Alamethicin, a Transmembrane Channel

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Peptides possessing antibiotic activity, isolated from microbial sources, have been the subject of intensive structural and biological investigation over the past two decades. While few are clinically useful, many chelate alkali and alkaline earth metal ions, interact strongly with artificial and natural membranes and serve to transport cations across lipid bilayers (ionophore activity).[1] Hence, these peptides have been extensively used to study transmembrane ion transport in model lipid membranes, cells, and organelles.[1] The major impetus for the study of cation transport is derived from the crucial role played by cation fluxes, across biological membranes, in modulating cellular functions.[2]

Peptide ionophores may be broadly classified into two groups (Figure 1), carrier ionophores and transmembrane channels. Carrier ionophores, which are generally cyclic peptides, function by selective chelation of metal ions whose complexes are lipophilic enough to diffuse through the lipid bilayer. Examples in this category include the depsipeptides valinomycin and enniatin, whose structures and cation binding properties have been thoroughly investigated.[1] Linear, acyclic polypeptides function as transmembrane channels for ion transport.[3] Examples include the linear gramicidins[4] and the α-aminoisobutyric acid (Aib) containing polypeptides like alamethicin,[5] suzukacillin,[6] antiamoebins,[5] emerimicins,[5] trichotoxin A-40,[7] and hypelcins[8] (Figure 2).

The observation by Mueller and Rudin[9] that alamethicin induces excitability in artificial lipid bilayer aroused tremendous interest in the study of alamethicin–lipid interactions. Studies on alamethicin-modified electrical conductance across lipid bilayers[9-14] indicate that the mechanism by which ions are transported across lipid bilayers is by the formation of channels or pores, as in the case of gramicidin A. However, unlike gramicidin A, alamethicin shows a strong voltage-dependent conductance. Studies on the concentration dependence of the channel conductance suggest that 6–8 molecules may be involved in an aggregate.[14] Further, the cation conductance by the peptide does not show any specificity. In spite of numerous reports,[3,15] a clear picture regarding the molecular structure of the pores and the manner in which they modulate passage of ions through the bilayer has not yet emerged. Attempts to develop structure–function relationships have in part been obscured by an early proposal of a cyclic structure for alamethicin.[16,17] Recent 270-MHz ^{1}H NMR[18] and field-desorption mass spectrometric studies[5] have provided firm support for the acyclic sequence in Figure 2. Synthetic studies have also established that the cyclic sequences lack pore-forming activity whereas the acyclic sequences are active.[19,20] Recent work on the other Aib-containing antibiotics[5-8] indicate that they modify electrical properties of lipid bilayers in a manner similar to alamethicin. Other membrane-modifying properties of alamethicin include its ability to cause fusion of lipid vesicles[21] and lysis of natural membranes like erythrocytes[22] and leucocytes.[23] The ability of alamethicin to form channels and act as a detergent has been used to study sidedness of natural systems like lymphocyte plasma membranes[24] and cardiac muscle cell membranes.[25]

While the structure and properties of the linear gramicidins have been intensely investigated for a number of years,[26-33] attempts to study alamethicin

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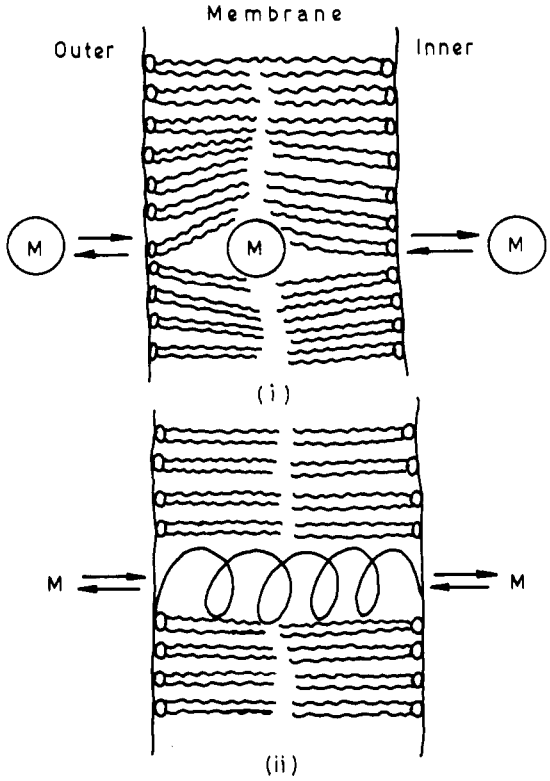

Figure 1. Passage of metal ions through membranes modulated by (i) carriers or (ii) channels or pores.

