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ZERVAMICINS, A STRUCTURALLY CHARACTERISED PEPTIDE MODEL FOR MEMBRANE ION CHANNELS

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Voltage dependent membrane channels are formed by the zervamicins, a group of α-aminoisobutyric acid containing peptides. The role of polar residues like Thr, Gln and Hyp in promoting helical bundle formation is established by dramatically reduced channel lifetimes for a synthetic apolar analog. Crystal structures of Leu[1]-zervamicin reveal association of bent helices. Polar contacts between convex faces result in an 'hour glass' like arrangement of an aqueous channel with a central constriction. The structure suggests that gating mechanisms may involve movement of the Gln[11] carboxamide group. Gln[3] may play a role in modulating the size of the channel mouth.

Voltage gated protein channels across cellular membranes can exist in closed or open states. In the former, the pore is sealed, whereas in the latter a continuous aqueous path is available for ions to traverse the hydrocarbon barrier. The molecular events underlying gating processes, which allow switching between the two states are unclear (1,2). Peptide models which mimic protein channels in artificial bilayers, provide a means of investigating structural events at high resolution (3-5). Several fungal peptides (6), of which alamethicin is the best example (7-11), impart excitability properties to lipid membranes, offering an attractive system for examining structure-function relationships. The zervamicins are a related class of peptides (12,13), rich in α-aminoisobutyric acid (Aib) a feature

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that constrains them to adopt 3_{10} or α-helical conformations (14,15) which are too narrow to allow passage of ions through the interior of a single helix (16). In addition, the zervamicins contain several polar, hydrogen bonding residues (Thr, Gln, Hyp) interspersed through the sequence, in contrast to alamethicin. We describe voltage dependent channel forming activity of the zervamicins, demonstrate the role of polar functions in stabilising channel clusters and relate recently determined crystal structures to channel gating and dynamics.

MATERIALS AND METHODS

Zervamicins IIB (ZIIB), the major polypeptide component and Leu^1-zervamicin (ZL) a minor peptide component, were isolated by reverse phase HPLC, from a crude isolate of zervamicin obtained from cultures of Emericellopsis salmosynnemata (17). Purified fractions were shown to be homogeneous by analytical HPLC on a C_{18} (5 μ) reverse phase column. The synthetic apolar analog ZA16 was synthesised by conventional solution phase procedures, purified by silica gel column chromatrography and crystallised from methanol-water (18). All peptides were completely characterised by one and two dimensional NMR at 270 MHz. Crystal structures have been determined for peptides ZA16 (19) and ZL (20).

Single channel conductances induced in diphytanoylphosphatidylcholine bilayers were measured as described earlier (21). The planar bilayers were formed across the tips of glass microelectrodes (diameter ca. 1 μm) (22,23). The electrolyte used was 0.5 M KCl.

RESULTS AND DISCUSSION

Representation of the zervamicin IIA(B) (ZIIA(B)) sequence (Fig. 1) on a helical wheel diagram reveals a clear segregation of most polar residues on the same helix face. Intermolecular hydrogen bonding has been proposed as a means of stabilising amphipathic helical bundles in lipid bilayers (6,9,16). Chemical synthesis of an apolar zervamicin analog (ZA16, Fig. 1), was effected in order to replace hydrogen bonding groups with apolar equivalents (Hyp ->Pro, Thr->Val, Gln->Ala, Phol->PheOMe) (18). Discrete channel opening events for ZIIB channels are observed in lipid bilayers, with channel formation being voltage dependent. Switching between discrete conductance levels is observable (Fig. 2B). Conductance induced by alamethicin channels (7,10,11), under similar conditions are compared in Fig. 2A. Kinetics of switching between conductance levels are approximately an order of magnitude faster for ZIIB as compared to alamethicin. Channel activity is also detectable for the synthetic, apolar analog ZA16, but the openings are smaller and significantly shorter lived than for ZIIA (Fig. 2D). Furthermore, only a relatively small number (2 to 4) of conductance levels can be observed with ZA16, whereas with ZIIB upto 15 conductance levels are seen (21). Clearly, this finding supports a model that ascribes a role to polar residues in stabilising functional channels by stitching

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                    5                   10                  15
ZIIA :  Ac-Trp-Ile-Gln-Aib-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol
(B)                 (Iva)
ZL   :  Ac-Leu-Ile-Gln-Iva-Ile-Thr-Aib-Leu-Aib-Hyp-Gln-Aib-Hyp-Aib-Pro-Phol

