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Topical Review

Model Ion Channels: Gramicidin and Alamethicin

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I. Introduction

Ion channels control the flux of ions across lipid bilayers in an extremely sensitive and specific manner. From a description of selective permeability (Bernstein, 1912), made more explicit by Hodgkin and Huxley (1952), the concept of specific ionic pathways in membranes has evolved to the point where selectivity can now be discussed in terms of the three-dimensional fold of the primary sequence of ion channel proteins (e.g., Guy, 1988). The elucidation of ion channel function has now landed squarely in the realm of structural biology, and the problem may be viewed in terms of the more general one of membrane protein structure. Membrane proteins have been difficult to characterize structurally, primarily because the requirement for maintaining a membrane environment hinders purification and crystallization and complicates spectroscopic measurements. Furthermore, ion channels are particularly complex, multisubunit membrane proteins so that, although the whole range of techniques from X-ray crystallography and NMR spectroscopy to site-directed mutagenesis and conductance measurements are being applied, complete three-dimensional structures of these proteins are unlikely to be easily obtained. One approach which has been successful in elucidating the structure and function of soluble proteins is the study of small protein (peptide) analogues. Great simplifications in obtaining material, in manipulation of this material, and in the interpretation of spectroscopic and physicochemical measurements permit an unparalleled description of structure at a molecular level which in many cases can be related to structure and function in full-size proteins.

This article describes two small membrane-associated peptides—gramicidin and alamethicin—which have received considerable attention as models for ion channel proteins. Despite their small size (16 and 20 residues, respectively), they display a rich variety of channel behavior including: ion selectivity, voltage dependence, subconductance states and blocking, and modulation of channel properties by the lipid membrane. As there are distinct differences between the two peptides, the manner in which they are useful as ion-channel protein models is different. On the whole, the conformation of the conducting channel is much better established for gramicidin than for alamethicin. This detailed knowledge of gramicidin structure has permitted fundamental studies on ion channel dynamics and the physical origins of ion selectivity, subconductance states, and other channel properties. On the other hand, alamethicin forms channels which may be more closely related to full-size protein channels and offers insight into the physical origins of voltage-dependent gating. We shall focus here on conformational aspects of these best-characterized ion channels as they relate to function; we suggest throughout how this information relates to larger ion channels and to membrane proteins in general as well as noting open questions in the model systems.

II. Gramicidin

Gramicidin is a linear peptide synthesized by *Bacillus brevis* which is composed of alternating D- and L-amino acid residues.[1] It acts as an antibiotic, particularly against gram-positive bacteria (hence the

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[1] Gramicidin S, a cyclic peptide, is also synthesized by *B. brevis*. Its activity is quite different from the linear gramicidins, however, and will not be discussed here. For a review of gramicidin S structure and activity *see* Izumiya et al. (1979).

name), and finds commercial application as a topical bacteriostatic agent. The primary sequence of gramicidin A is shown below (Sarges & Witkop, 1965*a*):

HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$.

The N-terminus is formylated and there is an ethanolamine group at the C-terminus so that the peptide has no charged sites. Natural sequence variants occur at position 11 with substitution of Trp by Phe (gramicidin B) or Tyr (gramicidin C); the Val in the first position may also be substituted by Ile (Sarges & Witkop, 1965*c*,*d*). Often experiments are performed with the natural mixture (termed gramicidin D or A′) which is predominantly (80%) gramicidin A. Distinct effects of these sequence variations have been found, however (*vide infra*), and, depending on the experiment, separation of isomers or separate synthesis may be required. Efficient chromatographic procedures have been developed for the separation of gramicidins A, B and C from the natural mixture (e.g. flash chromatography (Stankovic, Delfino & Schreiber, 1990), high-performance liquid chromatography (HPLC) (Fields et al., 1989)) and methods of *de novo* synthesis and partial synthesis have been described (e.g.: Sarges & Witkop, 1965*b*; Prasad et al., 1982; Trudelle et al., 1987; Fields et al., 1989). Since gramicidin is synthesized nonribosomally in vivo (Kleinkauf & von Doehren, 1987) and contains D-amino acids not genetically coded for, site-directed mutagenesis using recombinant DNA methods is not possible. Chemical synthesis, however, permits the incorporation of amino acids and analogues other than the 20 specified by the genetic code for structure-function studies.

Gramicidin forms channels in lipid membranes which are selective for monovalent cations (for reviews *see* Finkelstein & Andersen, 1981; Andersen, 1984; Hladky & Haydon, 1984; Hladky, 1987). Water and protons also pass through the channel, but divalent cations and anions are essentially impermeant (Myers & Haydon, 1972; Urban, Hladky & Haydon, 1978). The macroscopic current-voltage (*I-V*) curve for gramicidin is approximately linear and nonrectifying; there is some effect of voltage on channel properties, but there is no voltage at which the channel is closed and the voltage effects are not so pronounced as, for instance, with alamethicin (*vide infra*) or with voltage-gated sodium or potassium channels (e.g., Andersen, 1983). Single gramicidin channels behave in an ohmic manner with a conductance of about 5.8 pS in 100 mM NaCl at 20°C (Hladky & Haydon, 1972*a*) (Fig. 1). This is similar to the conductances of sodium channels in biological systems which typically fall in the range of 4 to 18 pS (Hille, 1984). Gramicidin single-channel conductance events have lifetimes on the order of a second (compared to several milliseconds for sodium channels (Hille, 1984)). The current through gramicidin channels is observed to saturate at high ion concentrations (e.g., Hladky, 1988) and also when very high voltages are applied at lower ion concentrations (e.g., Andersen, 1983). The phenomenon of current saturation has also been observed with a variety of channels in biological systems (Hille, 1984).

A. Gramicidin Assumes Several Distinct Conformations in Solution

The presence of alternating D- and L-amino acid residues in gramicidin produces some secondary structures not generally encountered with peptides and proteins which contain L-amino acids exclusively. Thus, while the specific conformations deduced for gramicidin are unlikely to be encountered in protein ion channels, all of the factors besides those contained in the primary sequence which determine the conformation and dynamics (and thereby the function) of gramicidin will also determine conformation and dynamics in protein systems. Furthermore, the methods that have been used to elucidate gramicidin structure will also find use in protein systems.

The amino-acid sequence of gramicidin is very hydrophobic and results in the peptide being virtually insoluble in water. When dissolved in a 1 : 1 mixture of dimethylsulphoxide/acetone, a polar organic solvent, gramicidin is monomeric and appears to have no persistent periodic structure (Roux, Bruschweiler & Ernst, 1990). In more nonpolar solvents (e.g., alcohols) gramicidin has been shown to exist as a mixture of dimeric forms in equilibrium with monomers (Veatch & Blout, 1974). The relative populations of dimers and monomers in solution at equilibrium are affected by solvent type, temperature and peptide concentration. The rate of conversion between forms is also solvent and temperature dependent; the process may take from minutes (in methanol) to weeks (in dioxane) (Veatch & Blout, 1974; Braco et al., 1986). These dimeric forms have been studied extensively using a variety of techniques (e.g., Veatch, Fossel & Blout, 1974) of which perhaps multidimensional solution-state NMR has been the most effective (Bystrov & Arseniev, 1988; Bystrov et al., 1990). All of these forms appear to be double helices of the type originally proposed by

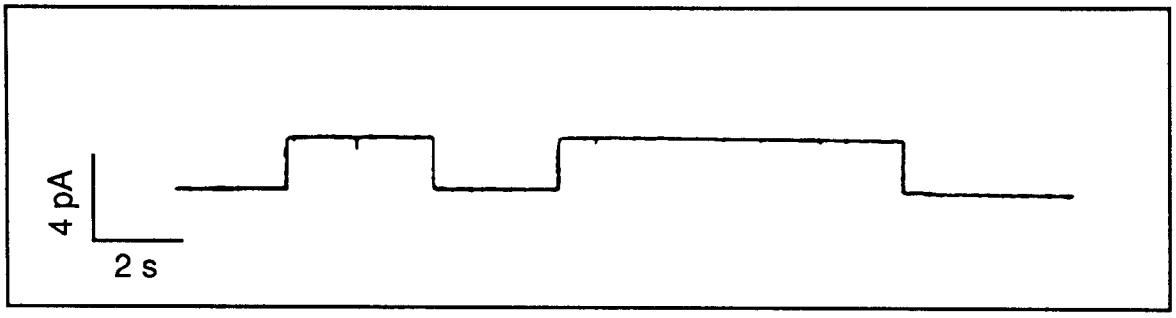

Fig. 1. Gramicidin single-channel events occurring in a glycerol monooleate/hexadecane membrane (100 mV applied potential; 1 M NaCl solutions). (After Busath et al., (1987) with permission).

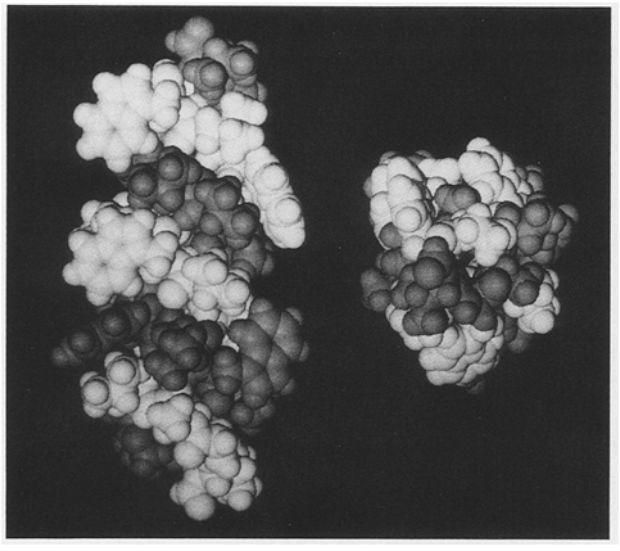

Fig. 2. Two views of the $\beta^{5.6}$-uncomplexed, left-handed, antiparallel, double helix crystal structure of gramicidin obtained by Langs (1988). Each gramicidin molecule is shaded differently (light grey and dark grey) to emphasize the handedness and the intertwined nature of the structure.

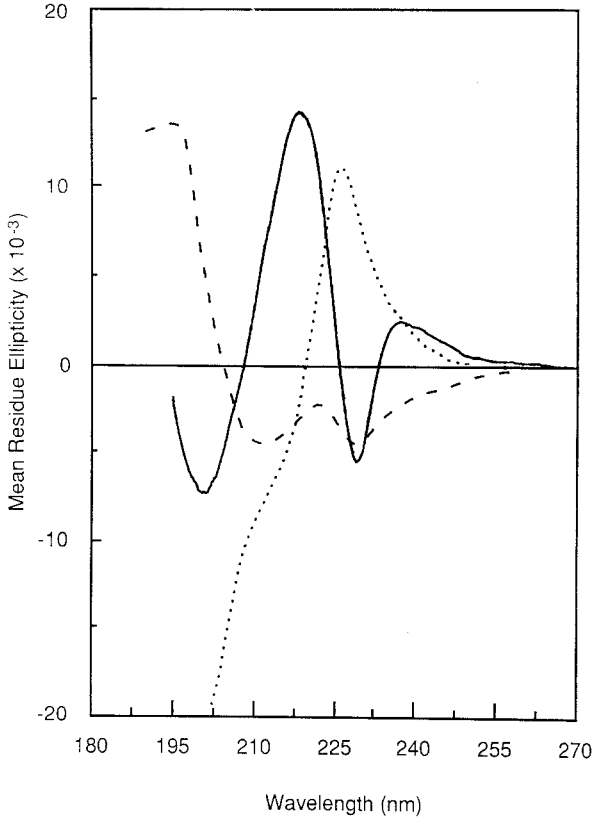

Fig. 3. Circular dichroism (CD) spectra of gramicidin in solution and membrane bound: in methanol (---); in methanol with the addition of Cs^+ ions (····); in dimyristoyl-PC vesicles (—). The addition of Cs^+ ions to membrane-bound gramicidin causes no change to the CD spectrum.

Veatch et al. (1974) in which two gramicidin peptide chains are interwound. They vary in handedness and in number of residues per turn, and parallel and antiparallel arrangements coexist. The most abundant species (but not the majority on a molar basis) is an antiparallel, left-handed $\beta^{5.6}$ double helical dimer, which is also the form found in the crystal structure (Langs, 1988; Langs et al., 1991)[2]. (Fig. 2)

The addition of ions to solutions of gramicidin causes conformational changes which can be observed by circular dichroism (CD) spectroscopy (Wallace, 1984) (Fig. 3) and NMR (Arseniev, Barsukov & Bystrov, 1985; Bystrov & Arseniev, 1988; Bystrov et al., 1990). The crystal structure of an ion-bound form of gramicidin has been solved (the CsCl complex) (Wallace & Ravikumar, 1988; Wallace,

[2] The β derives from the similarity of the hydrogen bonding pattern of the dimer to that of a β-pleated sheet. Because of the alternation of D- and L-residues in gramicidin, all side-chains are on one side in the β-sheet conformation. The sheet may then be rolled into a helix which contains a central cavity. The superscript 5.6 refers to the number of residues per turn.

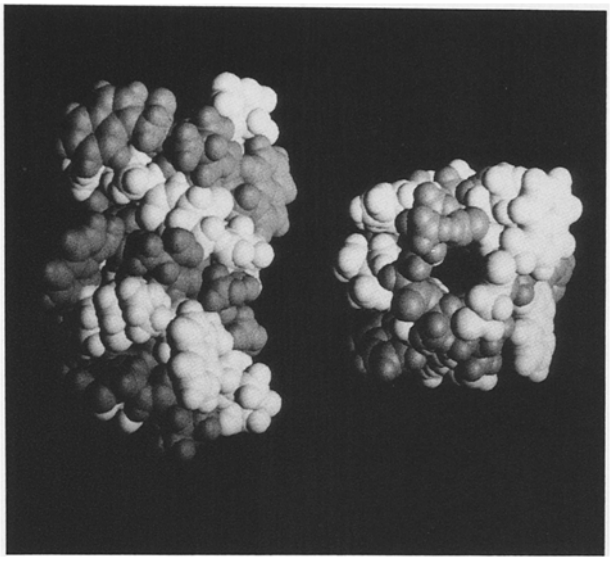

Fig. 4. Two views of the gramicidin-CsCl complex crystal structure determined by Wallace and Ravikumar (1988). Although also an antiparallel, left-handed, double helix, the CsCl form is shorter and wider than the uncomplexed form.

