ALAMETHICIN

A Rich Model for Channel Behavior

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ABSTRACT Alamethicin, a 20-amino acid peptide, has been studied for a number of years as a model for voltage-gated channels. Recently both the x-ray structure of alamethicin in crystal and an NMR solution structure have been published (Fox and Richards, 1982. Bannerjee et al., 1983). Both structures show that the amino end of the molecule forms a stable α-helix nine or 10 residues in length and that the COOH-terminal end exhibits a variable hydrogen bonding pattern. We have used synthetic analogues of alamethicin to test various hypotheses of its mode of action. As a result of these studies we propose a channel structure in which the COOH-terminal residues bond together as a β-barrel, leaving the α-helicies free to rotate under the influence of the electric field and gate the channel. Though the number of monomers per channel varies with experimental conditions, the gating charge per monomer stays close to that expected from an α-helical gate. We can also alter the sign of the voltage which turns on a channel by varying the charge on the alamethicin analogue. Channels are always slightly cation-selective even though formed by monomers with negative, positive, or zero formal charge. Channels are less stable in low ionic strength solutions than high. Finally, alamethicin conductance parameters vary systematically with changes in membrane thickness. We show how these results and others in the literature can be explained by a fairly detailed structural model. The model can be easily generalized to a form more suited to high molecular weight single-peptide–chain proteins.

INTRODUCTION

Alamethicin, a 20 amino-acid peptide, has been studied for a number of years as a model for voltage-gated channels. Because it and its analogues can be synthesized (Gisin et al., 1977; Balasubramanian et al., 1981), one can study the changes in channel properties produced by defined chemical changes. Each analogue can also be studied in a variety of lipid systems (Gordon and Haydon, 1972; Eisenberg et al., 1973; Boheim 1974; Latorre and Donovan, 1980). Changes in lipid composition, salt concentration, and other experimental variables markedly alter its conductance properties. Investigators have thus had good reason to hope that the structure and function of alamethicin could be understood in detail sufficient to provide a description of lipid-peptide interaction useful in clarifying the mechanisms by which voltage-gated channels in biological membranes operate.

Alamethicin promotes voltage-dependent lipid flip-flop and catalyzes its own movement across membranes (Schindler, 1979; Hall, 1981). It thus may provide useful models for control of lipid flip-flop and protein insertion and translocation in biological membranes.

New structural data from two laboratories, each using a different technique, has brought realization of some of these hopes a step closer. Fox and Richards (1982) have determined the crystal structure of alamethicin by x-ray analysis. Bannerjee and colleagues (Bannerjee et al., 1983) have measured the NMR-coupling constants between amide protons and α-carbon protons of alamethicin in methanol. From these constants they deduce a solution conformation. The two proposed structures differ, but the first nine or 10 residues from the amino end form a stable α-helix in both cases . In x-ray analysis, the ten residues at the COOH-terminal end show a variable hydrogen bonding pattern; in NMR they form a parallel β-sheet. This indicates that the COOH-terminus of the molecule has greater conformational flexibility than the NH_2-terminus. We believe this flexibility has important consequences for the mode of action of the alamethicin channel.

We shall show that the channel-forming characteristics of alamethicin depend in a systematic way on membrane composition, alamethicin chemical structure, and ionic strength of the solutions bathing the membrane. Next we shall discuss various models for the action of alamethicin and analogues of alamethicin, eliminating some models from consideration and showing how the available structural data lead to a molecular architecture for the alame-

thicin channel that can be generalized to a model for voltage-gated protein channels.

MATERIALS AND METHODS

Membranes were formed as described by Montal and Mueller (1972), and slightly modified by Vodyanoy et al. (1983). Membrane formation was monitored by measuring membrane capacitance.

Current-voltage (*I-V*) curves were taken by imposing a voltage on the membrane using either a 12-bit digital-to-analog converter (AD 575, Analog Devices, Norwood MA) or an eight-bit converter (Motorola 1408). The converters were under microcomputer control. Current was passed through chlorided silver wire electrodes also used to measure the voltage in symmetrical salt solutions. In asymmetric salt solutions, voltage was measured with chlorided silver wire electrodes joined to the solutions by 1.0 M-KCL 3%-agar bridges. Current was measured using a low bias current op amp (AD 48K; Analog Devices) in an ammeter configuration.

Relaxation times were measured using a fast sample and hold (SHM-2, Datel, Inc., Mansfield, MA) driving a 12 bit analog-to-digital converter MAS-1202, Analog Devices). Voltages were generated by an eight-bit digital-to-analog converter as described above. Data collection and pulse generation were under the control of a Z80 based computer system (Cromemco, Cupertino CA).

Phospholipids were purchased from Avanti Biochemicals, Birmingham, AL. Monoglycerides were purchased from Sigma Chemical Co., St. Louis, MO, and Nuchek Prep, Inc. Squalene was from Atomergic Chemetals Corp., Plainview, NY . *n*-Pentane; salts were from Mallinkrodt, Inc., St. Louis, MO.

Fraction 4 was purified from natural alamethicin, a gift of the Upjohn Company, Kalamazoo, MI. Synthetic derivatives were prepared using methods described by Balasubramanian et al. (1981). The derivatives used in this study were: Fraction 4, the major component of HPLC-purified Upjohn alamethicin; synthetic alamethicin, which gives experimental results identical to Fraction 4 and is thought to have the same structure; BG[1], a synthetic derivative; Boc 2-20, a synthetic derivative; and ALM-17, a synthetic derivative. The structures of these compounds are shown in Table I.

Peptide was usually added to only one side of a formed membrane, called by convention the *cis* side. The *trans* side was the electrical ground of the system. Analogues were added to the aqueous phase from ethanolic or methanolic stock solutions ranging in concentration from 10^{-3} to 10^{-6} g/ml. The total alcohol content never exceeded 4% and was usually much less. Control experiments established that alcohol in these concentrations had no effect on the properties of the membranes used in this study.

RESULTS

Number of Monomers in a Channel and Charge Moved Per Monomer

The number of monomers forming a channel and the charge per monomer that crosses the membrane are important constraints on models of channel function. Deducing these parameters from the power-dependence of conductance on aqueous peptide concentration can be risky, but systematic variation in these properties with changes in lipid composition and peptide chemical formula provides suggestive clues about the workings of alamethicin-like channels.

TABLE I
STRUCTURES AND NOMENCLATURE OF ALAMETHICIN ANALOGUES

Fraction 4. Component of natural alamethicin.

Ac—Aib[1]—Pro[2]—Aib[3]—Ala[4]—Aib[5]—Ala[6]—Gln[8]—Aib[8]—Val[9]—Aib[10]—Gly[11]—Leu[12]—Aib[13]—Pro[14]—Val[15]—Aib[16]—Aib[17]—Glu[18]—Gln[19]—Phol[20]
1
—COOH

Boc2—20

BOC—Pro[2]—Aib[3]———Aib[17]—Glu[18]—Gln[19]—Phol[20]
|
—BENZ

BG

Free —Pro—Aib[17]—Glu[18]—Gln[19]—Phol[20]
Amino |
Terminus ——BENZ

ALM—17

Ac—Aib[1]—Pro[2]—Aib[3]—Ala[4]—Aib[5]—Ala[6]—Gln[7]—
—Aib[8]—Val[9]—Aib[10]—Gly[11]—Leu[12]—Aib[13]—Pro[14]—
—Val[15]—Aib[16]—Aib[17]—COOH

Peptide-induced conductance as a function of voltage and concentration in steady state is given by

$$G = \Gamma C_p^n \exp(V/V_e) \quad (1)$$

where Γ is a parameter depending on salt type and concentration, lipid type, peptide chemical formula and other variables, C_p is the peptide concentration, V the applied voltage, V_e the voltage that produces an *e*-fold change in conductance at fixed concentration, and n the power dependence of G on peptide concentration. V_e is determined by measuring the logarithmic slope of the G-V curve. The shift of the current-voltage curve with peptide concentration is easier to measure accurately than the conductance at a fixed voltage. This shift obeys the experimental relation

$$\Delta V = -V_a \Delta(\ln C_p) \quad (2)$$

where ΔV is the shift in the I-V curve at a constant conductance, V_a the shift for an *e*-fold change in aqueous concentration, and C_p the aqueous concentration. It is easy to show that

$$n = V_a/V_e. \quad (3)$$

We have measured V_e and V_a for Fraction 4, Boc 2-20, and BG in bacterial phosphatidyl ethanolamine membranes. The results are shown in Table II.

To deduce the amount of charge moved we note that V_e can be modeled as

$$V_e = kT/en\alpha \quad (4)$$

where k is Boltzmann's constant, T the temperature, e the electronic charge, n the number of monomers, and α the

[1]BG is short for "Back Gating," the name bestowed on this peptide because of the I-V curve of BG-doped membranes.