ZA16 :  Ac-Trp-Ile-Ala-Aib-Ile-Val-Aib-Leu-Aib-Pro-Ala-Aib-Pro-Aib-Pro-Phe-OMe
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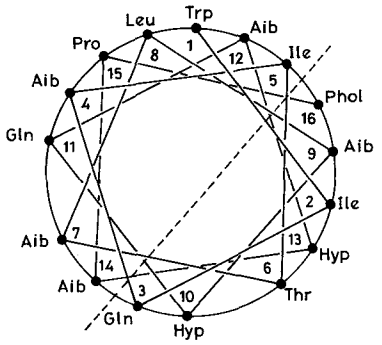
(A)



(B)

Figure 1. (A) Sequences of zervamicin IIA(B) (ZIIA(B)) (12), Leu^1-zervamicin (ZL)(17,20) and a synthetic apolar analog (ZA16) (17, 18). (B) Helical wheel diagram of the ZIIA sequence. The broken line demarcates the polar (left) and non-polar (right) faces. Gln^{11} which is positioned on the non-polar face adopts an unusual sidechain conformation, bending back to position the carboxamide function on the polar face in the ZL structure (see Figure 4).

together helical bundles (9,16). In particular, it suggests that polar residues are responsible for stabilisation of the higher order helical bundles made up of > 6 monomers which give rise to the higher single channel conductance levels observed with ZIIB (and with ZL and alamethicin) but not with ZA16.

A minor component of the microheterogeneous fungal polypeptide mixture, Leu^1-zervamicin (ZL, Fig. 1) containing a Trp^1->Leu^1 replacement has been isolated by reverse phase HPLC (17) and crystallised in four distinct crystal forms (20). ZL also forms channels, with a switching rate somewhat faster than for ZIIB (Fig. 2C). The crystal structures of the four ZL forms have been determined at resolutions ranging from 0.93 Å to 1.3 Å, with R values of 10.1 and 12.0% for the two refined structures. In all four cases a curved helical backbone, very similar to that observed for the analog ZA16 (19), is seen (Fig.3). In ZA16 the molecules are arranged front to back, with convex and concave faces fitted snugly to exclude solvent (19). In contrast ZL crystals show a more complex packing, with back to back arrangements resulting in aqueous channels running through the crystal, where the convex faces approach one another. The O atoms