1990, Wallace, Hendrickson & Ravikumar, 1990). This is also a left-handed, antiparallel double helix although the number of residues per turn is 6.4 and the overall length is 26 Å compared to 31 Å for the ion-free form; the ions are located in a central cavity formed by the double helix (Fig. 4).

If a gramicidin double helix spanned a lipid membrane so that the central cavity in the helix could act as an ion channel, one would expect both N- and C-termini of the gramicidin molecule to be at the membrane surface. Nuclear magnetic resonance studies which examined the effect of paramagnetic probes on isotopically-labeled gramicidin molecules indicated, however, that for the predominant conformation of gramicidin found in lipid vesicles only the C-terminus was at the membrane surface while the N-terminus was buried in the membrane interior (Weinstein et al., 1979, 1980, 1985). Furthermore, the CD spectrum of gramicidin in lipid vesicles was found to be significantly different from the spectrum in solution and could not be represented by any combination of the spectra of the different double-helical dimers or monomeric solution forms (Masotti, Spisni & Urry, 1980; Wallace, Veatch & Blout, 1981). In addition, while the CD spectrum of the solution form changes upon the addition of ions, that of the membrane-bound form is relatively unaffected (Wallace, 1984, 1990). (Fig. 3)

The conformational behavior of gramicidin in organic solution was originally investigated because the particulate and anisotropic nature of membrane systems complicates the use of many spectroscopic techniques. In addition, it was thought that organic solvents might mimic the hydrocarbon interior of lipid membranes. Indeed, the gramicidin structures obtained from crystals grown in these solvents appear quite reasonable as ion channels. However, there are various compelling reasons for believing these *do not* correspond to the structure of the predominant active membrane-bound channel as the CD and NMR studies described indicate and as will be detailed below. This finding emphasizes the critical effect of environment on structure and the difficulty in using simple organic solvents as membrane mimetics. It does not, however, imply that studies in such solvents are without value; indeed, they have been indispensable for our current understanding of gramicidin structure and function. Instead, these results stress the rather special nature of the lipid bilayer with its juxtaposition of hydrophobic and hydrophilic groups of fairly well-defined dimension. The strong effect of environment on both structure and dynamics and therefore, presumably, on function is an important general finding.

B. The Predominant Conformation in Membranes is a $\beta^{6.3}$ Helical Dimer

Clearly the structure of the membrane-bound form of gramicidin is of primary interest with regards to ion-channel formation, although it has been suggested that double-helix forms may comprise a minor fraction of especially long-lived ion channels (e.g., Durkin & Andersen, 1987; Koeppe et al., 1991). In addition, the structure(s) in organic solutions results in a significant solvent history dependence of the membrane-bound form which has been recognized only recently (Killian et al., 1988; LoGrasso, Moll & Cross, 1988; Bano, Braco & Abad, 1989, 1991). This solvent history dependence is particularly important for studies employing relatively high gramicidin concentrations and high peptide-lipid ratios but appears less important for conductance studies (black lipid membrane (BLM) and patch pipette), which employ much lower peptide concentrations (Sawyer, Koeppe & Andersen, 1990).

Early model-building studies (Urry, 1971; Urry et al., 1971) and energy calculations (Ramachandran & Chandrasekaran, 1972) suggested a single-helix structure for the gramicidin channel. In this model each gramicidin monomer adopts a $\beta^{6.3}$ (previously called $\pi^{6}_{L,D}$) helical conformation, resulting in a structure which spans about half the bilayer and contains a central cavity approximately large enough

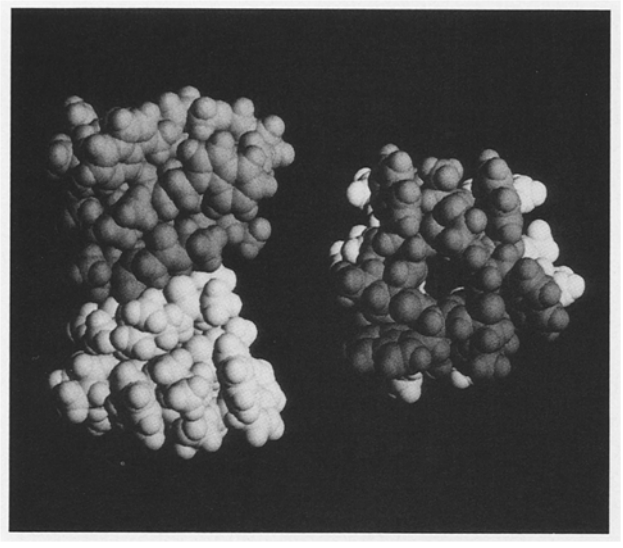

Fig. 5. Two views of the helical dimer model of the gramicidin channel. The channel is formed by two right-handed $\beta^{6.3}$ helices joined at their N-termini. The coordinates used to construct this model are based on the NMR data of Arseniev et al. (1985) for gramicidin in SDS micelles.

to pass monovalent cations (Fig. 5) The central cavity is lined by the groups of the peptide backbone, and all side-chains are on the outside of the helix in contact with the lipid membrane. The active channel was proposed to result from the coming together of two $\beta^{6.3}$ helical monomers, one from each half of the bilayer leaflet. A head-to-head arrangement (both N-termini at the center of the bilayer) would be stabilized by six hydrogen bonds and could form a continuous water-filled channel. This model for the structure of the gramicidin channel in membranes (referred to as the helical dimer model) appears to be correct in its essential features.

That two monomers are needed for a functional channel was deduced from a simultaneous measurement of gramicidin concentration (using a fluorescent gramicidin C analogue) and gramicidin conductance using a BLM apparatus (Veatch et al., 1975). A dimeric channel was also implied by the observation that the rate of channel appearance depended on the square of the total gramicidin concentration although this type of measurement is complicated by the insolubility of gramicidin in water (Hladky & Haydon, 1972*b*). Gramicidin analogues that display different single channel conductances can be added to the same lipid bilayer and hybrid channels are observed alongside pure channels of each analogue at a frequency predicted by the binomial distribution (Veatch & Stryer, 1977). If more than two monomers were required for an active channel then such mixing experiments would be expected to result in a wider variety of hybrid channels than that observed. Open-channel noise analysis and voltage-jump current relaxation experiments were also interpreted as evidence for a dimeric form of the active channel (Kolb & Bamberg, 1977).

A head-to-head association is supported by the observation that chemical modifications at the N-terminus (e.g. des-formyl (Morrow, Veatch & Stryer, 1979); acetyl (Szabo & Urry, 1979); pyromellityl (Bamberg, Apell & Alpes, 1977)) drastically affect channel formation, whereas similar modifications at the C-terminus do not. Measurements of fluorescence energy transfer from gramicidin Trp residues to acceptors covalently attached at different depths in the bilayer also support the head-to-head topology (Boni, Connolly & Kleinfeld, 1986). Covalent attachment of gramicidin N-termini through a malonyl linkage (Bamberg & Janko, 1977) or a tartaric acid linkage (Stankovic et al., 1989) results in functional channels, in many ways similar to native gramicidin channels although with much longer lifetimes. This combination of studies has essentially ruled out a double helical conformation as being the predominant form of the active channel in membranes.

Results from voltage- and temperature-jump experiments with the covalently-linked malonyl dimer have suggested that higher order (i.e., tetramer and above) aggregate formation may be required for channel activity (Stark, Strassle & Takacz, 1986). Experiments with other covalent gramicidin dimers (which were designed to be photomodulated by the incorporation of a photo-isomerizable crosslink) also show indications of an interaction of dimers (Stankovic, Heinemann & Schreiber, 1991). Autocorrelation analysis of gramicidin channel activity at high peptide concentrations has indicated that channels are affected by their neighbors (Kolb & Bamberg, 1977). Furthermore, in certain lipid systems (e.g., lyso-phosphatidylcholine (lyso-PC)) there is strong evidence for gramicidin supramolecular organization, albeit at rather high peptide concentrations (Spisni et al., 1983; Macdonald & Seelig, 1988). In other lipid systems, however, gramicidin dimers appear to move independently (Macdonald & Seelig, 1988). A head-to-head $\beta^{6.3}$ helical dimer appears to be a minimum requirement for channel activity, and the ensuing discussion of gramicidin structure and function is based on this model. Whether the occurrence of higher order aggregates is peculiar to the covalent dimer case or is of general importance remains to be clarified.

Veatch et al. (1975) were able to estimate the dimerization constant of gramicidin A in dioleoyl-PC BLMs to be about 2×10^{13} mol^{-1} cm^{-2}. This means that at the peptide concentrations generally

employed for spectroscopic experiments in lipid vesicles the fraction of monomers is negligible. Channel formation by gramicidin in lipid vesicles has been observed (e.g.: Cohen, 1982; Loew et al., 1985; Jyothi, Mitra & Krishnamoorthy, 1990), but conductance data are not so easily interpreted as with BLM systems and nonspecific leakage may occur when the peptide concentration is high (Classen et al., 1987). Despite the difficulty in simultaneously demonstrating ion-channel activity and performing spectroscopic measurements, the observation that the far-UV CD spectrum of membrane-bound gramicidin is not sensitive to peptide-lipid ratio (Wallace, 1986) (at least for those ratios experimentally accessible) suggests that the conformation of gramicidin is approximately the same for conductance studies (low peptide-lipid ratios) and spectroscopic studies (high peptide-lipid ratios). The CD-spectrum of gramicidin in lipid vesicles is often used as a 'signature' evidencing the presence of active $\beta^{6.3}$ helical dimers (Fig. 3). The membrane-bound monomer form of gramicidin has not been studied directly, although there is indirect evidence that more than one monomer conformation may exist (Ring, 1986; Killian et al., 1988; Sandblom & Theander, 1991). CD spectra of membrane-bound, N-terminally modified gramicidin and of gramicidin in vesicles made from long-chain lipids have been suggested to reflect the conformation(s) of monomeric peptide species (Wallace et al., 1981; Killian et al., 1988).

The membrane-bound gramicidin channel structure has been investigated using infrared spectroscopy which confirms the β-type hydrogen bonding pattern (Naik & Krimm, 1986). Solid-state NMR studies in multibilayers using isotopically labeled (^{15}N, ^{2}H, ^{13}C) gramicidin have confirmed the $\beta^{6.3}$ helical fold oriented with the helix axis perpendicular to the membrane surface (Smith et al., 1989; Hing et al., 1990*a*, *b*; Nicholson, Teng & Cross, 1991; Teng, Nicholson & Cross, 1991). The $\beta^{6.3}$ helix was found to be right-handed in dimyristoyl-PC multibilayers (Nicholson & Cross, 1989; Nicholson et al., 1991; Prosser et al., 1991; Teng et al., 1991). Two-dimensional NMR studies of gramicidin incorporated into sodium dodecyl sulphate micelles (where the CD pattern is similar to that seen in PC bilayers) have also indicated a right-handed $\beta^{6.3}$ helix (Arseniev et al., 1985; Bystrov et al., 1990). In lyso-PC sheets a left-handed helix was proposed (Urry, Walker & Trapane, 1982) based on an interpretation of NMR chemical shift changes which occur upon ion binding to ^{13}C-labeled carbonyl groups. Conceivably both helical senses might occur under difference conditions, although this would be expected to produce significant changes in CD spectra which have not been observed. An alternative interpretation of the ion-induced ^{13}C-carbonyl shift changes observed in the lyso-PC system consistent with a right-handed helical structure has been offered by Bystrov and Arseniev (1988). Interestingly, a molecule has been synthesized which is the mirror image of gramicidin A (i.e., all L-residues are replaced by the corresponding D-residues and vice-versa) (Andersen, Providence & Koeppe, 1990). A direct comparison of the CD and NMR spectra of this molecule with the parent compound should prove interesting.

X-ray diffraction of gramicidin in oriented multibilayers has been used to evaluate the overall dimensions and the location of ion-binding sites in the channel (He, Huang & Wu, 1991; Olah et al., 1991). Single crystals of gramicidin-lipid complexes have also been prepared and may ultimately permit a complete description of the three dimensional structure of the membrane-bound form of gramicidin at atomic resolution (Wallace & Janes, 1991).

C. Conformational Dynamics and Stability Influence Channel Properties

The preceding discussion may have implied a fixed conformation for the gramicidin channel. While gramicidin does appear to be less conformationally mobile than many peptides of its size (something which greatly simplifies the interpretation of spectroscopic data), probably because of the preponderance of rather bulky (e.g., Trp) side-chains and facile intramolecular hydrogen-bond formation, it is by no means rigid. In fact, motion of the peptide appears to be essential to its function. A static model of the $\beta^{6.3}$ helix has a central cavity with a diameter of about 4 Å. In order to solvate ions of different sizes effectively during permeation (e.g., Cs^+ and Li^+) some local movement of the peptide carbonyl groups is necessary. It has been proposed that tilting of the peptide unit between consecutive C^α carbons occurs (termed peptide libration (Urry et al., 1984*a*)). This libration or tilting toward and away from the central cavity can in fact be measured using the solid-state NMR methods mentioned above (Nicholson, Lo-Grasso & Cross, 1989). Specific ^{15}N-labeling of Ala^3 and Leu^4 amide groups has indicated rocking motions of $\pm 8°$ and $\pm 15°$, respectively, for these sites in the absence of ions (Nicholson et al., 1991). Molecular dynamics simulations have confirmed local distortions in gramicidin structure during ion transport (Jordan, 1987, 1990; Roux & Karplus, 1991) and have highlighted the flexibility of the channel structure (Roux & Karplus, 1988; Chiu et al., 1991).