TABLE II
CONDUCTANCE PARAMETERS FOR ANALOGUES IN BACTERIAL PHOSPHATIDYL ETHANOLAMINE MEMBRANES IN 1M KCL SOLUTIONS

Analogue	V_e	V_a	n	$n\alpha$	α
	mV	*mV*			
Frac 4	4.0 ± 0.1	42 ± 0.4	10.5	6.2	0.59
Boc 2-20	8.5 ± 1.5	80 ± 8	9.4	2.9	0.31
BG	9.2	52	5.6	2.7	0.48

product of the charge on one monomer and the fraction of the distance across the membrane this charge moves down the field during the gating event. $n\alpha$ is thus kT/V_e. $n\alpha$ is shown in column five of Table II. Finally we can calculate α for each analog from n and $n\alpha$. α does not differ significantly from 0.5 for any of the analogues tested.

Sign of the Voltage that Turns Channels On

If alamethicin is added to one side of a membrane, an asymmetric *I-V* curve usually results. The degree of asymmetry depends on lipid composition of the membrane (Vodyanoy et al., 1983). Some lipids allow alamethicin to cross the membrane rapidly compared to the rate at which it dissociates from the membrane, and membranes made from these lipids show nearly symmetric *I-V* curves. Other lipids apparently do not allow such rapid alamethicin translocation,for example, bacterial phosphatidyl ethanolamine. In membranes made from such lipids the *I-V* curve is very asymmetrical.

The sign of the asymmetry depends on peptide structure. For Fraction 4, synthetic alamethicin, Upjohn alamethicin, and the methyl ester of natural alamethicin, a positive voltage elevates the conductance more than a negative voltage of the same magnitude (Fig. 1 *a*, and see also Gordon and Haydon, 1975, Eisenberg et al., 1973, and Vodyanoy et al., 1983). The gating event thus must be associated with net movement of a positive charge from the *cis* side of the membrane toward the *trans*. As alamethicin itself has no positive charge, the gating charge must arise either from a bound cation or from a structural feature of the molecule having a net dipole moment. (This argument was first proposed by Eisenberg et al., 1973.) Alamethicin has a dipole moment of ~67 D (debye) in solution, which is adequate to account for the observed gating (Yantorno et al, 1982, Savko and Schwartz 1982). Since α-helices have appreciable dipole moments (Hol et al., 1981), this result is consistent with the structural observations of Fox and Richards (1982) and Bannerjee et al. (1983).

The voltage-dependent conductance induced by monazomycin has many similarities to the alamethicin-induced conductance. But the monazomycin gating charge is probably a formal positive charge rather than a structural dipole (Andersen and Muller, 1982; Muller and Andersen, 1982).

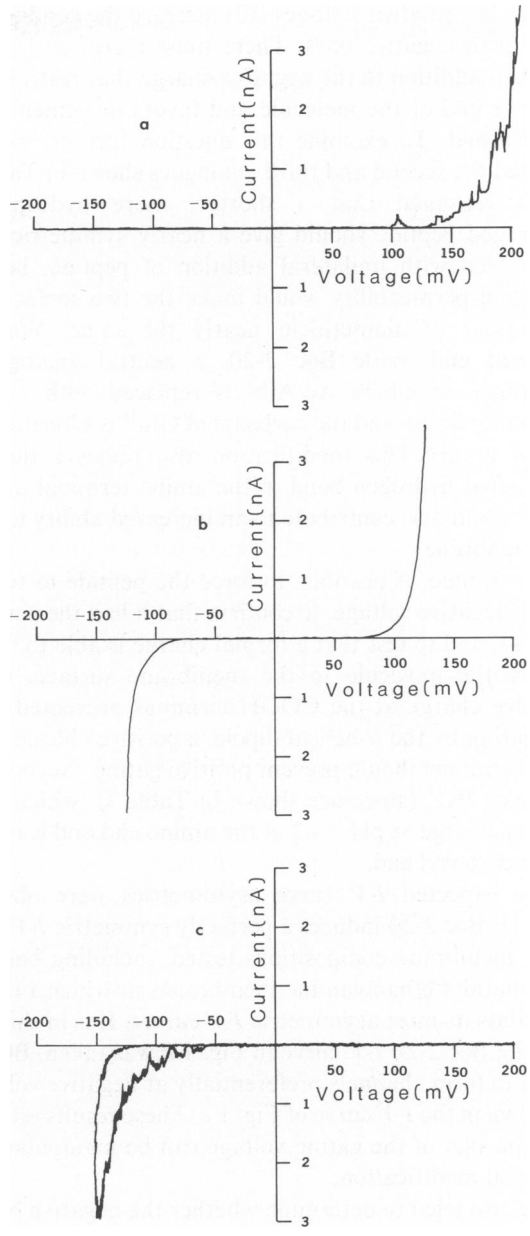

FIGURE 1 Current-voltage curves induced by different alamethicin derivatives added to *cis* side: *a*, Frac. 4; *b*, Boc2-20; *c*, BG. Membranes were formed from bacterial phosphatidyl ethanolamine with squalene in 1M KCL solutions (pH = 5.5) at room temperature.

If a change in the dipole orientation of an α-helix is the gating event, why does not the negative end of the dipole cross the membrane when a negative voltage is applied? Probably because the negative charge on the carboxyl fixes the COOH-terminal end of the molecule to the surface so that only the NH_2-terminal positive end of the molecule is free to cross the membrane under the influence of the voltage. Blocking the negative charge of the carboxyl, either by lowering the pH or esterifying it to a methyl or benzyl group, should then produce a symmetric *I-V* curve.

These manipulations do make the *I-V* curve more sym-

metric, but positive voltages still increase the conductance more than negative ones. There must therefore be some factor in addition to the negative charge that restrains the negative end of the molecule and favors movement of its positive end. To examine this question further, we constructed the second and third analogues shown in Table I.

We reasoned that a shorter, more hydrophobic, uncharged peptide should give a nearly symmetrical *I-V* curve even with unilateral addition of peptide, because increased permeability would make the two surface concentrations of alamethicin nearly the same. We thus designed and made Boc 2-20, a neutral analogue of almethicin in which Ac-Aib[1] is replaced with a *t*-Boc protecting group and the carboxyl of Glu[18] is esterified to a benzyl group. This modification also removes the only unsatisfied hydrogen bond in the amino terminal α-helix, which could also contribute to an increased ability to cross the membrane.

We wanted, if possible, to force the peptide to turn on with a negative voltage, to confirm that it had the potential to do so, and to test that a formal charge is able to fix one end of the molecule to the membrane surface. If the negative charge at the COOH-terminus prevented negative gating by the α-helical dipole, a positive charge at the NH_2-terminus should prevent positive gating. Accordingly we made BG[1] (structure shown in Table I), which has a positive charge at pH $>\sim 2$ at the amino end and is neutral at the carboxyl end.

The expected *I-V* curve asymmetries were observed (Fig. 1). Boc 2-20 induces a perfectly symmetric *I-V* curve in all membrane compositions tested, including bacterial phosphatidyl ethanolamine membranes in which Fraction 4 exhibits its most asymmetric *I-V* curves. It is in this lipid that the Boc 2-20 *I-V* curve in Fig. 1 *b* was taken. BG was found to form channels preferentially at negative voltages, as shown in the *I-V* curve of Fig. 1 *c*. These results establish that the sign of the gating voltage can be manipulated by chemical modification.

We also tried to determine whether the negative branch of the Boc 2-20 *I-V* curves arises from peptide which has diffused across the membrane or from reverse gating. In reverse gating the COOH-terminus crosses the membrane; in forward gating the amino terminus crosses the membrane. For alamethicin on the *cis* side, forward gating is promoted by a positive voltage; for alamethicin on the *trans*-side, forward gating is promoted by a negative voltage. The converse holds for reverse gating. To test for reverse gating we added a fixed ammount of Boc 2-20 to the *cis* side of the membrane, waited for steady state, and recorded an *I-V* curve. We then added the same amount of Boc 2-20 to the *trans* side, waited for steady state, and recorded the *I-V* curve again. The *I-V* curve shifts to lower voltages.