Gramicidin A[4] : HCO-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-CH_2-CH_2-OH

Alamethicin F50[5] : Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol

Suzukacillin[6] : Ac-Aib-Pro-Val-Aib-Val-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phol

Antiamoebin[5] : Ac-Phe-Aib-Aib-Aib-Iva-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp-Aib-Pro-Phol

Emerimicin III[5] : Ac-Phe-Aib-Aib-Aib-Val-Gly-Leu-Aib-Aib-Hyp-Gln-Iva-Hyp-Ala-Phol

Trichotoxin A-40[7] : Ac-Aib-Gly-Aib-Ala-Aib-Glu-Aib-Aib-Aib-Ala-Aib-Aib-Pro-Leu-Aib-Iva-Gln-Valol

Hypelcin A[8] : Ac-Aib-Pro-Aib-Ala-Aib-Aib-Gln-Leu-Aib-Gly-Aib-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Leuol.

Figure 2. Sequence of channel-forming antibiotics.

channels in molecular detail have been relatively more recent. Alamethicin and related polypeptides (Figure 2) provide simple systems for the study of gateable membrane channels.[34] An understanding of such systems would be extremely useful in developing molecular models for studies of the immensely more complex channel structures, formed by proteins in cell membranes.[35] Three basic issues need to be addressed: (1) What are the conformations favored by the Aib-containing channel peptides? (2) What are the structural requirements for the observation of membrane-modifying activity? (3) Can a definitive correlation be established between peptide conformation and channel-forming ability?

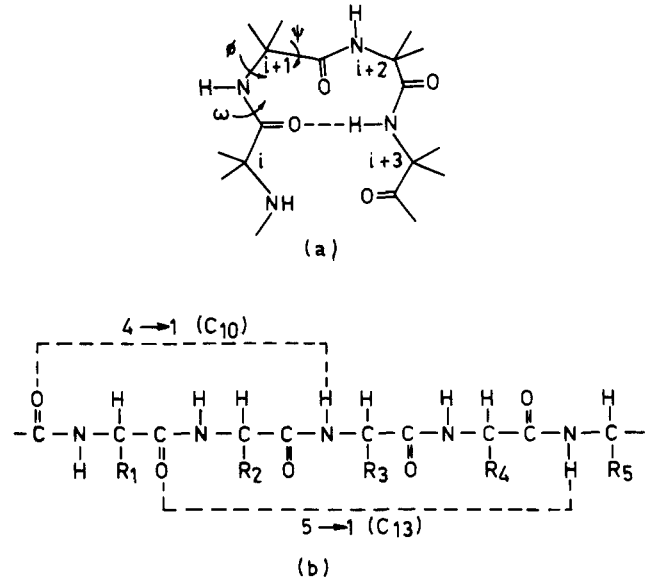

Figure 3. (a) Peptide chain folded to illustrate a β-turn conformation, stabilized by a 4→1 hydrogen bond. The C^α atoms of the four amino acid residues involved are designated by the symbols i to $i + 3$. The conformational angles ω (C^α–C–N–C^α), ϕ (C–N–C^α–C), and ψ (C–C^α–C–N) are indicated. (b) Hydrogen-bonding patterns frequently observed in peptide chains. Repetitive structures stabilized by 4→1 (C_{10}) hydrogen bonds are known as 3_{10} helices. The α helix is stabilized by 5→1 (C_{13}) hydrogen bonds.

In an attempt to provide answers to these questions, we have undertaken the synthesis and conformational analysis of alamethicin and related polypeptides and developed methods for the assay of the membrane activity of the synthetic fragments.[36-50] The results of our studies are summarized in this Account.

Conformations of Model Aib Peptides

Assuming rigid trans planar peptide units, the conformations of a peptide chain may be described in terms of two degrees of freedom, ϕ and ψ, which correspond

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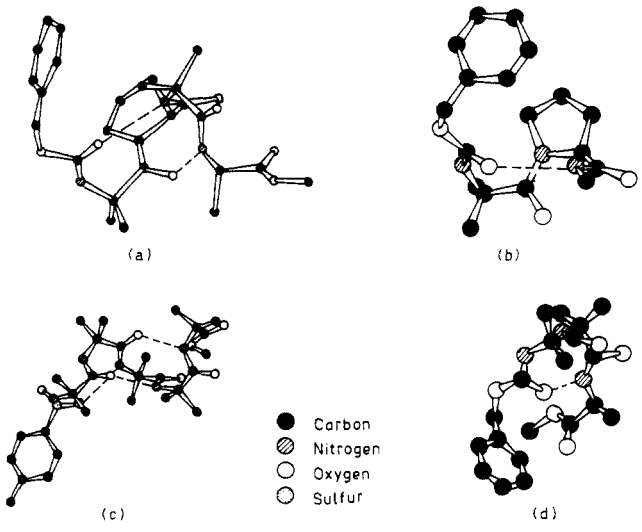