Deuterium NMR of gramicidin with selectively deuterated Trp residues has been used to probe the

rotational motion of the peptide in various lipid environments. In PC membranes gramicidin was found to rotate around its long axis (i.e., around an axis perpendicular to the plane of the membrane) at temperatures above that of the lipid phase transition (Macdonald & Seelig, 1988). This motion ceased in the gel phase although the channel is still active in gel-phase lipid (Krasne, Eisenman & Szabo, 1971; Boheim, Hanke & Eibl, 1980; Boheim et al., 1983*b*) and the structure does not seem to be much altered (Wallace et al., 1981; Cornell et al., 1988*d*; Cornell, Separovic & Smith, 1988*b*). Thus, peptide rotation does not seem to be a prerequisite for function.

Examination of a model of the $\beta^{6.3}$ helix reveals that for any residue in the primary sequence (residue *i*), the adjacent residue (residue $i + 1$) and the residue six removed (residue $i + 6$) are close together in space (Urry et al., 1981; Venkatachalam & Urry, 1983). An interaction is therefore possible between Trp^9 and Trp^{15}, and there is some fluorescence and CD spectroscopic evidence that these side-chains are stacked (Cavatorta et al., 1982; Masotti et al., 1986; Wallace, 1986; Woolley & Wallace, 1992). Such stacking interactions and the bulky nature of the side-chains may be expected to reduce the conformational flexibility and range of motion of side-chains in gramicidin. Conversions between side-chain rotomeric states may be concerted and have high activation barriers (Urry et al., 1981). Consistent with this suggestion, Macdonald and Seelig (1988) report that only small-amplitude motions of the Trp residues are detected by ^{2}H-NMR. Intrinsic fluorescence studies of gramicidin Trp residues have been carried out in a BLM system (which is something of a technical accomplishment) (Camaleno-Delgado, Zhao & Gendler, 1990) and in lipid vesicles (Scarlata, 1988, 1991). The presence of four Trp residues (in gramicidin A) per monomer complicates intrinsic fluorescence measurements, but the selective use of side-chain analogues may facilitate these studies. In the BLM system a direct comparison of Trp dynamics and channel functional properties may be possible. The role of side-chain dynamics in channel function is also being addressed by using molecular dynamics simulations (Brenneman, Chiu & Jakobsson, 1991).

The lifetime of gramicidin channels observed in conductance studies is directly related to peptide and lipid dynamics. Mean channel lifetimes of seconds are typically observed; this is believed to reflect the activation energy involved in breaking the six-hydrogen bonds holding the N-termini of the monomers together (Venkatachalam & Urry, 1983; Hladky & Haydon, 1984). The interaction of monomers (and thereby channel stability) is affected by a number of lipid properties. For instance, channels in thicker membranes, where 'pinching' of the membrane may be required for dimer formation, have shorter lifetimes (Hladky & Haydon, 1972*a*; Kolb & Bamberg, 1977; Elliott et al., 1983). Membrane interfacial tension is also a factor (greater tensions generally result in shorter channel lifetimes) (Elliott et al., 1983). Calculations of the energy of deformation of a lipid membrane surrounding a gramicidin channel have suggested that thermal fluctuations in lipid thickness may play a role in the docking of monomers (Huang, 1986; Helfrich & Jakobsson, 1990).

Variation of membrane thickness with voltage (electrostriction), which occurs primarily in membranes containing an organic solvent (e.g., decane), is in part responsible for the voltage-dependence of gramicidin channel formation (Hladky & Haydon, 1972*a*, 1984; Bamberg & Benz, 1976). Solvent-free membranes may also show a voltage-dependence of channel stability (Bamberg & Benz, 1976; Frohlich, 1979) which depends only on the magnitude and not on the sign of the potential. This voltage-dependence appears not to be due to an interaction of peptide bond (or side-chain) dipole moments of gramicidin with the field since an abrupt change in the sign of the potential should then cause a relaxation and reorientation of dipoles, which is not observed (Frohlich, 1979). The $\beta^{6.3}$ helical monomer has no large net dipole moment because peptide planes are alternately approximately parallel and antiparallel to the helix axis. Furthermore, the head-to-head helical dimer is a symmetrical structure so that the gramicidin channel should have no net moment in the transbilayer direction (Durkin, Koeppe & Andersen, 1990). Stabilization of individual peptide bond moments by the transmembrane electric field would be easily overcome by thermal energy. It is possible that the voltage-dependence of dimer stability arises from an effect of voltage on ion occupancy of the channel (Frohlich, 1979; Ring & Sandblom, 1988*b*) since channel stability has been demonstrated to be dependent on ion occupancy (Kolb & Bamberg, 1977; Ring & Sandblom, 1988*a*), although other possibilities have been discussed by Bamberg and Benz (1976). The covalently-linked malonyl dimer of gramicidin does not show this voltage-dependent behavior (Bamberg & Janko, 1977).

An increase in temperature causes increases in both the rate of gramicidin monomer association and of dimer dissociation (Bamberg & Läuger, 1974) with the result that the ratio of these rates (the fraction of time spent in the open state) is approximately independent of temperature. Lateral diffusion of fluorescently-labeled gramicidin C has been measured using the technique of fluorescence photobleaching recovery (Tank et al., 1982). A diffusion

coefficient of 3×10^8 cm^2 s^{-1} was calculated for the peptide in fluid phase membranes, indicating a rather rapid diffusion approximating that of lipid molecules themselves. This means that in fluid bilayers individual gramicidin channels move an average distance of several thousand ångstroms laterally during the lifetime of one open state. This motion is severely restricted in gel-phase membranes (Tank et al., 1982).

D. Gramicidin Channels Provide a System for Understanding Ion Selectivity in Molecular Terms

Gramicidin channels are permeable to monovalent cations in the order $H^+ \gg Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ as determined by measuring biionic potentials (Myers & Haydon, 1972). This order of ion selectivity corresponds to Eisenman sequence I (e.g., Eisenman & Horn, 1983), which indicates that ion dehydration energies may be important in determining selectivity. The high proton permeability is believed to result from the presence of a continuous row of water molecules in the channel so that protons move as they do in bulk water by a 'hopping' or 'tunnelling' mechanism (e.g., Akeson & Deamer, 1991). The presence of water in the gramicidin channel is confirmed by the observation of streaming potentials (a potential difference arising from an osmotic pressure difference) (Levitt, Elias & Hautman, 1978; Rosenberg & Finkelstein, 1978) and electroosmosis (water movement driven by ion movement through the channel) (Finkelstein & Andersen, 1981).

The selectivity ratio of gramicidin for K^+ over Na^+ is about 4 : 1 at a concentration of 100 mM (Myers & Haydon, 1972). This may be compared to the delayed rectifier K^+ channel (20 : 1 to 100 : 1 (Hille, 1984)) and to valinomycin, a carrier type ionophore (more than 1000 : 1 (Ovchinnikov, 1979)). Although the ion selectivity of gramicidin is not dramatic and the apparent binding constants rather weak, ion binding has been demonstrated (e.g., Veatch & Durkin, 1980; Shungu et al., 1986; Hinton et al., 1988). A series of ^{13}C-carbonyl-labeled gramicidin analogues incorporated into lyso-PC sheets was investigated for the effects of ion binding on the NMR signals of the labeled sites (Urry, Trapane & Prasad, 1983). Chemical shift changes induced by the presence of ions were seen at Trp^9, Trp^{11}, Trp^{13}, Trp^{15} selectively (not at positions Val^1, Ala^3, Ala^5, Val^7), suggesting a role for these carbonyls in ion coordination. Solid-state ^{13}C-NMR in oriented multibilayers has indicated an interaction of sodium ions with D-Leu^{12} and D-Leu-^{14} carbonyls with no effect on Val^7, D-Leu^4, Ala^3 or Gly^2 sites. These studies were interpreted as indicating a movement of the carbonyl groups of D-Leu^{12} and D-Leu-^{14} toward the channel center upon ion complexation (Smith et al., 1990).

Conductance measurements have shown the gramicidin channel to be impermeable to anions and divalent cations (Myers & Haydon, 1972; Urban et al., 1978). Theoretical calculations indicate that anions would be quite stable inside the channel but that the energy required for passing the channel entrance creates a kinetic barrier to anion transport (Sung & Jordan, 1987). Interestingly, anions are found within the channel in the crystal structure of the double-helical pore form of gramicidin (Wallace & Ravikumar, 1988). Divalent ions, although they have appropriate radii, are presumably impermeant because their charge cannot be adequately solvated by the peptide or because their hydration shells exchange too slowly (Hille, 1984; Pullman, 1987). In fact, they act as channel blockers (*vide infra*). The behavior of Tl^+ ions is rather unusual. While Tl^+ alone is more permeant than K^+ or Na^+, it competitively blocks Na^+ currents even when present at low concentrations (Neher, 1975). Similar effects have been noted with Ag^+ ions (McBride & Szabo, 1978) and are also evident when Tl^+ is added to sodium and potassium channels in vivo (Hagiwara et al., 1972; Hille, 1973). A variety of studies using conductance methods have indicated that gramicidin channels may be multiply occupied (e.g., Eisenman, Sandblom & Neher,1977). Multiple occupancy has a number of consequences for ion transport (e.g., concentration-dependent permeability ratios) aspects of which have been reviewed (Finkelstein & Andersen, 1981; Andersen, 1984; Hladky & Haydon, 1984; Hladky, 1988).

A major goal of many computational studies of ion transport using Eyring rate theory on multiple barrier/site models, molecular dynamics and other methods (*see* Eisenman & Alvarez (1991) for a review) has been an understanding of the ion selectivity of gramicidin under various conditions. The relevance of such studies for the interpretation of function in terms of molecular structure derives primarily from the possibility of deducing the number of coordination sites and barriers to transport to be expected for different ions, and their relative locations with respect to one another and the long axis of the channel. Often these predictions are model dependent (Andersen, 1983, 1984; Pullman, 1987). At the same time, of course, these studies are facilitated by the level of detail to which the channel structure of gramicidin is known, and thus conductance, computational and conformational studies often complement one another. The gramicidin system is the only one known for which detailed theoretical

simulations of ion transport may be directly compared with experiment (e.g., Pullman, 1987).

E. Blocked States and Conductance Sub-states May Also be Understood in Molecular Terms

The gramicidin channel is blocked by divalent cations (e.g., Ca^{2+}, Ba^{2+} and to a lesser extent Mg^{2+} and Zn^{2+}) (Bamberg & Läuger, 1977). Divalent cation block is a common feature in ion channel proteins where, for instance, the delayed rectifier potassium channel is blocked by Ba^{2+} and chloride channels are blocked by Zn^{2+} (Hille, 1984). Divalent cation block of gramicidin is apparently of the 'fast' type (Hille, 1984) since single channel conductances are decreased uniformly as the concentration of the blocker is increased; blocked and unblocked states are not seen (Bamberg & Läuger, 1977). Divalent cations also affect gramicidin channel lifetimes perhaps through effects on ion occupancy which in turn affect channel stability (Ring & Sandblom, 1988*a*,*b*). Interpretation of blocking is facilitated by knowledge of the gramicidin structure. X-ray diffraction of gramicidin in oriented multibilayers has indicated that Ba^{2+} ions bind near the mouth of the channel 13 Å from the center of the bilayer (He et al., 1991; Olah et al., 1991). The effects of Ba^{2+} ions on ^{13}C-carbonyl chemical shifts in the lyso-PC system has been interpreted as evidence for Ba^{2+} binding near residue D-Leu^{14} (Urry et al., 1982). Nuclear magnetic resonance studies on the effects of Mn^{2+} ions on gramicidin proton relaxation times in sodium dodecyl sulphate micelles have indicated that the Mn^{2+} ion binds at a position 6.4, 8.6, and 8.8 Å (± 2 Å) away from the carbonyl oxygen atoms of residues D-Leu^{12}, D-Leu^{14}, and D-Leu^{10}, respectively (Golovanov et al., 1991). Divalent cations do not appear to have significant effects on the overall conformation of gramicidin in membranes as judged by CD measurements (Wallace, 1984), which would seem to rule out an allosteric blocking mechanism. Thus, block by divalent cations may simply reflect binding near the channel mouth and occlusion of the channel, although effects of these ions on membrane surface charge can also affect channel conductance (Masserant & Starzak, 1991).

Gramicidin channels are also blocked by iminium ions. Blockage by iminium ions is of the 'flicker' variety; that is, open channels show a series of short-lived conductance decreases. Flicker block is commonly observed with protein ion channels as, for instance, when local anesthetics are added to acetylcholine receptor channels (Neher & Steinbach, 1978) and when Cd^{2+} or Mg^{2+} ions are added to calcium channels (Lansman, Hess & Tsien, 1986). The flicker block of gramicidin caused by iminium ions appears to result from a transient association of these ions with the channel interior during permeation (Busath et al., 1988; Hemsley & Busath, 1991). Whether this mechanism could apply in other cases where flicker block is observed or to the interaction of similar functional groups with protein ion channels (e.g., nonylguanidine with the sodium channel (Morello et al., 1980)) remains to be seen.

Gramicidin channel flickering may also be observed in the absence of blockers; this has been suggested to result from transitions to low conductance states in which the two $\beta^{6.3}$ monomers are partly dissociated (Ring, 1986; Sigworth & Shenkel, 1988). Consistent with this suggestion is the observation that an increase in membrane thickness increases the frequency of these transitions. Furthermore, these low-conductance states are observed quite commonly as intermediates in the opening and closing transitions of normal channels. The lifetimes of these states range from 20 μsec (about the limit of resolution of the recording apparatus) to about 1 msec (Sigworth & Shenkel, 1988).