The amount of shift depends on the changes in the alamethicin surface concentrations on the two sides of the membrane. These in turn depend on the rate constants for

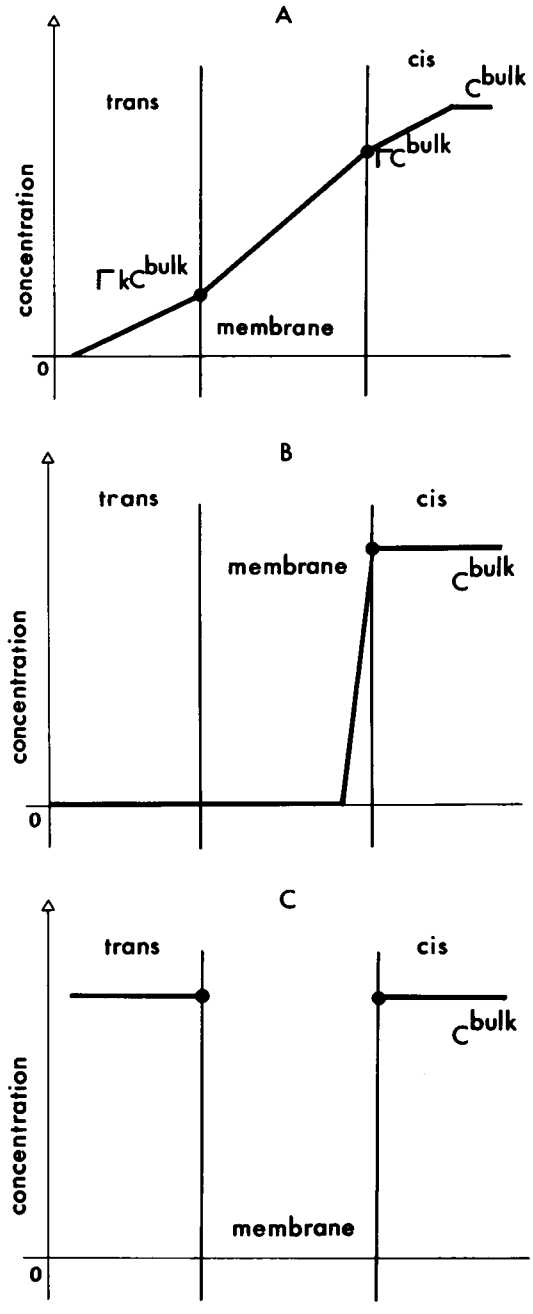

FIGURE 2 Idealized concentration profiles for alamethicin added to one or both sides of a membrane. Case *A*, alamethicin is added to the *cis* side and membrane is permeable to it; therefore, some alamethicin leaks to the *trans* side. This *trans* alamethicin could rotate its amino end across the membrane under the influence of a negative voltage accounting for the negative branch of the current-voltage curve. Case *B*, alamethicin is added to the *cis* side and the membrane is impermeant to it; therefore, the negative branch of the current-voltage curve can arise only from the COOH-terminus crossing the membrane under the influence of a negative voltage. Case *C*, symmetric surface concentrations with bilateral alamethicin addition. This profile would hold whether or not the membrane is permeable to alamethicin.

transfer of peptide into and across the membrane and the diffusion constant of peptide through the unstirred layers. For our purposes it is necessary to consider only two limiting cases: In Case *A*, the peptide crosses the membrane rapidly compared to all other process, adsorption or desorption for example; whereas in Case *B*, peptide is confined to one side of the membrane only. Idealized peptide concentration profiles for these cases are illustrated in Fig. 2. The black dots correspond to the surface concentrations of peptide; these are the concentrations effective in channel formation. Figs. 2 *a* and 2 *b* show concentration profiles for case *A* and case *B*, and Fig. 2 *c* shows the profile that would result if the same concentration as that originally added to the *cis* side were then added to the *trans* side. (For simplicity we have assumed a membrane-water partition coefficient of one, an assumption that does not alter the final conclusion.)

Fig. 3 shows two Boc 2-20 *I-V* curves before and after *trans* addition. The curve at higher voltage is for *cis* addition alone; that at lower voltage is for identical *cis* and *trans* additions. *Trans* addition shifts the *I-V* curve by 30 mV. This shift provides a critical test of whether Case *A*, Case *B*, or some case inbetween holds, because regardless of the mechanism by which alamethicin forms channels, the peptide surface concentration determines the voltage at which channel formation takes place. If Case *B* holds, the conductance at any voltage after *trans* addition should be exactly twice that with only *cis* peptide addition, provided that monomers rotated from the *cis* side cannot interact with monomers rotated from the *trans* side. Case *B* would give a shift of V_e ln 2 in the *I-V* curve. For Boc, 2-20 V_e is 8.5 mV, so the shift should have been ~4 mV if Case *B* obtained. Pure Case *A*, however, would give a shift of V_a ln 2 or 55 mV. (V_e and V_a are shown for all the analogues in Table II.) The observed shift of 30 mV can only be explained by a mixture of the two cases and provides indirect evidence that reverse gating can occur.

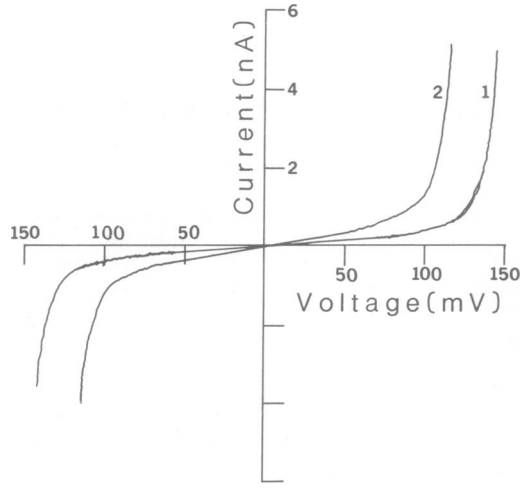

FIGURE 3 Current-voltage curves of PE(bacterial)/squalene membrane doped with Boc2-20. 1 M KCL (pH 5.5) at room temperature. *a*, Boc2-20 ($1.8*10^{-6}$ g/ml) added to *cis* side of the membrane only. *b*, Boc2-2 ($1.8*10^{-6}$ g/ml) added to the *trans* side of the same membrane 8 min after *cis* addition.

No Influence of Peptide Charge on Selectivity

Natural alamethicin has a negative charge at pH above ~5.2. Thus it is not surprising that its channels are slightly cation selective. But the negative charge of alamethicin cannot be entirely responsible for the cation selectivity because both the methyl ester and the benzyl ester of alamethicin, which are uncharged, show cation selectivity.

The voltage-dependent part of the conductance due to BG is also cation selective, even though BG is a positively charged molecule. We determined the selectivity of BG by forming a membrane with 1.0 M KCl on one side and 0.1 M KCl on the other. We measured the reversal potential of the channel by applying a pulse of voltage to turn on a large number of channels. The voltage was then stepped from the initial value to a series of lower values and the decay of conductance with time followed (see Fig. 4). Between 40 and 60 mV, the current decay reverses sign, indicating that the channel is cation selective. Some structural feature other than charge must therefore contribute to the cation selectivity.

Shorter Channel Lifetime in Low Ionic Strength Media

Channel lifetimes are shorter at low ionic strength than at high (Hall, 1975; Boheim et al, 1983). Fig. 5 shows how the rate at which channels disappear depends on ionic strength for Fraction 4 in dioleolyl phosphatidylcholine/squalene membranes. The time constant at a fixed voltage changes by two orders of magnitude in going from 1.0 M salt to 0.01 M salt. This means that the open channel has a much higher energy with respect to the closed in low ionic strength solutions than in high ionic strength solutions. We

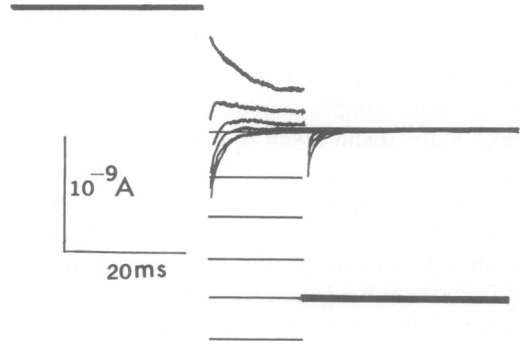

FIGURE 4 Selectivity of BG in a membrane (PE(bacteial)/squalene) with 0.1 M KCL on the *trans* side and 1.0 M KCL on the *cis* side. Upward currents are negative, upward voltages are *cis* side negative. The prepulse is 120 mV and the test pulses from 80 to −20 in 20-mV steps. The reversal potential of the channel is ~40 mV.

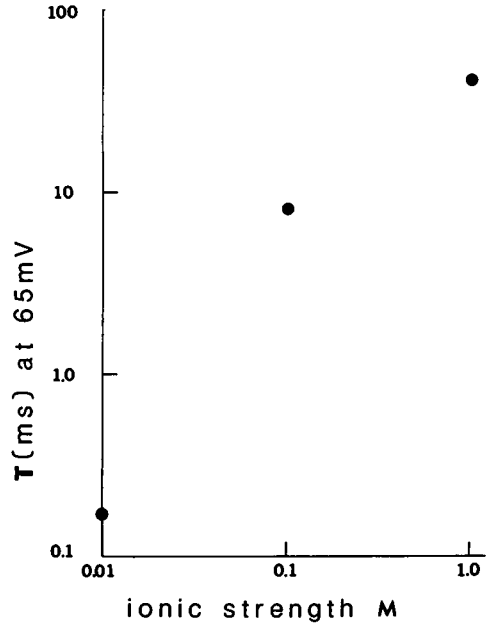

FIGURE 5 Time constants for the turn-off of alamethicin conductance at 65 mV as a function of ionic strength (0.01 M point extrapolated to 65 mV with exponential fit).