Figure 4. Crystal structures of (a) Z-Aib-Pro-Aib-Ala-OMe, (b) Z-Aib-Pro-NHMe, (c) tosyl-$(Aib)_5$-OMe, and (d) Z-Aib-Aib-Ala-OMe.

to rotations about the N–C^α and C^α–CO bonds, respectively (Figure 3). Peptide conformations may then be represented on a two-dimensional (ϕ,ψ) map in which sterically allowed regions can be defined.[51] For acetyl-Aib-*N*-methylamide, only a very small region of ϕ,ψ space is "allowed", corresponding to the regular 3_{10} and α-helical conformations.[52-54] These repetitive structures are characterized by different intramolecular hydrogen-bonding schemes. In the α helix the NH of the $i + 4$ residue is hydrogen bonded to the CO of residue i (5→1) whereas in the 3_{10} helix[55] the $i + 3$ NH group is involved in a 4→1 hydrogen bond (Figure 3). It may be noted that the 3_{10} helix is formed by a repetition of a short-range structural feature known as the β turn (Figure 3).[56] While an isolated β turn reverses the direction of peptide chain propagation, consecutive β turns generate an incipient helical structure. Various types of β-turn conformations have been classified on the basis of the ϕ,ψ angles at the corner residues.[56,57]

An initial study[37] of the solution conformation of Z-Aib-Pro-Aib-Ala-OMe (Z = benzyloxycarbonyl, OMe = methyl ester) by ^{1}H NMR indicated that the tetrapeptide adopted a highly folded incipient 3_{10} helical conformation stabilized by two intramolecular 4→1 hydrogen bonds. An X-ray crystallographic study revealed that this conformation is indeed maintained in the solid state (Figure 4a). Two consecutive type III β turns[56] are detected with Aib(1)-Pro(2) and Pro(2)-Aib(3) as the corner residues. The conformational angles (Table I) are close to those expected for a 3_{10} helix.[51] Similar features have been observed in the crystal structure of Boc-Pro-Aib-Ala-Aib-OBz (Boc = *tert*-butyloxycarbonyl, OBz = benzyl ester).[58] The propensity

Table I
Backbone Dihedral Angles from Crystal Structures of Aib-Containing Peptides[a]

peptide[a]	residue	ϕ	ψ	ω
Z-Aib-Pro-Aib-Ala-OMe[37]	Aib(1)	−50.7	−45.6	−171.0
	Pro(2)	−54.9	−36.0	−170.1
	Aib(3)	−71.7	−11.0	−173.1
	Ala(4)	−67.9	155.5	
Z-Aib-Pro-NHMe[39]	Aib(1)	−51.0	−39.7	−174.0
	Pro(2)	−65.0	−25.4	179.7
Tos-$(Aib)_5$-OMe[38]	Aib(1)	61.6	25.0	179.1
	Aib(2)	50.6	38.6	173.1
	Aib(3)	54.3	35.7	173.2
	Aib(4)	64.2	24.1	171.7
	Aib(5)	−53.3	−37.5	
Z-Aib-Aib-Ala-OMe[40]	Aib(1)	58.1	36.8	175.8
	Aib(2)	68.3	18.6	−177.7
	Ala(3)	−136.2		
Boc-Leu-Aib-Pro-Val-Aib-OMe[b]	Leu(1)	−103.8	−29.5	178.2
	Aib(2)	−46.3	−41.5	−171.5
	Pro(3)	−64.5	−15.5	173.7
	Val(4)	−59.2	−37.8	174.4
	Aib(5)	51.2	43.0	

[a] Convention recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry* 1970, *9*, 3471. [b] Ch. Pulla Rao, unpublished results.