Flickering may be distinguished by its timescale from the appearance of subconductance states of the gramicidin channel. Subconductance states or 'mini-channels,' which may constitute from 5 to 40% of the channel events in a single channel recording, have lifetimes similar to those of normal gramicidin channels (i.e., seconds) (Busath & Szabo, 1981). Busath and Szabo (1981; 1988*a*; 1988*b*) have made a detailed study of these subconductance states. They do not appear to be due to degradation of gramicidin or heterogeneity of the peptide primary sequence since they are observed even with highly purified, synthetic peptide preparations and also will occasionally arise directly from a normal channel without an intervening closed state. The percentage of 'minis' is not dependent on the concentration of gramicidin in membranes which indicates they are of the same molecularity as normal channels. Since mini-channel lifetimes are similar to the lifetimes of normal channels, the dimer junction is probably unchanged. The percentage of minis is not dependent on pH or ionic strength; it may, however, be influenced by the presence of detergents, lyso-lipids or other surface active materials (Busath, Andersen & Koeppe, 1987; Sawyer, Koeppe & Andersen, 1989) or lipid type (Bamberg & Benz, 1976). It has been suggested that these subconductance states may be the result of different (less common) side-chain conformations which in turn affect the coordinating ability of the carbonyl groups on the ion transport pathway. If different side-chain conformers are responsible for this conductance dispersity then

these conformers must interconvert slowly (i.e., on the timescale of seconds) (*see* section II.C). The presence of detergents and lyso-lipids could alter the frequency of occurrence of mini-channels by loosening lipid packing and causing side-chain conformations to become accessible which are energetically competitive. The role of side-chains in the occurrence of subconductance states remains to be further elucidated. Why the variants should all be of lower conductance is unclear. Side-chain analogues (*vide infra*) have shed some light on this question, but it is an area which needs further work. Because of their frequent occurrence in protein ion channels (Fox, 1987), the structural origins of subconductance states, which may be addressed in the gramicidin model system, are of particular interest.

F. The Features of a Lipid Membrane Responsible for Selectively Stabilizing the $\beta^{6.3}$ Helix have not yet been Identified

A great deal of work dealing with gramicidin-lipid interactions has focussed on the effects of the peptide on lipid morphology rather than on effects of the membrane on gramicidin channel structure (for reviews *see* Cornell (1987), de Kruijff, Killian and Tournois (1988), and Tournois (1990)). Effects of gramicidin on lipid morphology depend critically on the specific lipids involved and begin to occur at peptide concentrations about a thousand-fold higher than those at which channel formation is observed (de Kruijff et al., 1988). Thus, although these effects are unlikely to be relevant for conductance measurements, they are of particular importance for spectroscopic studies using relatively high peptide concentrations and care must be taken to ensure that a bilayer morphology is present (e.g., using ^{31}P-NMR, freeze-fracture electron microscopy, or X-ray diffraction) if characterization of the functional channel is intended. We shall focus here on properties of lipids in bilayer form that could affect gramicidin channel structure and dynamics.

While the open gramicidin channel (i.e., the single channel conductance) appears on the whole to be little affected by membrane properties (Hladky & Haydon, 1972*a*, 1984), the stability and dynamics of channels most definitely are, presumably because channel formation necessitates lipid rearrangement. The effects of membrane thickness on channel dynamics and stability have been discussed above; besides the hydrocarbon length, the exact nature of the lipid tails does not seem to be critical. Channels form in both saturated and unsaturated lipid membranes and in both solvent-free and solvent-containing membranes. Branched chain (diphytanoyl-PC) membranes will also support channels. The chirality of the lipid head group does not seem to be a factor since nonchiral, R-, and S-lipids can all support channels (e.g., Providence et al., 1991). There are definite effects of the charge of the lipid head group on gramicidin channel conductance (Apell, Bamberg & Läuger, 1979), but this is likely a result of changes in membrane surface potential rather than changes in gramicidin conformation or dynamics.

The dipolar potential of membranes seems to have little effect on gramicidin function in contrast to its effect on ion-carriers such as valinomycin. Channels in bilayers of ether lipids or glycerol monooleate are very similar to channels in ester-lipid bilayers despite large (e.g., 150 mV) differences in the dipolar potentials of these membranes (Bamberg & Benz, 1976; Providence et al., 1991). Jordan (1983) has discussed how the membrane dipole potential may be shielded in the gramicidin channel. Studies using CD spectroscopy (G.A. Woolley and B.A. Wallace, *in preparation*) and solid-state NMR (Cornell et al., 1988*a*,*b*; Smith et al., 1989) have indicated a normal $\beta^{6.3}$ channel structure in bilayers of ether lipids. Subtle changes may occur, however; a modelling study has suggested that a constriction of the mouth of the gramicidin channel due to a change in the interaction of Trp^{15} with lipid head groups could occur when ester lipids are substituted by ether lipids (Meulendijks et al., 1989).

It is an intriguing fact that the $\beta^{6.3}$ helical dimer is stable in membranes while the double helical forms are not (Killian et al., 1988; Bano et al., 1991). Both structures are stabilized by the same number of hydrogen bonds and are of similar dimensions. It is difficult to pinpoint the difference between a lipid bilayer and organic solvents which selects the $\beta^{6.3}$ helical dimer form. Except in the case of lyso-PC (Killian et al., 1983) and perhaps cholesterol (Gasset et al., 1988; Schagina et al., 1989) there does not seem to be evidence of any complex formation between gramicidin and the lipid moiety which could stabilize a specific peptide conformation (Wang et al., 1988). Phospholipids added to organic solutions of gramicidin will interact with the molecule (Braco et al., 1986) but, at least in methanol, will not cause a transition to the $\beta^{6.3}$ form unless bilayers exist (Wallace et al., 1981). There is considerable evidence for effects of gramicidin on lipid chain order (Chapman et al., 1977; Lee, Durrani & Chapman, 1984; Davies et al., 1990), however, and, although the interactions between gramicidin and lipid hydrocarbon chains appear to be weak and rather nonspecific, they may nevertheless be an important factor in determining the membrane-bound conformation. The interactions of Trp residues, in particular, with lipid may be a key factor in determining the confor-

mational free energy since modifications to these residues strongly affect the stability of the $\beta^{6.3}$ form (Prasad et al., 1983; Jones, Hayon & Busath, 1986; Classen et al., 1987; Killian et al., 1988; Becker et al., 1991). Interestingly, Phe[9]-gramicidin A and gramicidin B (which has Phe[11] in place of Trp[11]) do not show characteristic channel CD spectra when added to lyso-PC, although they do show channel-type CD spectra in PC vesicles (Killian et al., 1988; Sawyer et al., 1990). Double-helix forms of gramicidin have surface arrangements of Trp residues which are quite different from those expected for the helical dimer form (Langs, 1988; Wallace & Ravikumar, 1988; Langs et al., 1991); different interactions with lipids are therefore to be expected and could conceivably drive the conversion to the helical dimer form in a membrane environment.

G. Specific Side-Chain Modifications have Revealed Relationships between Gramicidin Structure and Activity

Aside from affecting the stability of the membrane-bound gramicidin conformation, side-chain modifications can alter the conductance properties of gramicidin $\beta^{6.3}$ helix channels. This occurs despite the fact that the side-chains of gramicidin do not contact the permeating ions. The channel-forming properties of the naturally occurring analogues gramicidin B (Phe[11]) and gramicidin C (Tyr[11]) have been compared with gramicidin A (Bamberg et al., 1976; Sawyer et al., 1990). All of these channels were judged to be structurally equivalent by the criterion of hybrid channel formation when different peptides are mixed in the same bilayer (Durkin et al., 1990). Single channel conductances of 14.5 pS (gramicidin A), 9 pS (gramicidin B) and 13 pS (gramicidin C) were measured in 1 M NaCl. Average lifetimes were 1 sec (gramicidin A), 2 sec (gramicidin B) and 0.9 sec (gramicidin C). Ion selectivity was also affected; for instance, the ratio of the single-channel conductances of Cs^+ and K^+ was 1.8 : 1 for gramicidin A whereas for gramicidin B it was 2.3 : 1. The nature of the aromatic residue at position 11 thus has a small but measurable influence on channel properties.

A further series of analogues has examined the effect of replacing the amino acid at position one (usually Val[1]) with either Phe, Trp or Tyr for each of gramicidin A, B and C (Mazet, Andersen & Koeppe, 1984). Replacements at position one can be accomplished rather easily in a semi-synthesis which involves deformylation and removal of the N-terminal amino acid through an Edman degradation (e.g., Morrow et al., 1979). Formylated amino acids can then be coupled using standard peptide bond formation methods (e.g., Weiss & Koeppe, 1985). These peptides again formed structurally equivalent channels (i.e., $\beta^{6.3}$ dimers) as judged by hybrid channel formation except for analogues with tyrosine in position one. Fairly large differences in single channel properties were observed as well as altered selectivity ratios (Mazet et al., 1984).

Heitz et al. (1988) have reported the synthesis of a variety of gramicidin analogues in which Trp residues have been substituted by tyrosine, tyrosine-O-benzyl, naphthylalanine and quinolylalanine residues. Distinct effects on single channel conductances, lifetimes and channel voltage-dependence were noted.

Changes in channel properties resulting from side-chain modifications may be due to conformational changes or to electrostatic effects (either through-space ion-dipole interactions or inductive electron shifts). To distinguish between these possibilities, gramicidin analogues with isosteric side-chains were prepared (e.g., valine, trifluorovaline and hexafluorovaline at position one) (Russell et al., 1986). Since these residues have significantly different polarities and dipole moments but similar sizes, any changes in channel properties must presumably be due to electrostatic rather than conformational (steric) effects. As the polarity of the side-chains increased, channel lifetimes and single channel conductances were found to decrease. Interestingly, the effects were more pronounced for sodium currents than for cesium currents. Further studies have implicated ion-dipole interactions (rather than inductive electron shifts) as the dominant cause of the observed effects (Koeppe, Andersen & Maddock, 1988; Koeppe, Mazet & Andersen, 1990). These data can be incorporated into calculations of the energetics of ion transport and contribute to the detailed mathematical descriptions possible with this well-defined ion channel (e.g., Sancho & Martínez, 1991).

Another area in which studies with side-chain analogues have been informative is in the investigation of the structural basis of the dispersity of single channel conductances (subconductance states; section II.E). As mentioned above, it has been proposed that different subconductance states represent molecules with different side-chain conformations. Since in a $\beta^{6.3}$ helix steric contacts may occur between residues i and $i + 6$ in the chain (Urry et al., 1981; Venkatachalam & Urry, 1983; Etchebest & Pullman, 1988), replacement of Ala[5] by the bulkier Leu[5] (residue i) was expected to reduce the conformational space accessible to Trp[11] (residue $i + 6$) (Urry et al., 1984*b*). As expected, the Leu[5] analogue was found to have a reduced dispersity of subconductance states. On the other hand, the Ala[7] (in place of Val[7]) ana-

logue was synthesized which might be expected to have increased conformational mobility of Trp^{13} and show an increased channel dispersity. Instead, a narrow range of channels with particularly high single channel conductance was found (Prasad et al., 1986). It was suggested that this substitution created a new, highly favorable, orientation for Trp^{13}. A systematic analysis of such analogues in conjunction with molecular dynamics simulations (Brenneman et al., 1991), with due consideration of possible environmental influences on mini-channel frequency (Busath et al., 1987), might serve to further elucidate the structure-function relationships of gramicidin channels.

III. Alamethicin

Alamethicin is an antibiotic peptide produced by the fungus *Trichoderma viride*. The fungus is a common inhabitant of soils and its secretions (including alamethicin) are believed to affect the quality of pasture land through effects on the bacterial contents of ruminant animal stomachs (Jen et al., 1987). The voltage-gated channel-forming properties of alamethicin were recognized a number of years ago (Mueller & Rudin, 1968), but a complete structural characterization of alamethicin has been slower to emerge. The alamethicin monomer is a relatively small molecule (mol wt ~ 2000) consisting of 19 amino acids and 1 amino alcohol. Although cyclic structures and structures with different linkages in the C-terminal region have been proposed (Payne, Jakes & Hartley, 1970; Martin & Williams, 1976), there is now general consensus that the structure of the major form of alamethicin is the one shown below (Pandey, Cook & Rinehart, 1977; Fox & Richards, 1982):

Ac-Aib-L-Pro-Aib-L-Ala-Aib-L-Ala-
L-Gln-Aib-L-Val-Aib-Gly-L-Leu-Aib-L-Pro-L-Val-
Aib-Aib-L-Glx-L-Gln-L-Pheol.

Aib refers to α-aminoisobutyric acid (or α-methyl alanine) and the preponderance of this rather unusual amino acid is the reason for the peptide's name. The amino terminus is acetylated and the C-terminal residue is an amino alcohol (as with gramicidin), in this case phenylalaninol.

As with gramicidin, there is some heterogeneity in the naturally occurring substance; for instance position 18 may be either Glu or Gln. These two isomers have differing mobility on thin-layer chromatograms and have been designated R_f30 (Glu^{18}) and R_f50 (Gln^{18}). In addition, the residue at position six may be either Ala or Aib (Pandey et al., 1977). Other primary sequence variants may occur in minor amounts depending on *Trichoderma viride* culture conditions and these require HPLC for purification (e.g., Balasubramanian et al., 1981; Brueckner & Przybylski, 1984)). Most experiments have used either the R_f30 (originally purified by the Upjohn company (1969)) or R_f50 fractions, both of which may be microheterogeneous. As with gramicidin, the in vivo synthesis of alamethicin is nonribosomal (Kleinkauf & von Doehren, 1987) so that production of the peptide using recombinant DNA methods is not possible. There have been a number of chemical syntheses of alamethicin (Balasubramanian et al., 1981; Nagaraj & Balaram, 1981; Schmitt & Jung, 1985). These are not straightforward, however, since Aib residues have a low reactivity in peptide bond forming reactions and the Aib-Pro linkage is acid labile (Schmitt & Jung, 1985; Leplawy et al., 1990).