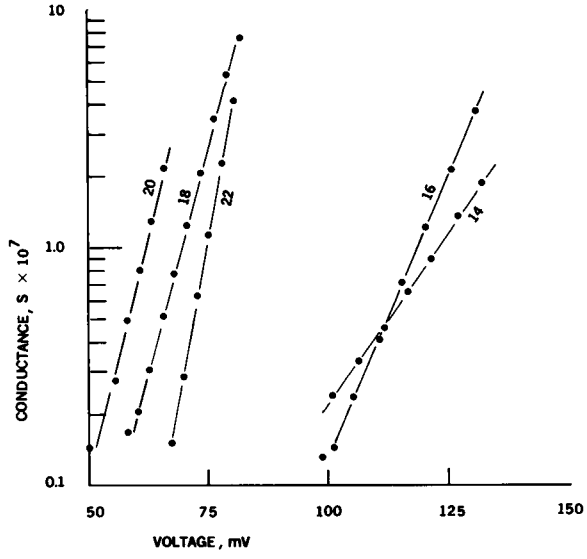

FIGURE 6 Monoglyceride/squalene membranes conductance-voltage curves due to Fraction 4 addition to *cis* side. 1 M KCL solutions at room temperature. Numbers on curves correspond to the number of carbons in monoglyceride chain. There is one double bond in each chain. 14:1-monomyristolein, 16:1-monopalmitolein, 18-monoolein, 20:1-monoeicosaeinoin, 22:1-monoerucin.

believe this is a consequence of the change in debye length with ionic strength: when debye length is short compared to the dimensions of the channel, the electrostatic repulsion is minimal and channel lifetime long; as ionic strength is lowered and the debye length approaches the size of the channel, electrostatic repulsion between the parallel dipoles of the open channel increases, resulting in a shorter channel lifetime.

Dependence of Conductance Properties on Membrane Thickness

Changing lipid composition alters the conductance properities of alamethicin (Boheim, 1974; Latorre and Donovan, 1980; Latorre and Alvarez, 1981). To isolate a specific compositional variable, we varied membrane thickness systematically. To minimize variation of other properties we chose the monoglyceride/squalene membrane series well-characterized by Waldbillig and Szabo (1979).

Fig. 6 shows sample conductance-voltage curves for Fraction 4 alamethicin taken in membranes formed from monoglyceride/squalene membranes, where the chain length of the monoglycerides was varied in two-carbon steps from 14 to 22. (Peptide aqueous concentration varies.) Table III summarizes the properties of membranes formed from these lipids and the conductance parameters of Fraction 4 for each lipid composition. The principal finding is that the *G-V* curves induced by fraction 4 are progressively steeper in thicker membranes (see Fig. 6). The conductance for each membrane composition is still described by Eq. 1, but the parameters change systematically with thickness. In monomyristolein (C 14:1), V_e is 14 mV and in monoerucin (C 22:1) V_e is 3.6 mV, a much steeper increase of current with voltage.

Most of the increase in steepness arises from an increase in the number of monomers that form a channel. This can be seen in Table III from the variation of V_a as a function of thickness. Using V_e and V_a to calculate n shows that the apparent number of monomers in a channel varies from two (monomyristolein) to 11 (monoeicosaenoein, *C* 20:1).

The apparent charge per monomer, derived as was done earlier for different analogues, decreases with increasing thickness. This decrease may arise from an artifact in our estimate of the monomer number, a possibility suggested

TABLE III
CONDUCTANCE PARAMETERS FOR FRACTION 4 AND ALM-17 IN MONOGLYCERIDE MEMBRANES OF DIFFERENT THICKNESS IN 1 M KCL SOLUTIONS

Chain	*d*	V_e	V_a	*n*	Rounded *n*	$\alpha = 25/V_e$	α
	Frac 4	*mV*	*mV*				
	mm						
14:1		14.3 ± 4.0	26 ± 3	1.8	2	1.7	0.94
16:1	2.23	8.7 ± 1.2	27 ± 4	3.0	3	2.8	0.93
18:1	2.58	5.4 ± 0.7	40 ± 32	7.4	7	4.6	0.62
20:1	2.89	4.7 ± 0.7	52 ± 5	11.0	11	5.3	0.48
22:1	3.23	3.9	indeterminate				
	ALM-17						
14:1	1.95	13.4 ± 2.0	35 ± 3	2.6	3	1.9	0.72
16:1	2.23	8.3 ± 0.6	48 ± 5	5.7	6	3.0	0.53
18:1	2.58	7.6 ± 0.7	61 ± 8	8.1	8	3.3	0.41
20:1	2.89	4.4 ± 0.6	61 ± 9	13.9	14	5.7	0.41

by the concentration dependence of the voltage-independent part of the concuctance, or it may arise from the failure of the dipole to span thicker membranes completely. This latter would result in a reduced apparent charge per monomer both because of the reduced electric field in thick membranes and the reduced fraction of the distance down the field that the charge could move.

Amino Terminus as the Preferred "Gate" End of the Molecule

The steepness of the current-voltage curve depends both on the number of monomers in a channel and on the charge per monomer that crosses the membrane. To study the structure of the gate, we removed the three carboxyl amino acids (Glu-Gln-Phol). This left a 17-amino acid peptide ALM-17. Though a higher concentration of ALM-17 must be added to the aqueous phase to achieve a given conductance at a given voltage, the conductance parameters are very similar to those of Fraction 4 (Table III). These results indicate that removal of the three COOH-terminal amino acids has not much altered the gating structure. Indeed the range of charge-distance product for ALM-17 is about the same as that for Fraction 4, as expected if the NH_2-terminal dipole is the gating "particle."

Change in Power Dependence of the Voltage-Independent Conductance with Thickness

Fig. 7 shows a log-log plot of the dependence of the voltage-independent conductance induced by Fraction 4 and ALM-17 on aqueous concentration. The concentration for a given conductance is much higher for ALM-17 than for Fraction 4, but both peptides induce a zero-voltage conductance that has about the same power dependence on its aqueous concentration in membranes of the same thickness. In monomyristolein the power dependence is $G_0 \sim (C_B)^{0.5}$; as the membrane thickness increases, the power dependence increases to $G_0 \sim (C_B)^2$.

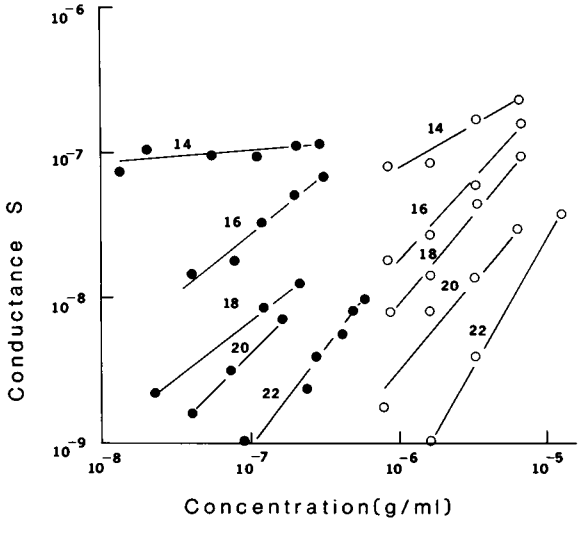

FIGURE 7 Dependence of voltage-independent conductance on thickness in monoglyceride/squalene membranes for Fraction 4 and ALM-17. •, Fraction 4. ○, ALM-17. Numbers on curves correspond to the numbers of carbons in monoglyceride.

It is interesting that the power dependence of G_0 is <1 in those membrane systems where the apparent α (charge moved) is nearer to 1.0 than to 0.5. A power dependence of less than unity suggests that the species closest to equilibrium between the membrane and the water may be not the monomer but a multimer, possibly the dimer. If in thin membranes the dimer is very unstable, the observed power would be half the true power.[2]

MODEL CONSTRAINTS: A SUCCESSIVE APPROXIMATION ALGORITHM

In this section, we shall use the results presented here along with those in the literature to develop constraints on models for alamethicin. The ultimate model should explain both analogue and lipid composition effects with a minimum of ad hoc assumptions. Previous work has established that all models must include the notion that a number of alamethicin monomers must aggregate together to form a channel. (For reviews see Hall, 1978, and Latorre and Alvarez, 1981.) The details of the aggregation process remain open but the data presented here will allow us to place preliminary constraints on the process.