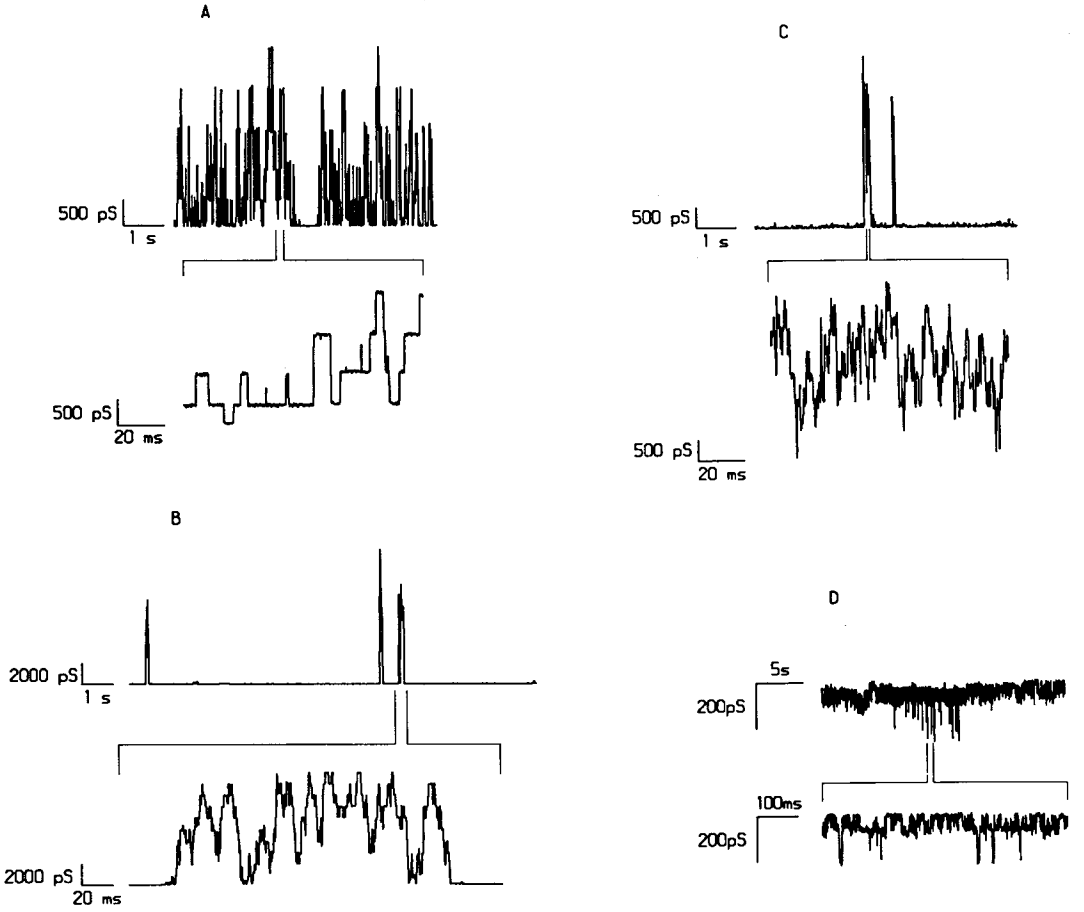


Figure 2. Single channel conductances induced in diphytanoylphosphatidylcholine bilayers by (A) alamethicin (B) zervamicin IIB (ZIIB) (C) Leu^1-zervamicin (ZL) and (D) synthetic analog ZA16. Membrane potentials refer to the cis compartment, the trans compartment being connected to ground. In (A), (B) and (C) the peptide was added to the cis compartment, at concentrations of 0.26, 1.1 and 0.57 μM, respectively. In (D) ZA16 was included in the bilayer forming solution (in pentane), at a peptide:lipid molar ratio of 1:100. The membrane potentials used in the recordings shown were (A) +125 mV, (B) +175 mV, (C) +100 mV and (D)-150 mV. For each peptide, a long timebase (1 to 5 s) was used for the upper trace and an expanded timebase (20 100 ms) for the lower trace.

(hatched) and N atoms (striped) of the side chains of polar residues Gln^3, Thr^6, Hyp^{10}, Hyp^{13}, as well as several exposed carbonyl oxygens and the hydroxyl of $Phol^{16}$ occur on the surface of the convex face and participate in hydrogen bonds with water molecules in the interrupted channel. The Gln^{11} residue does not obey the n+3 or n+4 distribution of polar residues as shown in a helical wheel (Fig. 1B); however, the long side-chain in Gln^{11} (shaded) makes a reverse turn and wraps around the helix backbone so that the dipolar carboxamide group augments the polar face. The aqueous

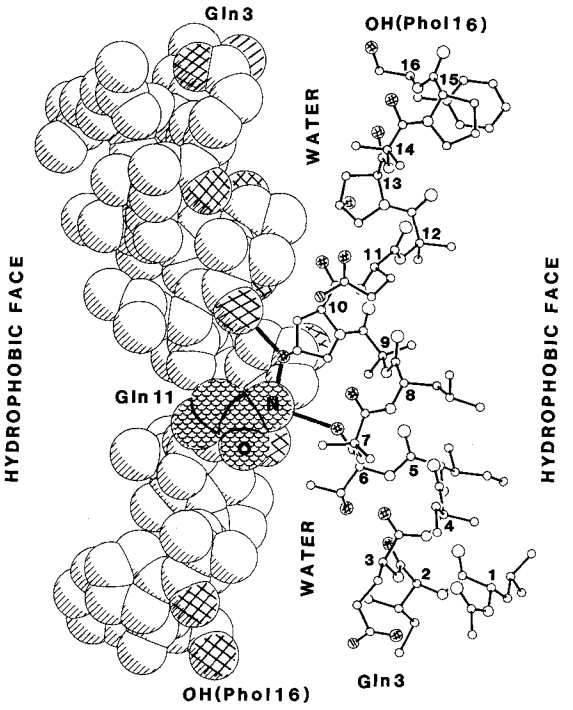


Figure 3. Interrupted water channel formed in the crystal of leucine-zervamicin by the antiparallel association of polar faces of the bent helices. The conformation of peptide ($P2_1$, a = 23.068 Å, b = 9.162 Å, c = 26.727 Å, β=108.69°, R = 10.1%, resolution 0.93 A) is shown in a ball and stick representation on the right and a space-filling representation on the left. A very similar channel is formed in four different polymorphs (space groups $P2_1$ and $P2_12_12_1$) in which the helix backbone has a different amount of bend (30°-45°) and consequently the water content in the channel increases with increasing value of the bend (20). Pairs of molecules are linked by hydrogen bonds shown by heavy lines: N^{ϵ} H(Gln^{11})...O=C(Thr^{6}); N^{ϵ} H(Gln^{11})...O^{δ}(Hyp^{10}); and OH(Hyp^{10})...O=C(Aib^{7}).