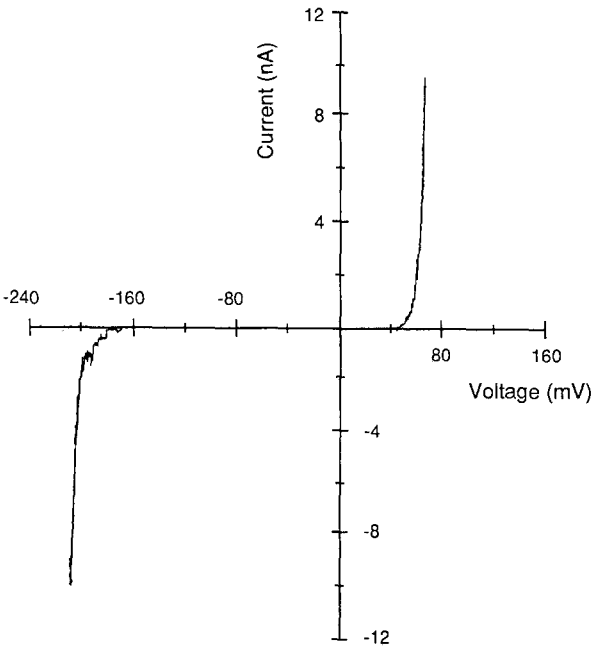

Fig. 6. Current-voltage (*I-V*) curve for alamethicin in glycerol monooleate/cholesterol/hexadecane membranes. The electrolyte is 2 M KCl and 1 μM alamethicin R_f30 was present on one side of the membrane only. The sign of the potential is given for the alamethicin side of the membrane. (After Gordon and Haydon (1975) with permission).

Despite its small size alamethicin can show quite complex channel-forming behavior (for a review *see* Latorre & Alvarez, 1981). Indeed, Mueller and Rudin (1968) were able to observe spikes in a voltage recording from an artificial bilayer containing alamethicin which resembled action potential spikes seen in voltage recordings from neurons. When added to bilayers alamethicin induces a macroscopic current which is strongly voltage dependent (Fig. 6).

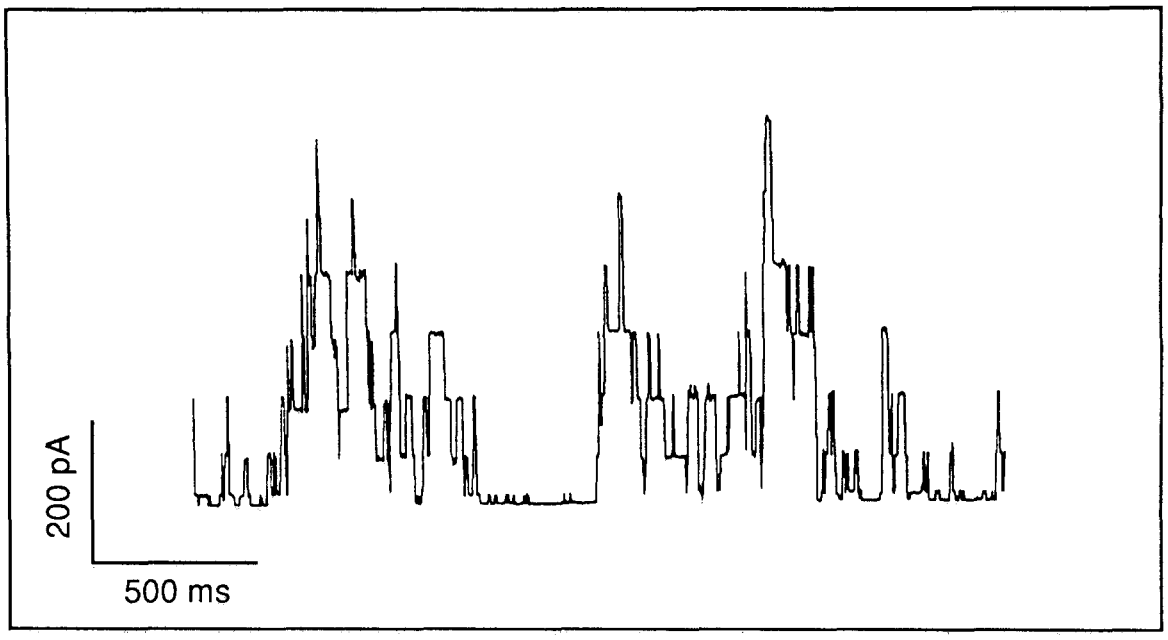

Fig. 7. Alamethicin (R_f50) single-channel events occurring in a diphytanoyl-PC membrane (125 mV applied potential; 0.5 M KCl solutions). (Reprinted with permission from *Prog. Biophys. Mol. Biol.* **55**:139–235, Sansom, M.S.P., The biophysics of peptide models of ion channels, copyright 1991, Pergamon Press PLC.)

Depending on the specific lipid-peptide combination (Vodyanoy, Hall & Balasubramanian, 1983; Hall et al., 1984) (*vide infra*) this current may be rectifying, being greater when the side opposite to that of alamethicin addition is made negative. Alamethicin conductance increases *e*-fold for every 4–5 mV increase in voltage (Eisenberg, Hall & Mead, 1973), a voltage-dependence similar to that observed with sodium and potassium channels in squid axons (Hodgkin & Huxley, 1952). Ion selectivity is minimal although cations are somewhat preferred over anions; some relative permeabilities are P_K/P_{Cl} = 2.7, P_{Na}/P_{Cl} = 1.6, P_{Ca}/P_{Cl} = 0.3 (Eisenberg et al., 1973). Saturation of currents through alamethicin channels at high ionic strengths or voltages is generally not observed (in contrast to gramicidin) (Gordon & Haydon, 1975, 1976). Observation at the single channel level reveals bursts of channel activity with each burst containing five or more well-defined conductance states (Fig. 7) ranging from 19 to 4400 pS or higher (1 M KCl) (Hanke & Boheim, 1980; Sansom, 1991). The conductance of any one of these states is not an integral multiple of the conductance of any other state. The observed pattern, therefore, is not a result of the simultaneous opening of several channels; instead the different levels must represent conductance substates of one channel. Lifetimes of individual substates are typically in the millisecond range and their conductance is approximately ohmic (Eisenberg et al., 1973); the channel as a whole (the burst of subconductance states) may last for seconds (Boheim & Kolb, 1978). The distribution of subconductance states is not greatly affected by voltage, nor is the channel mean open time (the average length of time between baseline current intervals). Therefore, the voltage-dependence of the macroscopic conductance must arise from voltage-dependent changes in the number of active channels in the membrane (Eisenberg et al., 1973; Gordon & Haydon, 1975; Latorre & Alvarez, 1981).

The macroscopic steady-state conductance (g_{ss}) depends on, in addition to voltage, the concentration of alamethicin and the salt concentration. These effects are summarized in the following relationship:

$$g_{ss} = \zeta \cdot [\text{salt}]^{\alpha} \cdot [\text{alm}]^{\beta} \cdot \exp(\gamma e \Delta\psi / kT) \quad (1)$$

where the constants α, β, and γ depend on membrane composition (values of 4, 9, and 6, respectively, are found for dioleoyl-PC/decane membranes (Eisenberg et al., 1973)); ζ is a proportionality constant, e is the electronic charge, $\Delta\psi$ the transmembrane potential, k is the Boltzmann constant and T the absolute temperature. Note that the dependence of g_{ss} on salt concentration is not simply due to an increase in the conductivity of the electrolyte solution (*see* section III.C.4).

The three-dimensional structure of the alamethicin channel is less well characterized than the gramicidin channel structure but may in fact more closely

mimic protein ion channels in several respects. The parallels between alamethicin channels and voltage-gated channels in vivo have been described (e.g., Hall, 1975). Furthermore, alamethicin has served as a prototype for describing the behavior of a variety of natural and synthetic channel-forming molecules (magainins, pGLA, paradaxin, cecropins, etc.) (e.g., Ojcius & Young, 1991; Sansom, 1991) including recently synthesized 'minimalist' ion channel-forming peptides (Lear, Wasserman & DeGrado, 1988; DeGrado & Lear, 1990) and synthetic pores (synporins) (Oiki, Danho & Montal, 1988; Montal, Montal & Tomich, 1990).

A. Alamethicin Monomers Show a Range of α-Helical Content in Solution

The presence of the unusual residue α-aminoisobutyric acid (Aib) in fairly high proportion severely restricts the conformational space available to the alamethicin molecule so that α- and 3_{10}-helices are preferred structures (Toniolo et al., 1982; Karle & Balaram, 1990; Marshall et al., 1990). In contrast to the gramicidin β-helices discussed above, α-helices and 3_{10}-helices (in short sections) are commonly found in proteins (Schulz & Schirmer, 1979; Toniolo & Benedetti, 1991). The Aib residue is achiral which means that left- and right-handed helical conformations are equally likely, and, indeed, a left- to right-handed helix interconversion has been observed for a pure poly-Aib peptide (Jung et al., 1988). However, the presence of amino acids with the standard L-configuration in alamethicin confers a preference for right-handed helices.

The crystal structure of alamethicin (crystallized from an acetonitrile/methanol mixture) was solved by Fox and Richards (1982) (Fig. 8). The asymmetric unit in the crystal structure was a trimer which did not contain an identifiable channel. The conformations of the three individual alamethicin molecules in the asymmetric unit were found to be rather similar, and a consensus conformation of the alamethicin monomer was proposed: essentially pure α-helix from the N-terminus to Pro^{14} followed by a bend in the helical axis and a second α-helix from Val^{15} to $Pheol^{20}$. Along with α-helical hydrogen-bonding patterns, some 3_{10} hydrogen bonds were observed to varying extents in the different monomers.

Nuclear magnetic resonance studies have been performed on alamethicin in methanol (Davis & Gisin, 1981; Banerjee & Chan, 1983; Banerjee et al., 1983; Esposito et al., 1987) and dimethylsulphoxide (Chandrasekhar et al., 1988). These are generally consistent with a large proportion of α-helix although some β-sheet formation in the C-terminal region was noted in one instance (Banerjee et al., 1983) and proposed to be a result of peptide aggregation. Fourier-transform infrared (FT-IR) spectroscopic studies (Haris & Chapman, 1988) of alamethicin in methanol gave an amide I absorption at an unusual wavenumber, assigned to the presence of some 3_{10}-type hydrogen bonds. Circular dichroism studies in a variety of organic solvents show a range (from 20 to 40%) of helical content (Jung, Dubischar & Leibfritz, 1975; Cascio & Wallace, 1984; 1988). The CD spectrum for a 3_{10} helix has been calculated theoretically (Woody & Tinoco, 1967); the calculated spectrum is similar to the pattern for an α-helix and would be difficult to resolve if it contributed a fraction to the overall CD pattern (Sudha, Vijayakumar & Balaram, 1983; Cascio & Wallace, 1988). Addition of ions to solutions of alamethicin does not cause conformational changes (Hauser, Finer & Chapman, 1970; McMullen, Marlborough & Bayley, 1971; Jung et al., 1975) (in contrast to gramicidin), although weak complex formation is observed

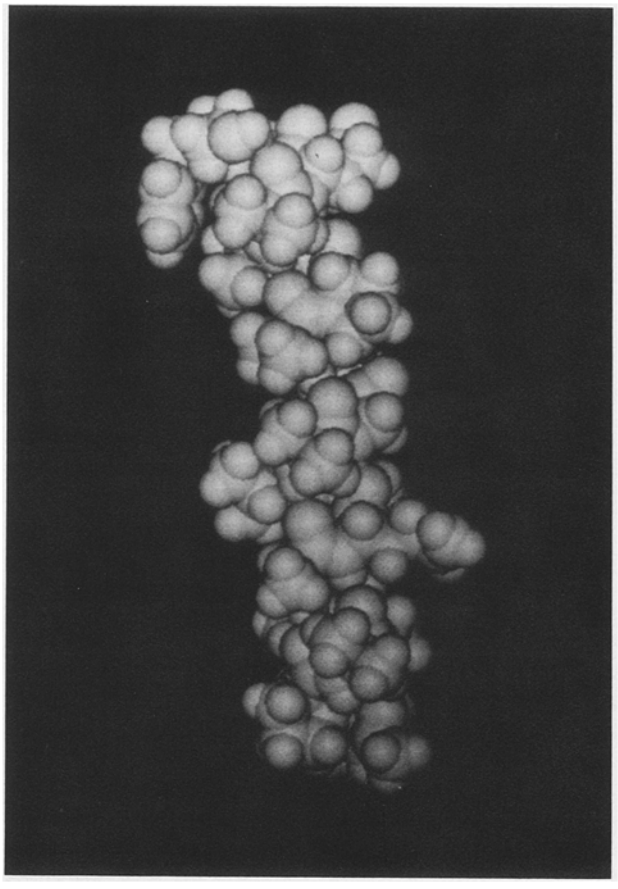

Fig. 8. Structure of an alamethicin monomer found in the crystal structure determined by Fox and Richards (1982).