The first successful model of alamethicin conductance was a kinetic one proposed by Baumann and Mueller (1974). In their now familiar scheme, alamethicin monomers, initially oriented parallel to the membrane surface, are rotated to span the membrane. These spanned monomers then aggregate and dissociate with various rate constants to form aggregates of differing sizes. The lumens of these aggregates, with monomers arranged around them like the staves of a barrel, form the conducting pores.

With proper choices of the various rate constants, this model will fit just about any known alamethicin data. The model does not, however, provide any physical interpretation of the rate constants and thus has no predictive power.

The Gate

There is solid evidence that the gate is associated with the α-helix of the NH_2-terminal portion of the molecule. Our

[2]For example, assume that the partitioning species is a dimer: C_p is the bulk concentration of peptide. $(A_{aq})_2$ is the aqueous concentration of dimer. $(A_m)_2$ is the membrane surface concentration of dimer, and (A_m) the membrane concentration of monomer, and $(A_m)_n$ the membrane concentration of n-mer, the species which forms channels.

$$C_p \rightleftharpoons (A_{aq})_2 \rightleftharpoons (A_m)_2 \rightleftharpoons (A_m) + (A_m).$$

If these equations are near equilibrium, mass action gives $(A_m)_n = \text{const}\ C_p^{n/2}$.

ability to reverse the sign of the gating voltage is inconsistent with a bound-cation gating charge. The magnitude of the observed gating charge, ~0.5 electronic charges, is quantitatively what would be expected from the movement of the amino end of the monomer from one side of the membrane all the way across to the other. That the charge moves all the way may explain an additional feature of the alamethicin conductance, namely channel stability. Unscreened charges in the membrane have a higher energy than on the surface (Parsegian, 1969). The gating "charge" is likely to have a lower energy on either surface than in the center of the membrane. Having a charge at each end, a dipole long enough to span the membrane would have two minimum energies: one with the charges on the same side of the membrane and one with the charges on opposite sides of the membrane, that is, spanning the membrane. Thus both the "off" state and the "on" state are in energy minima according to the dipole-gating scheme.

While we cannot yet describe the details of the process of channel formation, we can describe a sort of limiting case model based on our observation of reverse gating with BG and ALM-17. For clarity of arugment, we assume a first-order kinetic process, but it will be readily apparent that the same essential physics can give rise to more complicated kinetic schemes. We consider channels formed by either forward or reverse gating. (Forward gating is rotation of the amino end of the monomer across the membrane. Reverse gating is rotation of the carboxyl end across the membrane.) We shall not try to determine whether monomers aggregate first and then rotate, or rotate individually and then aggregate, or whether they rotate in groups of a certain size and the groups then aggregate (Kolb and Boheim, 1978). We consider now only which end most easily crosses the membrane.

We can obtain a rough estimate of the relative rates at which each end of the molecule crosses the membrane by treating each end as a single charge. The amino end of Fraction 4 has an apparent charge of $+0.5\ e$ and the carboxyl end has a charge of $-1.5\ e$ at pH above ~2, $0.5\ e$ being due to the effective charge at the end of the α-helix and $1.0\ e$ being due to the charge of the ionized carboxyl group. The rate at which a charge crosses the membrane will be proportional to $\exp(-A/kT)$, where A is the activation energy, k Boltzmann's constant and T, the absolute temperature. The activation energy will have the approximate form

$$A = \frac{q^2}{8\pi\epsilon_0\epsilon_{hc}\alpha} - f\,q\,V \tag{5}$$

where q is the charge, ϵ_0 the permittivity of free space, ϵ_{hc} the dielectric constant of the membrane, a the radius of the charge, f the fraction of the distance down the field where the peak of the activation barrier is located, and V the applied voltage. Clearly the activation energy for the crossing of the negative end of the molecule will be greater than that for the crossing of the positive end with no applied voltage. The magnitude of the negative voltage that must be applied to make the negative end crossing rate equal to the positive end crossing rate can be calculated by setting the activation energies for crossing equal and solving for the voltage. This gives

$$V = \frac{e}{8\pi\epsilon_0\epsilon_{hc}a^+f(l+m)}\left[\frac{a^+m^2}{a^-} - l^2\right] \tag{6}$$

where l is the electronic charge of the positive end, m the electronic charge of the negative end, a^+ the radius of the positive charge, and a^- the radius of the negative charge. If we assume that a^+ is ~0.2 nm and that a^- is three times a^+, V is ~1 V, a value unlikely to be obtained experimentally. Thus it is clear that at most voltages the rate of crossing of the low-charge end will be faster than the rate of crossing of the high-charge end.

Consider a simplified first-order scheme where peptide is confined to one side of the membrane. For positive voltages, channel formation will occur entirely by insertion of the low-charge amino end. Channel disappearance will occur entirely by return of the low-charge end to the *cis* side. For negative voltages, channel formation for alamethicin confined totally to the *cis* side will occur by insertion of the high-charge carboxyl end. But channel disappearance will occur by crossing of the low-charge positive end to the *trans* side, at least for all negative voltages of magnitude lower than predicted by Eq. 8. Since the *trans* side provides an infinite sink for alamethicin, the material from these channels will be lost. Such channels, once formed, will have the same lifetimes as channels formed at a positive voltage of the same magnitude (because they are disappearing by the same mechanism); but they will be formed at a much smaller rate.

To express these remarks quantitatively, we define: $\mu(V)$, the rate at which channels turn on for a positive voltage; $\lambda(V)$, the rate at which channels turn off for a positive voltage; and $\mu^-(V)$, the rate at which channels turn on for a negative voltage.

$$\begin{aligned}\mu^+(V) &= \mu_0^+ \exp(V/V_{\mu^+}) \\ \mu^-(V) &= \mu_0^- \exp(-V/V_{m^-}) \\ \lambda(V) &= \lambda_0 \exp(-V/V_\lambda)\end{aligned} \tag{7}$$

where $\mu_0{}^+$, $\mu_0{}^-$, and λ_0 are the rates at zero voltage and $V_\mu{}^+$, $V_\mu{}^-$, and V_λ the voltages that change the indicated rate e-fold. The rate of change in the average number of channels per unit time and the number of channels in steady state will then be of the following form for positive voltages:

$$\begin{aligned}\frac{dN}{dt} &= N'\mu^+(V) - \lambda(V) \\ N &= N'\frac{\mu^+(V)}{\lambda(V)}\end{aligned} \tag{8a}$$

where N is the average number of on channels per unit area and N' is the number of off channels on the *cis* side (V is a positive number in 8a). The expression for negative voltages is:

$$\frac{dN}{dt} = N'\mu^{-}(V) - \lambda(-V)$$
$$N = N'\frac{\mu^{-}(V)}{\lambda(-V)}. \tag{8b}$$

These expressions clarify the role of the formal charge, negative for Fraction 4, positive for BG. The formal charge does, as we intuitively argued in designing BG, fix the peptide's end to the membrane surface, but it does not necessarily prevent the end of the molecule from crossing the membrane. What this change does do is make it more likely that, if the charge crosses the membrane, it will stay there; the channel will turn off to the *trans* side rather than to the *cis* side from which the peptide originated.

These considerations thus eliminate all models that cannot provide an explanation of the I-V curve asymmetry, and they strongly support the α-helix gating hypothesis.

Channel Stability

The ionic strength dependence of the rate constant is consistent with the gating picture previously presented. Fig. 8 shows a simplified energy barrier diagram for transition from the open state to the closed state. The energy of the closed state is taken as the reference. We assume the energy of the transition relative to the closed state is not altered by changes in ionic strength. We further assume that the open state consists of parallel monomers arranged on the periphery of a cylinder as shown in Fig 9. The net interaction energy of these monomers consists of two parts: a relatively ionic-strength–independent part arising from hydrophobic interactions, hydrogen bonding and the like, and an electrostatic interaction term summing all the charge interactions. This term will be essentially zero in high-ionic–strength media where the debye length

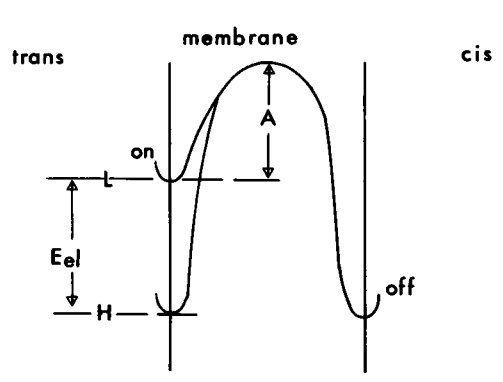

FIGURE 8 Idealized energy level diagram for the open channel state and the closed state at high and low ionic strengths. The difference in energy is assumed to be given by only the nearest-neighbor repulsions of the amino terminus. *Curve L* shows the energy profile at low ionic strength and *curve H* the profile at high ionic strength.

