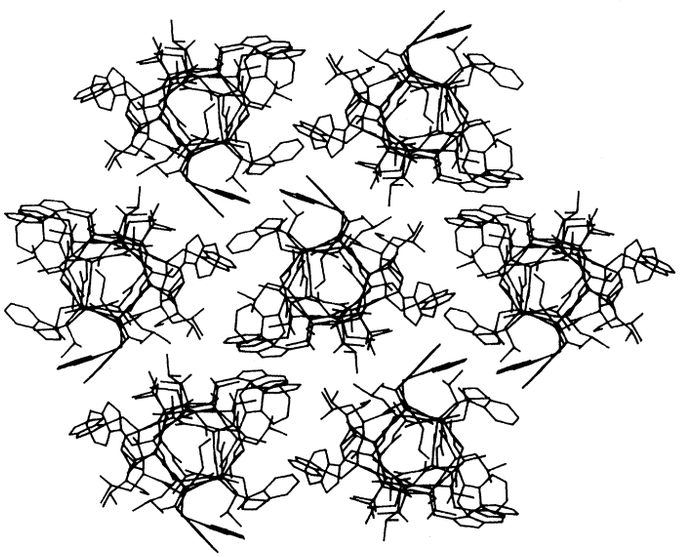

Fig. 4. A perspective of the hexagonal packing arrangement of the gramicidin channels. Solvent molecules have been excluded for clarity.

the *b* axis of the crystal to stack upon one another perpendicular to the hexagonal net. Only one direct interchannel hydrogen bond is seen to link these helices in an end-to-end manner, that being between an A-chain Gly^2 carbonyl oxygen and a B-chain Gly^2 peptide nitrogen. All of the other end-to-end hydrogen bonds between the channels involve the ethanol solvent structure. It seems unlikely that a pair of dimers would associate in this manner across a membrane if polar solvent molecules were required at the center of the lipid bilayer to stabilize the end-to-end association.

The uncomplexed form of the gramicidin A ion channel crystallizes as an empty pore, in contrast to the CsCl complex, which contains two Cs^+ and three Cl^- ions in the channel. The uncomplexed form, although empty, is predisposed with a number of sufficiently large potential binding sites in the channel to accommodate an unsolvated ion. The presence of lipid stacking against the sides of the channel may be an important factor for enforcing ion specificity by indirectly limiting the size of these binding sites. The selectivity of these channels for monovalent cations may be a consequence of the difficulty of stripping solvent molecules from polyvalent cations, such that they are excluded from entering these channels. These binding sites can be induced to travel the length of the channel by a cooperative flexing of the peptide backbone, but the mechanism by which these channels are induced to flex in a peristaltic manner remains to be determined.

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29. The refined atomic coordinates of the uncomplexed gramicidin A structure have been deposited with the Protein Data Bank, Brookhaven National Laboratory, and are available in machine readable form from the Protein Data Bank at Brookhaven or one of the affiliated centers at Melbourne or Osaka.

30. I thank M. G. Erman and W. A. Pangborn for their technical assistance and S. A. Potter, R. H. Blessing, and G. D. Smith for their computational and graphics expertise. W. L. Duax has been responsible for initiating and directing this research project, which was supported in part by PHS grants GM32812 and HL32303.

28 March 1988; accepted 18 May 1988