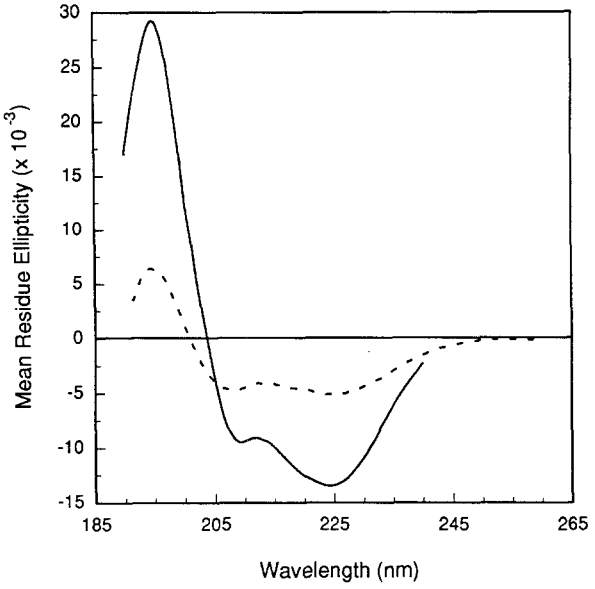

Fig. 9. CD spectra of alamethicin in water (– – –) and bound to dimyristoyl-PC vesicles (—).

(Feinstein & Felsenfeld, 1971). Sedimentation studies have demonstrated that alamethicin is monomeric in most common organic solvents, but in aqueous solutions peptide aggregates have been found to occur when the alamethicin concentration is above about 10 μM (depending on temperature and ionic strength) (McMullen & Stirrup, 1971; Jung et al., 1975; Rizzo, Stankowski & Schwarz, 1987; Archer, Ellena & Cafiso, 1991). The number of monomers per aggregate is not well defined in contrast to the situation with gramicidin. The monomeric form of alamethicin in water is largely disordered but retains some helical content as judged by CD measurements (Fig. 9).

B. Channels are Formed in Membranes by Bundles of Helical Monomers

In the same way that many experiments with gramicidin were aimed at distinguishing the model proposed by Urry et al. (1971) from that proposed by Veatch et al. (1974), many experiments with alamethicin have been based on an early model proposed by Baumann and Mueller (1974) (*vide infra*) in which opening of an alamethicin channel corresponded to movement of alamethicin molecules from the membrane surface to a transbilayer position. This model and early measurements of alamethicin partitioning into lipids (Chelack & Petkau, 1973) coupled with the fact of the peptide's limited (but not negligible) water solubility, focussed attention on membrane-bound forms of alamethicin. The movement of peptide molecules between membrane and aqueous domains was largely ignored. Furthermore, the possible effects of different peptide-lipid ratios and absolute concentrations on the relative populations of different membrane-bound and solution forms was not generally accounted for. As a consequence, there is a rather confusing literature on alamethicin-membrane interactions with apparently contradictory findings by authors using different techniques. Ideally, one would like to simultaneously demonstrate channel formation and analyze structure in order to be sure of studying the 'active' conformation of the alamethicin channel. However, as with gramicidin, this is a difficult task because the techniques most useful for demonstrating channel formation (BLM and patch pipette conductance measurements) generally employ peptide concentrations below spectroscopic detection limits. Voltage-dependent channel formation by alamethicin in a phospholipid vesicle system has been demonstrated, however (Lau & Chan, 1976; Woolley & Deber, 1988; 1989; Archer & Cafiso, 1991), and some progress has been made in using spectroscopic techniques to follow this process (Woolley & Deber, 1988; Brumfeld & Miller, 1990; Archer & Cafiso, 1991). Nevertheless, virtually all conformational studies of alamethicin in membranes have been performed in the absence of a transmembrane voltage.

Fourier-transform infrared spectroscopic studies of alamethicin in oriented multibilayers (Fringeli & Fringeli, 1979) were interpreted as indicating an extended conformation for the peptide, while more recent studies using lipid dispersions (Haris & Chapman, 1988) have suggested an α-helical structure with some 3_{10}-helix contribution. CD studies of alamethicin in oriented multibilayers (Vogel, 1987; Wu, Huang & Olah, 1990) show it to be helical with the helix axis perpendicular to the plane of the membrane when the lipid is fully hydrated. X-ray diffraction studies of alamethicin in dioleoyl-PC oriented multibilayers (P.E. Fraser, *personal communication*) give reflections typical of α-helices, where again the helix axis is perpendicular to the membrane plane. Freeze-fracture electron microscopy and X-ray diffraction using lipid vesicle suspensions have indicated an interaction of alamethicin with both hydrophobic and hydrophilic regions of the bilayer and show some evidence of peptide aggregation in the membrane (McIntosh, Ting & Zampighi, 1982). Raman spectroscopic experiments (Knoll, 1986; Vogel, 1987) and experiments with hydrophobic photolabelling reagents have also demonstrated an interaction of alamethicin with the core of the lipid bilayer (Quay & Latorre, 1981). Ion leakage experiments using lipid vesicles have been used to further characterize aspects of alamethicin-lipid interactions (e.g.,

Miller & Doll, 1990; Portlock, Clague & Cherry, 1990; Schwarz & Robert, 1990). In contrast to gramicidin, alamethicin does not appear to induce nonbilayer lipid morphologies, at least in those lipid systems for which this has been investigated (Banerjee et al., 1985). There is evidence, however, of increased rates of vesicle fusion at high alamethicin concentrations (Lau & Chan, 1975).

CD studies (Jung et al., 1975; Cascio & Wallace, 1984, 1988; Rizzo et al., 1987) show a marked increase in negative ellipticity near 220 nm when alamethicin moves from an aqueous to a membrane phase (Fig. 9) consistent with an increase in helix content at the expense of unordered structure. In addition, the shape of the CD spectrum of the membrane-bound form of alamethicin (in contrast to the situation with gramicidin) appears to depend on peptide-lipid ratio (Cascio & Wallace, 1988). The binding of alamethicin to lipid vesicles is actually a rather complex process; a detailed study has been published by Schwarz and colleagues (Schwarz, Stankowski & Rizzo, 1986; Rizzo et al., 1987). These workers measured the CD spectrum of alamethicin in the presence of varying amounts of unilamellar dioleoyl-PC vesicles. As the peptide binds lipid, the molar ellipticity near 220 nm becomes more negative; there is, however, a clear dependence of the size of the molar ellipticity change on the total alamethicin concentration. That is, as the total concentration of alamethicin increases, its apparent affinity for lipid increases, i.e., there is marked positive cooperativity. A likely cause of this positive cooperativity is aggregation of the peptide in the membrane (where the aggregate cannot dissociate from the membrane as easily as monomers do) (Schwarz et al., 1986; Rizzo et al., 1987). Archer et al. (1991) have questioned this interpretation, however, on the basis that the ESR signal of spin-labeled alamethicin derivatives does not appear to be sensitive to peptide-lipid ratio (*see* section III.C.4).

Inspection of molecular models of alamethicin derived from X-ray or NMR data (Fig. 8) reveals some amphipathicity in the structure. There is a polar face of the alamethicin helix which contains the Gln^7 and Glu^{18} residues and a corresponding nonpolar face containing Val, Aib, Ala and Leu residues. While the length of an individual alamethicin molecule in an α- or 3_{10} helical conformation is sufficient to traverse the lipid bilayer, such a structure contains no central cavity like the gramicidin $\beta^{6,3}$ helix through which ions could flow. However, an aggregate of alamethicin helices may be envisaged in which all of the polar faces are at the center of a bundle and the nonpolar faces make up the exterior of the bundle in contact with the lipid membrane (Fig. 10). Ions could then flow through the center of the aggregate. These helix-bundles (or 'barrels' since each monomer is like a stave in a barrel (Baumann & Mueller, 1974; Boheim, 1974)) have been incorporated into every model for alamethicin channel formation to be discussed below. They provide a straightforward explanation for the strong peptide-concentration dependence of channel formation (Eq. 1). Bundles of helices appear ubiquitous in membrane proteins generally (e.g., bacteriorhodopsin (Henderson et al., 1990) and the photosynthetic reaction center (Deisenhofer et al., 1985)) and have been proposed to occur widely in ion channel proteins in particular (Unwin, 1986; Guy & Conti, 1990; Montal, 1990).

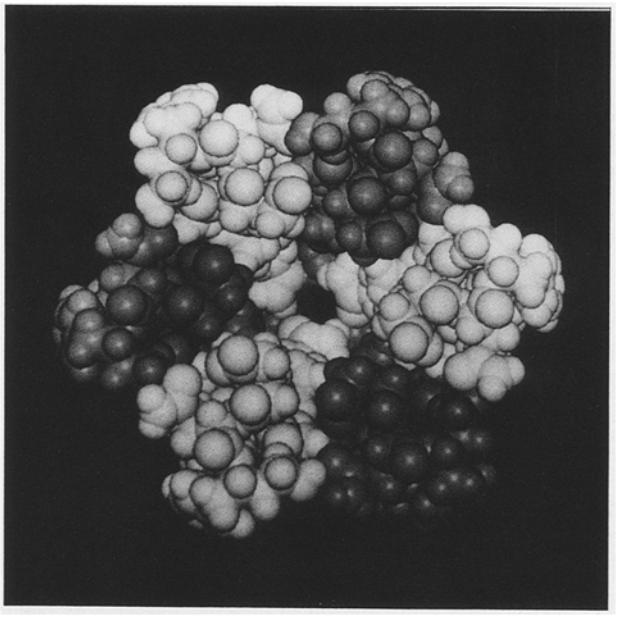

Fig. 10. Barrel-stave model of the alamethicin channel viewed from above. Alamethicin monomers associated to form a bundle of helices with a central lumen through which ions can flow. Six monomers (shaded alternately dark grey and light grey) are shown in this representation but a variety of aggregate sizes are possible.

Before addressing possible mechanisms of voltage-gating it is important to consider the alamethicin molecule from an electrostatic point of view. As Hol et al. (1981) have pointed out, the α-helix has an excess of negative charge at the C-terminus and positive charge at the N-terminus due to alignment of the dipole moments of individual peptide bonds. Thus, a peptide in the α-helical conformation may be thought of as a macro-dipole. The experimentally measured dipole moment of alamethicin in octanol (Schwarz & Savko, 1982) and in a 40% ethanol/dioxane mixture (Yantorno, Takashima & Mueller, 1982) is about 75 Debye. Although there are a number of assumptions and approximations involved which make these

numbers inexact, it is clear that alamethicin has a large dipole moment.

As mentioned previously the R_f30 component of natural alamethicin has glutamic acid at position 18. If the glutamate side-chain is ionized it dominates the electrostatic character of the molecule. The measured pH of this group in H_2O/MeOH has been variously reported from 5.2 to 6 (McMullen, 1970; Payne et al., 1970; Pandey et al., 1977) but may be significantly different in a membrane environment. In any case, this possible charged site does not appear to be essential for voltage-sensing since alamethicin R_f50 and a methylester of the Glu^{18} form are also able to form channels (Eisenberg et al., 1973; Boheim, 1974; Gordon & Haydon, 1975) (although *see* section III.C). As well, the pH dependence of channel formation is negligible (Cherry, Chapman & Graham, 1972).

It is generally agreed that a cation or anion from the electrolyte is not involved in the gating mechanism of alamethicin since an extremely wide variety of ions can be used as charge carriers with little difference in the voltage gating (Gordon & Haydon, 1975). As well, alterations in alamethicin chemical structure which would be expected to alter an ion binding site (e.g., $Glu^{18} \rightarrow Glu^{18}OMe$) do not prevent voltage-dependent gating (Baumann & Mueller, 1974; Boheim et al., 1987). Finally, although *trans*-negative voltages are usually more effective, alamethicin channels can be activated by voltages of either sign; this observation is difficult to reconcile with any model in which a bound ion acts as the voltage sensor (Gordon & Haydon, 1975). It appears then that the voltage-sensor is associated with the molecule itself and presumably arises from the macro-dipole created by a helical conformation. Note that unordered conformations have negligible net dipole moments and β-sheets have much lower dipole moments than helices (Hol et al., 1981). This is indirect evidence that in a membrane the active form of alamethicin is helical.

C. A Variety of Mechanisms of Alamethicin Channel Formation are Possible

Several models have been proposed for the molecular mechanism of channel formation by alamethicin (Cherry, 1972; Baumann & Mueller, 1974; Boheim, 1974; Smejtek, 1974; Gordon & Haydon, 1975; Fox & Richards, 1982; Boheim, Hanke & Jung, 1983*a*; Hall et al., 1983; Mathew & Balaram, 1983; Rizzo et al., 1987; Cascio & Wallace, 1988). We will briefly review the major models proposed:

i) Baumann and Mueller (1974) proposed that alamethicin molecules are adsorbed to the membrane surface in the absence of an electric field. Application of a voltage tilts the monomers (each acts as a dipole) into the membrane; these then aggregate to form channels.

ii) Fox and Richards (1982) proposed that a nonconducting preaggregate exists with a structure based on their crystal structure for alamethicin. In the absence of a voltage the C-terminus of each monomer in the aggregate is relatively disordered. A voltage causes a transition of each C-terminus to a helical conformation, the aggregate inserts more deeply into the membrane and becomes conducting.

iii) Hall and co-workers (1983) also proposed a nonconducting preaggregate except that the conformation involves a β-barrel formed by the peptide C-termini while the N-terminus of each monomer is α-helical. This conformation is based on the NMR data of Banerjee et al. (1983). A voltage is proposed to cause a movement of the helical domains hinging around the Gly^{11}-Pro^{14} section of the sequence such that the aggregate then traverses the membrane and becomes conducting.

iv) Boheim et al. (1983*a*) proposed a model that does not require a conformational change. Alamethicin monomers traverse the membrane in helical conformation and form aggregates of various sizes in which monomers come together in an antiparallel fashion (this is the most favorable arrangement for two dipoles side by side). A voltage causes monomers to flip in the membrane so that all dipoles become aligned with the field. The now parallel dipoles destabilize the helix bundle and permit ions to flow.

v) If alamethicin monomers can aggregate in the membrane in the absence of a potential, then as Rizzo et al. (1987) point out, a simple model for voltage gating presents itself. That is: since the membrane-incorporation of a suitably-oriented helix dipole will be stabilized by the transmembrane electric field, voltage may simply affect the extent of association of alamethicin with the membrane; if a sufficient concentration of membrane-bound peptide is reached, aggregates will form and these aggregates may, by their nature, be conducting and act as ion channels.