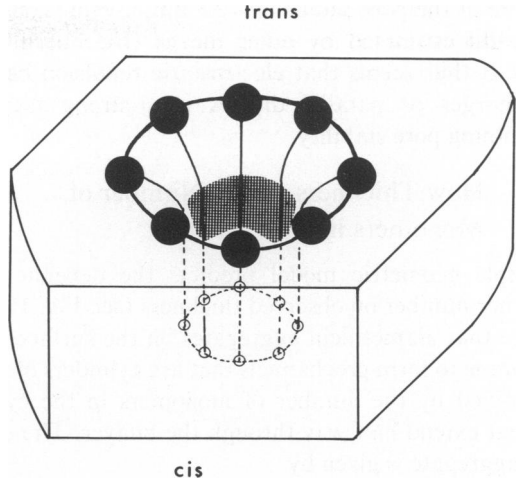

FIGURE 9 Geometry of the open channel showing how the radius of the pore is related to the distance between the effective charges. The negative charges at the amino end of the α-helix are assumed to be on a larger circle than those at the carboxyl end of the molecule.

is short compared to the dimensions of the channel. In low-ionic–strength media it will be the sum of all the coulomb interactions between the charges. We are interested principally in repulsive terms, and it is clear that adjacent charges repel most strongly. We therefore consider only nearest-neighbor repulsive interactions. We discard repulsive interactions at the negative end of the molecule on grounds that they are nullified by a structural feature of the molecule. Thus the energy of the open channel at low ionic strength has an electrostatic term of the form

$$E_{el} = \frac{nq^2}{4\pi\epsilon_0\epsilon_{hc}r_m}\exp(-r_m/\lambda_0) \tag{9}$$

where n is the number of monomers in an aggregate, q the gating charge of one monomer, r_m the separation of nearest neighbors, λ_D the debye length, ϵ_0 the permittivity of free space, and ϵ_{hc} the membrane dielectric constant. This term will be zero at high ionic strength.

We assume the time constant is given by an expression of the form

$$\tau = \tau_0\exp(-A/kT) \tag{10}$$

where τ is a parameter, A the ionic-strengt—independent part of the activation energy, E_{el} the repulsive energy from Eq. 9, k Boltzmann's constant, and T the temperature.

The ratio of τ_h at high ionic strength, where E_{el} is zero, to τ_l at low ionic strength, where E_{el} is full strength, is then

$$\frac{\tau_h}{\tau_l} = \exp(E_{el}/kT). \tag{11}$$

Since this ratio is experimentally found to be about 900, E_{el} has a value of ~6.9 kT. If we assume 10 monomers and a charge of 0.5 electronic charges, this value of E_{el} gives an

estimate of the pore radius of ~0.8 nm, a value consistent with radii estimated by other means (Eisenberg et al., 1973). It thus seems that electrostatic repulsion between like charges or parallel dipoles is a strong factor in determining pore stability.

How Thickness Alters Number of Monomers in a Channel

A simple geometric model predicts the dependence of monomer number on observed thickness (see Fig. 10). We assume that alamethicin aggregates on the surface of the membrane to form prechannels that are cylinders of radius determined by the number of monomers in the cylinder and that extend half way through the bilayer. The energy of an aggregate is given by

$$E = E_A - n E_M \tag{12}$$

where E_A is a destabilizing energy depending on the surface area of the cylinder exposed to water and E_M is an attractive energy assumed to be due to the binding of one monomer to the aggregate.

The surface area exposed to water, not counting the ends, which are assumed to be hydrophilic, is

$$A = \frac{2r^2}{d}\sqrt{4l^2 - d^2} \tag{13}$$

where l is the length of the cylinder, d is the thickness of the membrane, and r is the radius of the cylinder. We estimate the radius of the cylinder, r, as

$$r = \frac{C}{2\pi} = \frac{n r_m}{\pi} \tag{14}$$

where C is the circumference of the cylinder, r_m is the radius of a monomer, and n is the number of monomers. If H is the hydrophobic energy per unit exposed surface area,

$$E_A = n^2 H\left\{\frac{2r_m^2}{d}\sqrt{4l^2 - d^2}\right\}. \tag{15}$$

Minimizing the total energy with respect to n gives the most likely aggregate size as

$$n = Q\, d\sqrt{4l^2 - d^2} \tag{16}$$

where Q is a parameter made up of E_s, H and r_m. If we choose Q = 5.9 nm and l = 1.7 nm, Eq. 18 predicts the numbers of monomers shown in Table IV. The values of n deduced from experiment are also shown for comparison. If we assume that the values of n for the two thinnest membranes are off by a factor of two for reasons discussed earlier, the agreement between this simple model and the observed results is quite good. The model breaks down at the point where half the membrane thickness becomes equal to the α-helical length of the first 10 residues of alamethicin, that is d = 3.4 nm. The only membrane thicker than 3.4 nm is monoerucin, where experimental difficulties make it too difficult to obtain reliable values for V_a and thus the number of monomers.

TABLE IV
COMPARISON OF NUMBER OF MONOMERS IN A CHANNEL OBSERVED AND CALCULATED FROM GEOMETRIC MODEL.

d	n	n^+	n_{model}
nm			
2	1.8	4	4.3
2.23	3.0	6	5.4
2.6	7.4	7	7.0
2.8	11.0	11	11.06

Note n^+ is the number of monomers assuming the gating charge per monomer is 0.5e. n_{model} is calculated from Eq. 18 with q = 5.9 and l = 1.7 nm.

Significance of Cation Selectivity of Charged and Uncharged Analogues

Because both the charged and uncharged analogues of alamethicin are weakly cation selective, the cation selectivity, as suggested earlier, must reside in structural features of the molecule other than its formal charge. Somehow the lumen of the pore must be lined with effectively electronegative groups. Fox and Richards (1982) find that two carbonyl groups, which would be located near the center of the membrane, are solvent accessible. These groups may contribute to the cation selectivity. The shape of the channel may also be a factor. If the channel were cone-shaped with at the amino end radius larger than the carboxyl end radius, it would be cation selective because of the radial component of the gating dipole. (See, for example, Fig. 10 and our proposed structure in Figs. 11 and 12.) This model predicts interaction of the gate and the permeant ions and that the channel should be more stable with ions in it than otherwise.

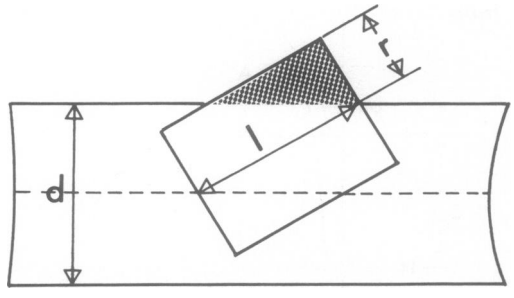

FIGURE 10 Geometry of a simple model to explain the decrease of number of monomers in a channel with membrane thickness. r is aggregate radius, d is membrane thickness, and l is the length of the off-state channel. The shaded area is exposed to the water, and the energy of exposing that surface is assumed to be proportional to the surface area (Reynolds et al., 1974).

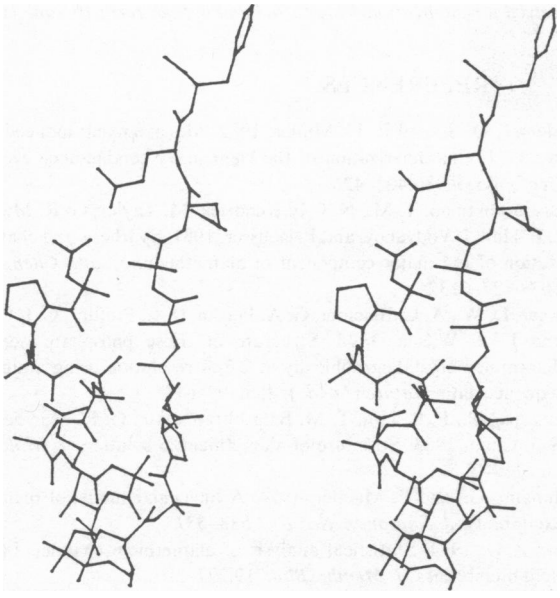

FIGURE 11 Stereo view of a monomer of alamethicin in the closed channel configuration. We have no direct evidence for the number of monomers in a closed channel; indeed this number probably varies with membrane composition. The photolabeling experiment of Latorre et al., 1981, suggests that there would have to be at least three monomers in the closed channel to prevent photolabeling of the carboxyl amino acids.

A Plausible Structure Incorporating These Features

As a specific proposal incorporating most of the points previously discussed, we offer a structure for the alamethicin pore. This structure has been formulated with both electrical measurements and recent structural results in mind and has been designed to include a gate and a stabilizing end. We began at the amino terminus with an α-helix 10 residues in length. The helix gives the molecule a gate having an effective charge 0.5 and is consistent with the available structural data. Latorre, Miller, and Quay, (1981), found that photoactivated groups attached to phospholipids at the terminal methyl position of the fatty-acid chain reacted only with the amino half of the alamethicin molecule. We believe this result requires that the first 10 residues from the amino terminus must be in the membrane.