channels are discontinuous because of a constriction, involving close approach of Hyp^{10} and Gln^{11} sidechains on adjacent antiparallel molecules, with the latter adopting an unusual gauche⁻-gauche⁻ conformation ($\chi^1 \sim -52°$, $\chi^2 \sim -52°$ in the four structures). A conceivable component of the gating mechanism would then involve movement of the dipolar carboxamide group under the influence of an applied potential to open a continuous channel. The role of Pro residues in kinking helices (24,25) and the possibility of funnel shaped channel aggregates, with a central constriction have been considered recently (26). In the Leu^1-zervamicin structure, Hyp^{10} serves to bend the helix and also provides, via the γ-OH, an intermolecular hydrogen bonding site for closing the channel. A recent report suggests that replacement of Pro^{14} in melittin by Ala, straightens the helix and results in diminished ability to induce voltage dependent

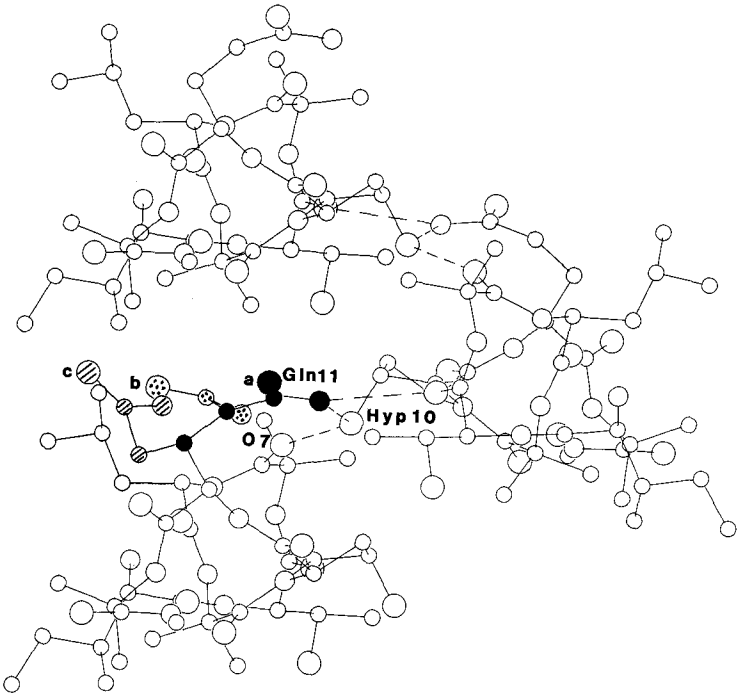


Figure 4. A view into the restricted portion of the channel. The view is perpendicular to that of Figure 3 showing only the middle segments of three adjacent helices, i.e.,the two antiparallel molecules of Figure 3 and the molecule in front of the left molecule. The Gln^{11} side chain, shown in black (a) as it occurs in the crystal, closes the channel. The three hydrogen bonds indicated by dotted lines are the same as those shown in Figure 3. Hypothetically, upon application of voltage, the Gln^{11} side chain can rotate about the $C^{\beta} - C^{\gamma}$ bond to assume a position such as (b), dotted atoms, or alternatively rotate about the $C^{\alpha} - C^{\beta}$ bond to assume a position such as (c), striped atoms, which would open the channel.

conductance in lipid bilayers. (27). The possibility of a funnel shaped channel, with a role for a central Gln in gating has been considered in a model for alamethicin, although the aggregation pattern in crystals did not reveal such an arrangement (8). The crystal structure of Leu^{1}-zervamicin does indeed suggest the possibility of accomodating such a movement. A view of a putative channel bounded by three helices in the open and closed states is shown in Fig. 4. A rotation of 120° about the $C^{\alpha} - C^{\beta}$ bond appears to be sufficient to create a continuous pore (Fig. 4c). Other positions of the Gln^{11} sidechain obtained by rotation about the $C^{\beta} - C^{\gamma}$ bond (Fig. 4b) also serve to open the channel. In these positions van der Waals contacts with neighbouring atoms are within allowed values confirming that sufficient space is present in the crystalline arrangement for sidechain movement. Detailed models for channel opening events must undoub-

tedly, consider cooperative motions of other proximal residues, notably Thr^6. The crystalline trimeric aggregate could serve as the starting point for molecular dynamics simulations of channel gating events. Gln^3 also plays a major role in constructing the channel mouth and may influence selectivity and sieving properties. Fig. 3 illustrates the positioning of this residue at the mouth. In the four crystal forms the mobility of the Gln^3 sidechain results in the separation, between the alcohol OH group of the C-terminal phenylalaninol and N of Gln^3 of an adjacent molecule, that varies from 3.7 Å to > 8 Å. This motion dramatically alters the size of the channel at the mouth. The zervamicins promise to be a good system for delineating molecular details of channel events at high resolution, providing a basis for understanding the more complex protein channels which still await three-dimensional characterisation (28). The observed `hour-glass' like association of the convex polar faces of the bent amphipathic helix of zervamicin, with a centrally located channel constriction may indeed be an attractive model for the transmembrane pore lining of protein channels (29).

Acknowledgments

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