1. Surface to Transbilayer Reorientation Model

As mentioned previously, the surface to transbilayer reorientation model of Baumann and Mueller (1974) provided the impetus for many experiments aimed at distinguishing surface and embedded topologies for alamethicin. It appears from a variety of studies (*vide supra*) that alamethicin may be associated with the lipid core even in the absence of a transmembrane voltage. There are situations, however, where

alamethicin may be predominantly on the membrane surface (e.g., in dehydrated membranes or at low temperatures) (Vogel, 1987; Wu et al., 1990) and there appears to be a dependence of topology on peptide-lipid ratio (Wu & Huang, 1991). Boheim and Benz (1978) have used the charge-pulse technique (in which a voltage pulse is applied to a membrane and the discharging process is recorded) to study alamethicin-membrane interactions with a time resolution of 2 μsec. They observed no measurable (i.e., $> 10^{-14}$ mol $\cdot$ cm^{-2}) charge movement before channel formation occurred which was interpreted as evidence against the Baumann and Mueller (1974) model since a voltage-induced tilting of surface-associated alamethicin dipoles into the membrane prior to channel formation should have been detected. These conclusions depend, however, on the estimate of surface alamethicin concentration and they also rest on the assumption that the processes of membrane insertion and of aggregation into channels proceed at significantly different rates. If this supposition is not made then the models of Baumann and Mueller (1974) and of Rizzo et al. (1987) (*vide infra*) are conceptually very similar; the difference is essentially to what extent the surface-associated alamethicin molecule is a stable intermediate on the pathway to channel formation.

2. *Pre-aggregate and Conformational Change Models*

The models of Fox and Richards (1982) and Hall et al. (1983) are analogous in that both propose that a preaggregate of alamethicin molecules exists which changes conformation and becomes conducting when a voltage is applied. The model aggregate proposed by Hall et al. (1983) is extremely similar in three-dimensional structure to the enzyme triosephosphate isomerase (the latter determined crystallographically by Banner et al. (1975)) which suggests, at least, that such a structure could be stable. The model aggregate proposed by Fox and Richards is based on an analysis of possible packing arrangements of the crystallographically-determined alamethicin monomer structure.

The interpretation of voltage-jump and autocorrelation BLM experiments (Boheim & Kolb, 1978; Kolb & Boheim, 1978) is more straightforward if preaggregates are assumed to exist, but these experiments do not directly prove the existence of such preaggregates. If preaggregates do not exist then channel formation must be tied to monomer aggregation and the time constant describing the onset of current might then be expected to have a strong dependence on alamethicin concentration. The observation that this time constant is virtually independent of alamethicin concentration in glycerol-monooleate (GMO) and GMO-cholesterol membranes (Latorre & Donovan, 1980) has been used as an argument in favor of preaggregate models (although *see* section III.D).

3. *Flip-Flop Models*

The models of Boheim et al. (1983*a*) and Mathew and Balaram (1983) also incorporate the notion of preaggregates. In these models, however, the essential characteristic of the preaggregate is that it is composed of alamethicin monomers associated in an antiparallel fashion. The relative occurrence of parallel and antiparallel orientations of alamethicin molecules in membranes in the absence of a voltage has not been determined experimentally, although this may be addressed through the use of specifically-labeled fluorescent alamethicin derivatives (G.A. Woolley and B.A. Wallace, *in preparation*). Theoretical calculations have indicated, however, that helix-helix dipole interactions are not the major factor in helix-bundle stability (Furois & Pullman, 1988; Gilson & Honig, 1989). Gilson and Honig (1989) have calculated that the all-parallel arrangement of a four-helix bundle is only marginally less stable than the arrangement in which nearest neighbors are antiparallel. Although these calculations refer to soluble proteins, they apply to membrane proteins in which the helix termini are at the membrane surface, as is likely the case here. Both parallel and antiparallel arrangements of adjacent helices have been found in X-ray crystal structures of a variety of Aib-containing peptides related to alamethicin (Karle et al., 1990). The dominant factor in helix-bundle stability appears to be how well side-chains of adjacent helices can pack together in a compact structure (Chothia, 1984; Pullman, 1988; Gilson & Honig, 1989; Popot & Engelman, 1990).

The helix flip-flop models of Boheim et al. and Mathew and Balaram may appear intuitively unlikely since the activation energy required for such a process would probably be high. Moreover, a covalently-linked bundle of shortened (residues 1–17) alamethicin molecules has been found to form channels (Vodyanoy, Marshall & Chiu, 1989); this would seem to require the concerted flip-flop of four helices if the flip-flop model is generally applicable. Nevertheless, increased rates of lipid flip-flop induced by alamethicin have been observed (Hall, 1981) and long-chain quaternary ammonium ions can permeate alamethicin channels (Donovan & Latorre, 1978), which suggests that an aggregate of alamethicin molecules may provide pathways for flip-flop which are energetically

reasonable. Furthermore, time-dependent changes in the asymmetry of channel *I-V* relationships observed when alamethicin is added to one side of a membrane are a direct indication that alamethicin molecules can move across bilayers in an autocatalytic fashion (Schindler, 1979; Hall et al., 1984).

This ability of alamethicin molecules to move across bilayers is dependent on lipid type, however, and is affected by variations in peptide structure (Hall et al., 1984). For instance, alamethicin R_f30 does not appear to cross bacterial phosphatidylethanolamine (PE) membranes as gating is only observed when *trans*-negative voltages are applied across these membranes (i.e., when the side opposite to that of alamethicin addition is made negative). It has been argued that a *trans*-negative voltage would cause the N-terminus of alamethicin (the positive end of the macro-dipole) to move across the membrane while the presence of a formal negative charge on Glu^{18} may tend to anchor the C-terminus at the membrane surface and prevent the formation of channels when a *trans*-positive voltage is applied (Vodyanoy et al., 1982, 1983). The R_f50 version of alamethicin (Gln^{18}) and the methyl and benzyl ester of alamethicin R_f30 show decreased asymmetry of the *I-V* curve in PE membranes (Vodyanoy et al., 1982), indicating that for these analogues either end of the molecule may enter the membrane. In addition, an alamethicin analogue in which there is a formal positive charge at the N-terminus (and Glu^{18} is benzylated) preferentially forms channels when *trans-positive* voltages are applied, presumably since the C-terminal, negative end of the macrodipole can enter the membrane while the N-terminus is held at the membrane surface (Hall et al., 1984). The flip-flop gating model proposes that bundles of alamethicin helices are present before the application of a voltage with monomers arranged in an antiparallel fashion. This implies that either orientation of the molecule would be equally likely (i.e., there is symmetry across the membrane before the application of a voltage). Asymmetry such as that observed with alamethicin R_f30 in bacterial PE membranes would not then be expected.

It has recently been observed (Taylor & DeLevie, 1991) that sudden reversal of the applied voltage when a channel is open, particularly when large voltages are employed, can result in the formation of new, very long-lived alamethicin channels. These experiments were carried out using alamethicin R_f30 and diphytanoyl-PC/cholesterol membranes, a system in which channels normally form only when *trans*-negative voltages are applied. It is possible that the so-called 'reversed' alamethicin conductance (seen when the voltage is abruptly switched to *trans*-positive) results from channels formed by peptide molecules which flip-flopped as the voltage changed sign—flip-flop in this somewhat unusual case being facilitated by a large voltage and the presence of an open channel.

Interestingly, such voltage-reversal experiments have further suggested that the *open* alamethicin channel is composed of an all-parallel bundle of helices. If the sign of the applied voltage is suddenly reversed while a channel is open (and if the 'reversed' channels just described are not formed), ions flow in the opposite direction until the channel closes. The conductances observed under these conditions, however, are smaller than before the voltage reversal, which indicates that the energy profile an ion experiences is not symmetric across the bilayer (Boheim et al., 1983*a*; Taylor & DeLevie, 1991) (this is not observed for gramicidin channels). An all-parallel bundle of helices is an asymmetric structure, whereas an antiparallel bundle is not.

In summary, although there appear to be situations where alamethicin flip-flop is likely to occur, it has not been convincingly demonstrated that flip-flop is a necessary requirement for alamethicin channel formation.

4. Voltage-Dependent Partitioning Model

The voltage-dependent partitioning model proposed by Rizzo et al. (1987) is appealing in its simplicity; however, it has not been firmly established that alamethicin aggregation occurs in the absence of a voltage, and it has not been demonstrated that the aggregates which may form in vesicles are in fact channels. There is, however, some evidence that voltage can affect the extent of association of alamethicin with membranes. Vodyanoy et al. (1988*b*) have demonstrated, using measurements of bilayer capacitance, that voltage can affect the membrane adsorption of (charged versions of) alamethicin at concentrations below those required for channel formation. These experiments further showed evidence of an interaction of alamethicin molecules on the membrane surface (Vodyanoy, Hall & Vodyanoy, 1988*a*; Vodyanoy et al., 1988*b*). An interaction between peptide molecules is also implied by the observation that the CD spectrum of membrane-bound alamethicin is sensitive to peptide-lipid ratio (Cascio & Wallace, 1988; Wu & Huang, 1991). On the other hand, Archer et al. (1991) using spin labeled alamethicin in egg-PC vesicles report no evidence of peptide-peptide interaction, a surprising result in light of the fact that peptide-lipid ratios as high as 1 : 5 were employed. Further studies using different techniques will be necessary to resolve this issue.

Effects of salt and cholesterol on alamethicin binding to vesicles correlate with bilayer conductance data and constitute indirect evidence in support of the voltage-dependent partitioning model (Stankowski, Schwarz & Schwarz, 1988; Stankowski & Schwarz, 1989). For instance, increased ionic strength causes an increase in membrane partitioning of alamethicin (Gordon & Haydon, 1975; Stankowski et al., 1988) and a decrease in the solubility of the peptide in water (McMullen & Stirrup, 1971). Increases in ionic strength also facilitate alamethicin channel formation (Eisenberg et al., 1973; Boheim & Kolb, 1978; Stankowski et al., 1988) as would be predicted by the voltage-dependent partitioning model. Stankowski et al. (1988) have shown that the presence of cholesterol decreases the affinity of alamethicin for lipid vesicles. The voltage-dependent partitioning model would then predict higher voltages to be required for channel formation in cholesterol-containing membranes in order to drive a sufficient concentration of alamethicin into the membrane. Consistent with this expectation, the presence of cholesterol causes a shift in the macroscopic alamethicin channel *I-V* curve, indicating that a higher voltage is required to establish any particular current level (Latorre & Donovan, 1980; Stankowski et al., 1988). The observation of Eisenberg et al. (1973) that a voltage-independent conductance occurs when the concentration of alamethicin is high enough is also in keeping with the voltage-dependent partitioning model. These observations suggest that there is no special role of voltage in creating an active alamethicin conformation.

It is perhaps worth noting that if the interaction of alamethicin with lipid membranes involves the penetration of the membrane potential gradient by an alamethicin macro-dipole, then voltage-induced redistributions of alamethicin between membrane and aqueous phases must inevitably occur. It is possible, however, that voltage-induced redistributions occur too slowly to be of consequence for channel formation.

D. Voltage May or May Not Affect Alamethicin Conformation Directly

In order to distinguish unequivocally between models that propose an effect of voltage on the conformation of membrane-bound alamethicin and models in which a voltage causes channel formation through effects on the binding (or insertion) reaction, it will be necessary to have some method for distinguishing free (aqueous) alamethicin, closed channels and open channels. A detailed kinetic analysis may prove revealing. If the binding or aggregation step is demonstrably slower than the rate of channel opening then these processes could not account for gating. Mueller (1975) and Boheim et al. (1976) have solved numerically the set of differential equations which describe the kinetics of pore formation for the Baumann and Mueller model (note that these can also describe the model of Rizzo et al. if peptide in the aqueous phase is included). It is observed experimentally that the onset of current after a voltage step generally obeys first-order kinetics (Boheim & Kolb, 1978). First-order kinetics is predicted if monomer insertion is fast compared to aggregation (and the total monomer concentration in solution and surface-associated is large compared to the aggregate concentration). If the insertion step is slow then the onset of alamethicin current is predicted to be *S*-shaped (Boheim et al., 1976). *S*-shaped time courses have also been observed in BLM studies under certain conditions (Mauro, Nanavati & Heyer, 1972; Baumann & Mueller, 1974; Boheim & Kolb, 1978).

As mentioned above, the observation of a concentration-independent time constant describing the rate of current onset (τ_s) in GMO and GMO-cholesterol membranes (Latorre & Donovan, 1980) has been used as an argument in favor of the preaggregate models of Fox and Richards (1982) and Hall et al. (1983). Note, however, that a time constant that is insensitive to alamethicin concentration might also be observed if incorporation of monomeric alamethicin into the membrane was the rate-limiting step in channel formation. If this was the case then current onset would be expected to be *S*-shaped as indeed it is in GMO membranes (Latorre & Donovan, 1980). In membranes of different lipid composition, where current onset is observed to be first-order, the time constant describing onset is found to depend on the second or third power of the alamethicin concentration (Boheim & Kolb, 1978; Latorre & Alvarez, 1981).