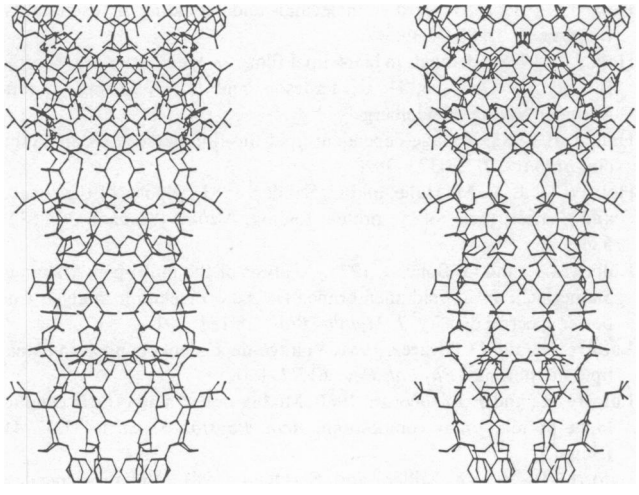

FIGURE 12 Stereo view of alamethicin channel model in the open state (eight monomers). The bar to the right of the figure is 1.8 nm long, and the dashed lines (---) show where the surfaces of a 3.6 nm membrane would be located. In membranes made of monoglycerides, channels are most stable when membrane thickness is 3.0 nm. The single-channel level may change because monomers enter and leave the barrel-like aggregate, or it may change because the conformation of the aggregate changes. There is at present no convincing evidence ruling out either possibility.

Electrical measurements require that the gating charge and thus the NH_2-terminus itself be at the *cis* surface of the membrane. Because the COOH-terminus must also be at the surface of the membrane, we concluded that the molecule must bend. The residue sequence –Gly–Leu–Aib–Pro from residues 11–14 is a sequence very likely to produce produce a β-bend (Chou and Fasman, 1978). We thus decided to bend the alamethicin molecule at that point and extend the peptide chain back to the surface where the COOH-terminus must be. The next question is the conformation of the part of the chain going back to the surface. Here we considered both the electrical measurements comparing Fraction 4 and ALM-17 and the ability of alamethicin to form parallel β-sheet dimers in methanol. We reasoned that a plausible structure for this part of the chain is a parallel β-sheet. This structure is suggested by the NMR results of Bannerjee et al., 1983, and can also stabilize the open channel against the strong electrostatic repulsion of the parallel dipole moments of the α-helices. The monomers thus form a two-layered barrel with β-sheet on the inside and α-helix on the outside.

This model can be consistent with the electrical results only if the energy of the negative charge near the geometric center of the membrane does not change during gating. This would be the case if in the off-state the half channel were filled with water, making the positive charge and the negative charge at the same electrical potential. When the channel opens, some of the β-sheet might be converted to α-helix, thus keeping the negative charge near the *cis* side of the membrane. Alternatively, when the channel is open, the negative charge may be close enough to the *cis* side that it sees very little of the electric field. If one of these does not happen, the applied voltage will raise the energy of the negative charge in the open channel form, and reduce the effective gating charge.

Fig. 11 shows a stereo view of the closed-form monomer we propose. This form is not totally arbitrary from the point of protein structure. It does in fact exist as a structural element of triose phosphate isomerase (TIM), and our structure was constructed by taking the conformation of residues 110 to 130 of TIM (Banner et al., 1975) and replacing the amino acids with the alamethicin sequence. We then allowed a computer program that

minimizes residue interaction energy to operate on this comformation. The algorithm makes small variations in the torsion angles and searches for a downhill path on the multidimensional energy surface. By making only very slight alterations in the torsion angles, the program lowered the calculated interaction energy by several hundred kilocalories per mole, indicating that our monomer conformation is in a deep local mimimum.

For the "on" state of the pore, we propose that the voltage across the membrane causes the rotation of the dipole so that the amino terminus crosses the membrane and the helix rotates across the membrane by forcing the β-bend to become helical, a transformation usually forbidden but here made possible by the large electric field. Our view of the open-channel conformation is shown in Fig. 12 as a stereo pair. Note the cone angle of the α-helices and the location of the membrane. We believe there is some possibility that some of the residues shown here in the β-conformation may be converted to the α-conformation in the "on" state. The possibility of field-induced shift from β to α-form has been considered by Latorre, Miller, and Quay, 1981, and by Boheim, 1975.

The number of monomers in the both the "off" and the "on" configuration varies, and is affected by membrane composition. But even in a membrane of a given composition, there is probably a distribution of aggregate sizes. To agree with the Latorre, Miller, and Quay photolabeling experiment, and to satisfy all β-sheet hydrogen bonds, our "off" channel would have to have at least three monomers. We have not addresssed the question of whether an "off" n-mer is converted directly to an "on" n-mer of the same size or builds up its size by postinsertion aggregation. The essential features of our structure can accommodate both possibilities. We are presently testing this question by synthesizing a covalent dimer of alamethicin, which, according to our structure, should be able to form channels much as Fraction 4 does.

It has not escaped our notice that the structure we propose for alamethicin can be easily generalized to a form more suited to high molecular weight single-chain proteins which might form voltage-gated channels. Triose phosphate isomerase has an eight-chain β-barrel surrounded by eight α-helices (Banner et al., 1975). If a protein like TIM were situated in a membrane, its β-barrel, if perpendidular to the membrane, would span the membrane, and the α-helices would see all of the applied electric field. By changing the orientation of the α-helices, the electric field could alter their mutual electrostatic repulsion and thus exert radial force on the β-barrel, thereby changing its shape and producing the gating action.

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DISCUSSION

Session Chairman: V. Adrian Parsegian *Scribes*: Michael Cascio and Ross Flewelling

ANDERSEN: Does the model you presented allow you to account for the multi-conductance levels that are observed in the open state? If you look at the analogues of alamethicin do you see changes in the pattern of transitions within each open event? And do such changes fit into your notions of how the channel should behave?

HALL: The single channel conductances could be determined by the alteration in the cross-sectional area of the β-barrel due to intramolecular interaction. The channel would probably not open until all the α helices flipped down. This model does not explain the single channel levels very well. However, Gunther Boheim, Gunther Jung, and Wolfgang Hanke (1983. Alamethicin-pore formation—voltage-dependent flip-flop of α-helix dipoles. *Biophys. Struct. Mech.* 9:181–191) have proposed a model that has a number of advantages over ours. Perhaps it can explain the single channel levels better. Their model does not address in the way ours does the problem of what holds the channel together when it is open. We may be able to merge the models and come up with a model that really works.

In Boheim's model, a few of the monomers flip across to the other side of the membrane so that they point in opposite directions and aggregates are built up which are held together by electrostatic attraction of antiparallel dipoles. When an electric field is applied, the monomers rotate to a parallel configuration. The single channel conductances can then be explained: one flip, one level change. We agree with Boheim that as the ionic strength is decreased the channel lifetime diminishes, and the probability distribution is shifted from the lower channel levels to the higher channel levels.

In answer to your question, yes, we can sort of hoke-up an ad hoc explanation of why the single channels are there, but it is not all that satisfying. We think the COOH-terminus sticks the molecule together and the α-helical amino-terminus is responsible for the gate.

RUDY: Does the number of single levels change as you change the thickness?

HALL: Yes. For natural alamethicin there is a correlation between the apparent monomer number in the macroscopic observations and the number of single channel levels that we observed in one pore.

RUDY: And what about the case for the derivatives?

HALL: Alamethicin BG, the back-gating derivative, does show good single channel levels, but we have not studied the thickness dependence. The levels are very different from natural alamethicin in that there are multiple levels and the channel is much noisier. So we cannot pull the same tricks that we can with natural alamethicin to separate statistically which levels are due to a single pore and which are due to multiple pores.

Alamethicin BOC 2-20 is even worse than BG because it does not show good, crisp single channels at our frequency resolution. It is interesting that analogues showing quite different single-channel characteristics have the same kind of voltage dependence. So there is some decoupling between the voltage dependence and single channel properties.

RUDY: Is there a power relation for the salt concentration?

HALL: There is, but it is probably due to changes in the adsorption of the alamethicin to the membrane surface (Gordon and Haydon, 1975).

MISLER: Has your purification procedure helped alleviate the problem of the multiplicity of states?

HALL: No. For natural alamethicin there are inherently five to seven states in an open pore, and a channel fluctuates amongst those states. That is a fundamental characteristic of the conductance. However, purification does remove noisy trash.

DONOVAN: You mentioned that the BOC 2-20 channels are much faster than the two charged types. Perhaps they are fluctuating so fast that you are just getting a time average and hence your I-V curve looks smoother?

HALL: Yes, that would certainly explain our data.

DONOVAN: Is your time resolution good enough to distinguish the fast fluctuations?

HALL: We don't know how good our resolution needs to be to see single channels.

DONOVAN: What happens if you mix the different types of alamethicin?

HALL: We have done a few preliminary mixing experiments to see if the different classes of alamethicin, F4, BOC 2-20, and BG interact with one another. If you add reverse-gating (BG) alamethicin alone to one side of the membrane, you get an I-V curve which shows strong rectification (Fig. 1 *C*). And if you add the same concentration of forward-gating (natural) alamethicin to the same side of the membrane you get an I-V curve that looks like that in Fig. 1 *A*. There is no change in the single channel characteristics and thus no apparent interaction between these two polypeptides when they are added to the same side of the membrane. We think that for BG the COOH-terminus goes down into the membrane to gate. That would mean that the amino groups of the natural peptide cross the membrane and that the amino groups of the BG remain on the surface.

On the other hand, if you add the peptide to opposite sides of the membrane you do see a minimal interaction between them. If F4 is added to one side of a membrane and BG is added in increasing amounts to the opposite side of the membranes, the time constant of F4 decreases as a single exponential function. The conductance also decays faster as you add more BG. If the two conductances were independent, you would

expect two time constants. This was not the case. We have preliminary evidence that when the two forms are added to the opposite sides of the membrane they form a hybrid channel, but when they are added to the same side of the membrane they do not.

FINKELSTEIN: In going from the thick membrane to the thin membrane, are you seeing a change in the molecularity, i.e., the number of monomers per channel?

HALL: Just looking at the raw data and not making any assumptions, the molecularity increases from two monomers in the thin membrane to eleven in the thick.

FINKELSTEIN: Are you seriously proposing that two monomers form a channel?

HALL: No, we aren't proposing that. We can deduce the apparent gating charge because we know the number of monomers and we know the slope of the current voltage curve. If we assume the charge moves across the membrane, that gives us the apparent gating charge. α helices look as though they have half an electronic charge at each end, positive at the amino end, and negative at the carboxyl end. Disregarding the two thinnest membranes, for all the analogues we always get an apparent gating charge of about half, which would be consistent with an α helix being the gate. As we go to the thinner membranes, especially monomyristolein where, supposedly, we see two monomers forming a channel, the gating charge doubles and it becomes 0.9 (Table III). This may be an artifact due to aqueous chemistry problems. In the thin membranes for some reason the dimer or a higher monomer may be in partition equilibrium with the membrane. If this were the case, we would be looking at the power dependence of the dimer or the monomer going into the membrane and we would be thrown off in estimating the molecularity.

FINKELSTEIN: What do you think is the molecularity of the channel formed in the thin and thick membranes?

HALL: My guess is that in the thin membrane it is four, and in the thick membrane it is eleven (see Table IV).

FINKELSTEIN: What would be the diameter of the channel in the lowest and highest conductance states predicted by your model?

HALL: The diameter of the lowest state would be <4 Å. In the highest state it may get up to 20 Å.

FINKELSTEIN: It is my understanding that these two levels show very little difference in selectivity of permeant ions. You show a factor of five difference in diameter, which should be a very striking difference, and you are getting this effect simply by changing the thickness of the lipid. This is something I do not understand in the modeling.

KOLB: There is an appreciable zero-voltage conductance and excess noise at zero millivolts. How can you explain this with your model and how does it change with membrane thickness?

HALL: As you say, not only does alamethicin exhibit a strongly voltage-dependent conductance but in some membranes there is a zero-voltage or voltage-independent conductance (cf., Fig. 3). In our monoglyceride series of membranes, we found the power dependence of this zero-voltage conductance also changed with thickness. It was quite different from the apparent power dependence of the voltage-dependent conductance. In the thinnest membrane, that is the monomyristolein, the power dependence was essentially flat; the zero-voltage conductance changed less than a power of one with the aqueous concentration of the alamethicin. This could be explained by the previous argument concerning the change of partition of monomer and dimer. As you go up to the thickest membrane you get a maximum power dependence of ~2. Guy Roy was the first to publish information on this zero-voltage coductance and he also found a lower power dependence of the zero-voltage conductance on the aqueous concentration of alamethicin. (Roy, G. 1975. Properties of the conductances induced in lecithin bilayer membranes by alamethicin. *J. Membr. Biol.* 24:71–75). This voltage-independent conductance may be due to a half channel of alamethicin surrounded by only a monolayer. Igor Vodyanoy has measured the thickness dependence of a bare bilayer conductance: it's very large. For a factor of two change in thickness, one can get a 1,000-fold change in conductance. It is possible that a large population of closed half channels may appreciably increase the conductance of the whole bilayer. Alterations in the dielectric constant of a membrane could result in the increased conductance across a membrane. Insertion of monomer or antiparallel aggregation in thin membranes could raise the dielectric constant.

I. VODYANOY: One can see the dependence of our model on changes in monomer number and size of the pore by lookng at Fig. 12. There is an 18-Å bar which is approximately the diameter of this eight monomer structure. Taking four monomers, the diameter decreases more than twice, which accounts for the cross-sectional area of the channel being much less than that of eight monomers.

LATORRE: Experiments by Hanke and Boheim showed that the zero level conductance is almost the same as that gramicidin channels but that the selectivity is much more pronounced. I think this is a very important experiment that supports the aggregation model.

MAKOWSKI: I wanted to ask you about the model of Fox and Richards based on the crystallography. There are some good structural reasons for thinking that alamethicin will have a different structure in a bilayer than in a crystal. Are your data consistent with their model? In particular, one problem with their model might be the electrostatic repulsions between alamethicin monomers. Could you elaborate on the ionic strength dependence of alamethicin?

HALL: I think that Fox and Richards' structural work is very valuble and an extraordinarily useful tool for people who are trying to build models of alamethicin. However, it seems intuitively obvious that the structure of alamethicin in a bilayer bears an unknown relationship to the structure of the crystal. Much of the structural work on alamethicin, including that of Fox and Richards, shows the amino end of the molecule to be α-helical in almost any environment while the COOH-terminus shows variable structure even in the crystal. Work done in organic solvents, NMR studies by Chan and Bannerjee, and CD studies by Cascio and Wallace (*NY Acad. Sci.*, in press), all find quite variable conformation of the COOH-terminus.

In response to your question about ionic strength, the pore is large enough to contain a fair amount of water. When the Debye length is dropped down to <4 Å, the effective charges on the end of the molecule in the open channel form (Fig. 9) don't see each other much. At low ionic strength, the Coulomb interaction between the monomers increases and the structure is destroyed (Fig. 8). I think that both Boheim's and our data show a shift to the higher levels of conductance with decreased ionic strength. These levels are presumably pores with larger diameters. We also see an increased rate of channel closings.

CASCIO: Recent work in our lab shows that the organic solvent systems used in the crystallographic studies of Fox and Richards and the NMR studies of Bannerjee and Chan are inappropriate. Looking at the circular dichroism of alamethicin in both the above-mentioned solvent system and in DMPC vesicles, we saw significant secondary structure differences in the two environments.

BLANK: The potent effects of charge in these systems are generally interpreted in the context of channel research, but many other systems are also governed by slight changes in charge. For example, it is possible to

conceive of channel mechanisms on the basis of oligomeric equilibria in which there is an asymmetric distribution of the charge (see Blank. 1982. *Bioelectrochem. Bioenerg.* 9:615–624). In such a system, the large charge on one side of a polarized membrane causes that part of the system to "disaggregate" or be in an open configuration, while the lower charge on the other side causes the molecules to be more tightly associated, or in a closed configuration. Slight changes in polarization will lead to a shift of the charge, and the low charge surface may reach a point where it will cause the channel to open up. In the dissociation of hemoglobin, the charge density at which tetramers become dimers is equivalent to that on the inner face of the resting squid axon. It is quite possible that the effects you are getting with alamethicin channels can be explained by the aggregation-disaggregation phenomenon. Our oligomeric channel model has been used to account for the responses of a membrane to voltage clamp (see Blank. 1983. *Bioelectrochem. Bioenerg.* 10:451–465).

SACHS: I wonder if the molecular dynamics of the multimeric channels are not very important for determining selectivity. There is usually a great amount of unresolved variation about a mean conductance, but this variation is often at frequencies too large to measure. The channel is actually changing size, so it may be very hard to pick up sieving differences of conductance levels since it is only the mean that is fixed and there is actually a lot of variation during that time. Do you have any feeling for this?

HALL: It is very clear that you can see large fluctuations in the single-channel conductance. The fluctuation of a single level is much greater than instrumental noise. We know that the alamethicin channel is a dynamic structure, and the single-channel conductance is determined by this structure that's changing a lot.