Bruner and Hall (1983) performed a series of experiments using a BLM system held under high hydrostatic pressures (up to 1000 atm). Reaction rates are affected by pressure according to the activation volume associated with them; equilibria may be affected if a significant overall volume change is associated with the reaction. Steady-state alamethicin conductance was found to be unaffected by pressure but the onset of current after a voltage step became increasingly slowed and *S*-shaped as pressure was increased. These authors proposed a three-state model for alamethicin channel formation in which a species formed prior to the open channel has a significant activation volume. This model is not inconsistent with what might be expected if channel opening involved the movement of an alamethicin monomer from solution through a surface-associated state to an incorpo-

rated and aggregated state, but other possibilities exist as discussed by Bruner (1985).

The time constant describing the onset of current, in addition to pressure and peptide concentration (as noted above), also depends on voltage, temperature and lipid composition (Latorre & Alvarez, 1981). Although it varies considerably, an approximate value for this time constant is 200 msec. Stopped flow measurements in which fluorescently labeled alamethicin was mixed rapidly with lipid vesicles (Schwarz et al., 1987) indicated an association time not inconsistent with a role of voltage-induced membrane association in the gating event.

The effects of different lipid types on channel properties (Latorre & Donovan, 1980), the effects of voltage on membrane properties (Bamberg & Benz, 1976; Elliott et al., 1983) and the complexity of subconductance state patterns complicate the interpretation of these macroscopic kinetic experiments (Eisenberg et al., 1973; Sansom, 1991). It is possible that aggregation of molecules prior to insertion, on the one hand, or insertion followed by aggregation, on the other, are simply limiting cases of a complex reaction which depends on a variety of conditions.

We may ask, however, whether there is a special effect of voltage on the conformation of membrane-bound alamethicin; i.e., are there conformational states that are significantly populated only when a voltage is applied? We would have an answer if a unique spectroscopic 'signature' arose when a voltage was applied. Attempts in this direction have been made. Wille et al. (1989) have used a spin-labeled alamethicin derivative in a thylakoid membrane system in which a transmembrane voltage can be created by exposing the preparation to light. They report a suppression of the ESR signal of membrane-bound peptide when a voltage is applied and suggest this is due to a deeper insertion of the alamethicin helices in the membrane.

Obviously, a clear answer to this question will be required in order to develop more detailed models for the alamethicin channel and the physical basis of voltage gating. It is perhaps worth noting in this regard that voltage gating in protein ion channels could also involve voltage-induced movement of channel segments between membrane and aqueous domains with accompanying changes in conformation (e.g., Guy & Conti, 1990). The voltage sensors in protein ion channels, however, appear to be charged groups rather than helix dipoles (Stühmer et al., 1989; Papazian et al., 1991) and the reaction is intramolecular instead of intermolecular.

E. Patterns of Subconductance States Reveal Aspects of Channel Dynamics and Stability

The preaggregate models of Hall et al. (1983) and Fox and Richards (1982) attribute the range of subconductance states observed in single channel recordings (Fig. 7) to different conformations of the alamethicin channel. The models of Boheim et al., (1983*a*) Mathew and Balaram (1983), Baumann and Mueller (1974) and Rizzo et al. (1987) incorporate the appearance of subconductance states in a natural way: different levels simply correspond to aggregates containing different numbers of alamethicin monomers and hence having different lumen diameters. Using estimates of the membrane concentration of alamethicin made by Gordon and Haydon (1975), Boheim and Kolb (1978) have calculated the mean separation of alamethicin monomers (assuming these predominate over aggregates) to be about 10 nm in a typical conductance experiment. With a diffusion coefficient of about 10^{-8} cm^2 sec^{-1} collisions could occur sufficiently fast to explain the pattern of subconductance states as a process of uptake and release of alamethicin monomers.

If alamethicin monomers are represented as simple cylinders then the diameter of the channel formed by a bundle of these cylinders may be readily calculated. The increases in channel size calculated as monomers are added to the bundle approximately follow the increases in conductance seen in single channel recordings (Baumann & Mueller, 1974; Hanke & Boheim, 1980; Sansom, 1991). It is intriguing that any observed subconductance state always arises from the state either one level higher or one level lower. This suggests that alamethicin molecules never move in pairs and a large channel never breaks up into smaller channels without completely closing. Alternatively, if the different subconductance states are due to conformational transitions in an aggregate then some sort of defined pathway of transitions must presumably occur. The observation of well-defined subconductance states is the strongest argument against peptide-induced disruption of lipid packing as the basis for alamethicin channel formation. Although single channel events may be seen in pure lipid bilayers during fusion events (Woodbury, 1989) or phase transitions (Antonov et al., 1980) or upon addition of certain detergents to membranes (Blumenthal & Klausner, 1982), these do not in general show well-resolved subconductance states. Thus, it would seem reasonable to attempt to model aggregates of alamethicin monomers as fairly well-defined dimers, trimers, tetramers, etc.

The number of subconductance states observed

depends on the lipid type as well as voltage (to some extent), alamethicin concentration, ionic strength and temperature. Although the lowest subconductance state (19 pS in 1 M KCl) is impermeable to Ca^{2+} and Cl^- ions, upper states can have conductances in the nS range (i.e., lumen diameters of 25 Å or more) and have virtually no ion selectivity. It is this characteristic of alamethicin channels which has prevented the type of gating charge experiments that have been useful in probing the gating mechanisms of protein ion channels since no suitable compound has been found to block alamethicin currents. Interestingly, high ionic strengths have been found to stabilize lower subconductance states (Boheim et al., 1983*a*) and increase channel lifetimes (Hall et al., 1983). These effects have been suggested to arise from a shielding effect on the electrostatic repulsion of alamethicin monomers.

A process that involves the association and dissociation of monomers from an aggregate might be expected to involve lipid rearrangements. Membrane properties such as tension and fluidity as well as lipid type might then be expected to alter the dynamics and stability of alamethicin helix bundles (e.g., Gordon & Haydon, 1976). The presence of cholesterol has been found to increase the mean lifetime of alamethicin subconductance states (Latorre & Donovan, 1980). Alamethicin channel lifetimes in a biological membrane preparation were also found to be unusually long (Sakmann & Boheim, 1979), further suggesting effects of the lipid environment on alamethicin helix-bundle stability and dynamics. Alamethicin channel formation has been demonstrated in bilayers below the lipid phase transition temperature although current onset is much slower and lifetimes longer (Boheim et al., 1980; Hanke, Eibl & Boheim, 1981). Mean channel lifetimes decrease as temperature increases in fluid phase lipid (from 5.4 sec at 4°C to 200 msec at 25°C (Boheim & Kolb, 1978)).

Systematic studies of the effects of changes in lipid type and membrane properties on alamethicin channels have only recently been attempted (Stankowski & Schwarz, 1989). Unfortunately, the incomplete characterization of the alamethicin channel structure hampers detailed modelling of these interactions.

F. Dynamics and Stability are Particularly Sensitive to Side-Chain Modifications

There are a variety of naturally occurring analogues of alamethicin, several of which have been characterized extensively (*see* Brueckner & Przybylski, 1984; Sansom, 1991). These peptides have been termed 'peptaibols' since they all contain Aib residues and a C-terminal amino alcohol. Members of the family include trichorzianins (Molle, Duclohier & Spach, 1987), emerimicins (Toniolo et al., 1982), paracelsin (Brueckner, Graf & Bokel, 1984), antiamoebin (Das, Krishna & Balaram, 1988), trichotoxins (Hanke et al., 1983), suzukacillin (Jung et al., 1983) and zervamicin (Karle et al., 1991); the channel-forming properties of many of these have been characterized. Unfortunately, the differences among these peptides are not systematic and it is difficult to relate specific structural changes to changes in activity (although *see* Boheim et al., 1987). However, some insight has come from the work of Molle et al. (1988; 1989) who have prepared alamethicin analogues in which all Aib residues are replaced either by Leu or Ala and the C-terminal Pheol is replaced by Phe-NH_2. These peptides can be synthesized using solid phase methods much more easily than the parent molecule. The Ala-containing compound was found to be inactive and appeared to form intermolecular β-sheets. The Leu analogue, however, appeared to be helical and did form functional channels but the lifetime of channel subconductance states was much shorter than for alamethicin. The incorporation of serine at position 1 lengthened these lifetimes although they were still ten times shorter than those seen with natural alamethicin. Further studies by Molle et al. (1991) have investigated the effects of different C-terminal groups (Phe-NH_2, Pheol, Trp-NH_2) on the conductance behavior of the Leu analogue. Conductance amplitudes and open channel lifetimes did not appear to be significantly affected by these changes in structure. While still in its early stages, this systematic approach appears to be a promising one for establishing the factors involved in alamethicin channel stability and dynamics.

Helix-bundle formation has been analyzed theoretically by several authors (e.g., Crick, 1953; Dunker & Zaleske, 1977; Chothia, 1984; Gilson & Honig, 1989) and particularly by Pullman (1988) in the context of ion channel formation. These authors emphasize the importance of specific side-chain interactions and 'knobs into holes' or 'ridges into grooves' packing. Also, the importance of proline residues in helix bundles engaged in transport has been emphasized recently (von Heijne, 1991; Williams & Deber, 1991; Woolfson, Mortishire & Williams, 1991). The role of proline residues in conferring flexibility on protein segments has been discussed (Deber, Glibowicka & Woolley, 1990; Williams & Deber, 1991). Factors involved in the stabilization of helix bundles are of general relevance to membrane protein and ion channel function (Unwin, 1986; Oiki, Madison & Montal, 1990; Popot

& Engelman, 1990). Since the motif of bundles of helices seems to be shared between alamethicin channels and many protein channels, how is it that these latter channels acquire ion selectivity? Perhaps selectivity is simply achieved through the anchoring of pore-forming helices by the covalent structure of the protein such that only certain sizes and types of helix bundle are possible. Adjacent domains may then alter the electrostatic environment or provide 'selectivity filters.' Attempts to limit the size of alamethicin aggregates by covalent attachment to appropriate linkers or templates have begun. As mentioned above, Vodyanoy et al. (1989) have reported an alamethicin tetramer analogue, and we have prepared covalent dimers of alamethicin (Woolley, 1990). It should prove interesting to investigate the pattern of subconductance states and the voltage dependence of these analogues. This template-assisted approach (*see* Mutter, 1988) has already met with some success in the characterization of the ACh receptor channel (Montal, 1990).

IV. Summary and Conclusions

We have discussed in some detail a variety of experimental studies which were designed to elucidate the conformational and dynamic properties of gramicidin and alamethicin. Although the behavior of these peptides is by no means fully characterized, these studies have already permitted aspects of ion channel activity to be understood in molecular terms.

Studies with gramicidin in a variety of organic solutions have revealed conformational heterogeneity of this peptide; at least five major isomers exist, several of which have been characterized in detail using NMR spectroscopy and X-ray crystallography. When added to lipid membranes gramicidin undergoes a further conformational conversion. Although the conformation of gramicidin in membranes is not as well characterized as the solution conformation(s) and an X-ray structure is not yet available, detailed data, particularly from solid-state NMR studies, continue to become available and a right-handed $\beta^{6.3}$ helical conformation of the peptide backbone is now generally accepted. Two of these $\beta^{6.3}$ helices joined at their N-termini are believed to form the conducting channel. The conformational behavior of the side-chains of gramicidin in the membrane-bound form is not well established and several NMR, CD, fluorescence and theoretical studies are now focussed on this. Although the side-chains do not directly contact the permeating ions, they can have distinct effects on conductance and selectivity by altering the electrostatic environment sensed by the ion. The dynamics of both side-chain and backbone conformations of gramicidin appear critical to a detailed understanding of the ion transport process in this channel. As the description of the membrane-bound conformation of gramicidin becomes more detailed, simulations of ion transport using computational methods are likely to improve and will further our understanding of the processes of ion transport.

As well as internal motion of the backbone and side-chains, gramicidin undergoes rotational and translational motion in the plane of the membrane. These motions do not appear to be essential for the process of ion transport but can affect channel lifetime since lifetime is determined by the rate of association and dissociation of gramicidin monomers. Gramicidin-membrane interactions are also likely to be involved in the frequency of occurrence of channel subconductance states, the frequency of channel flickering and fundamentally in the stability of the membrane-bound gramicidin conformation.

Alamethicin forms channels in membranes which are strongly voltage-dependent. The molecular origin of voltage-dependent conductances has been a fundamental problem in biophysics for many years. The appeal of alamethicin as a model for voltage-dependent protein channels also derives from the observation that the active alamethicin channel appears to be composed of a bundle of transmembrane (predominantly α-)helices—a common motif in protein ion channels. In addition to being present as an aggregate (bundle) of helices, alamethicin may be associated with the membrane as a monomer (either transmembrane or surface associated) or it may be in solution. The relative occurrence of these different forms of the peptide appears to be a complex function of environmental conditions (*viz.* peptide-lipid ratio, lipid type, ionic strength, etc.). It has not yet been possible to decide whether application of a voltage opens an alamethicin channel by causing a specific conformational change in the membrane-bound peptide or simply by causing a redistribution of the peptide between aqueous and membrane phases (with concomitant aggregation) and thus promoting the occurrence of a conducting form.

The characteristics of a channel formed by the aggregation of transmembrane peptide helices will be affected by the number of monomers per aggregate and the details of their association. Alamethicin channels show a pattern of subconductance states which is most easily explained by a process of uptake and release of peptide monomers from a conducting aggregate. The lifetimes of these subconductance states are affected by factors such as lipid type, temperature, ionic strength and particularly by changes to the peptide primary sequence. Manipulation of alamethicin side-chain structure is beginning to permit a characterization of the factors involved

in helix-bundle stability and in the ion transport properties of these bundles.

In discussing ion channel proteins the molecular basis of activity is easily overlooked since the complexity of these systems has often necessitated their representation in terms of cubes and groups of cylinders between the parallel lines of a bilayer. Because of their ready availability and relative simplicity, gramicidin and alamethicin model systems can permit the search for explanations of ion channel behavior in molecular terms using a variety of physicochemical techniques.

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