of Aib residues to adopt 3_{10} helical conformations ($\phi \sim \pm 60°$, $\psi \sim \pm 30°$) is further established by the crystal structures of *p*-toluenesulfonyl-$(Aib)_5$-OMe[38] (Figure 4c), Z-Aib-Pro-NHMe[39] (Figure 4b), Z-Aib-Aib-Ala-OMe (Figure 4d),[40] Z-Aib-Pro-Aib-Pro-OMe,[47] and Boc-Leu-Aib-Pro-Val-Aib-OMe (unpublished). Conformational angles in these molecules are summarized in Table I. An unusual conformational feature observed in Aib-Pro sequences is the presence of an Aib-Pro β turn, which accommodates Pro in the $i + 2$ (right-hand corner) position. Such a feature has been found very infrequently in proteins[57] and has been suggested to be energetically unfavorable from theoretical calculations.[59] It appears that the stereochemical constraints imposed by the Aib residue, compel the pyrrolidine ring to occupy the $i + 2$ position.

The observed conformational preferences of Aib residues suggests that Aib-containing sequences cannot adopt the II (LD) type helical structure or the double helical conformations proposed for gramicidin A.[26,28] In these models the D residues would have values of $\phi \sim 105°$ and $\psi \sim 120°$. While Aib residues can take up positive ϕ,ψ values, generally adopted by D amino acids, the allowed regions of conformational space are restricted to values of $\phi \sim 60 \pm 20°$, $\psi \sim 30 \pm 20°$. These findings suggest that the Aib-containing channel peptides must adopt structures entirely different from those proposed for gramicidin A.

In studies of the conformation of Aib-containing peptides, excellent agreement has been obtained between the results of NMR[37,47,50] and IR[41,42] studies in solution and X-ray diffraction studies in the solid state.[36-40,47] This is presumably a consequence of the limited conformational flexibility of even small peptides containing Aib residues. Aib residues may therefore be used in synthesizing conformationally well-defined model peptides for use in the development of spectroscopic methods of conformational analysis[60] as well as in furthering our understanding of the forces determining peptide folding. Another possible application

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Table II
^{1}H NMR Parameters of Alamethicin Fragments

peptide	residue	NH chemical shifts, δ		$(d\delta/dT) \times 10^3$,[a] ppm/°C	$J_{HNC^\alpha H}$, Hz[b]		no. of intramolecular hydrogen bonds	
		$CDCl_3$	$(CD_3)_2SO$		$CDCl_3$	$(CD_3)_2SO$	NMR	IR[c]
Z-Aib-Pro-Aib-OMe[37]	Aib(1)	5.33	8.04	5.97	-	-	1	1
	Aib(3)	7.39	7.56	2.24	-	-		
Z-Aib-Pro-Aib-Ala-OMe[37]	Aib(1)	5.83	7.93	5.47				
	Aib(3)	7.21	7.75	4.92			2	2
	Ala(4)	7.52	7.49	2.96	7.0	7.3		
Z-Aib-Pro-Aib-Ala-Aib-Ala-OMe (1-6 fragment)[50]	Aib(1)	5.66	8.21	5.05				
	Aib(3)	7.52	7.75	3.41				
	Ala(4)	7.14	7.18	1.74	8.0	7.7	4	4
	Aib(5)	7.37	7.47	2.17				
	Ala(6)	7.53	7.55	1.24	8.0	8.1		
Boc-Gln-Aib-Val-Aib-Gly-Leu-Aib-OMe (7-13 fragment)[50]	Gln(1)	6.68	7.13	5.68	5.5	7.0		
	Aib(2)	7.09	8.25	5.68				
	Val(3)	7.54	7.36	2.50	5.5	7.7		
	Aib(4)	7.70	7.94	3.75			5	5
	Gly(5)	7.79	7.94	4.20				
	Leu(6)	7.74	7.57	2.47	8.8	7.7		
	Aib(7)	7.23	7.75	3.72				
Boc-Leu-Aib-Pro-Val-Aib-OMe (12-16 fragment)[42]	Leu(1)	4.98	6.87	6.23				
	Aib(2)	7.20	8.43	4.95				
	Val(4)	7.46	7.42	1.88	8.9	9.6	2	2
	Aib(5)	7.04	7.55	3.50				
Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (11-16 fragment)[42]	Gly(1)	5.97	7.06					
	Leu(2)	6.88	7.94	5.56	6.7	7.5		
	Aib(3)	7.63	8.29	4.49			2	3
	Val(5)	7.55	7.35	2.56	9.5	9.0		
	Aib(6)	7.23	7.51	3.13				

[a] The $d\delta/dT$ values are measured in $(CD_3)_2SO$. [b] Errors in J values are estimated to be ±0.4 Hz. [c] IR studies were done in dilute $CHCl_3$ solution (~5×10^{-3} M).

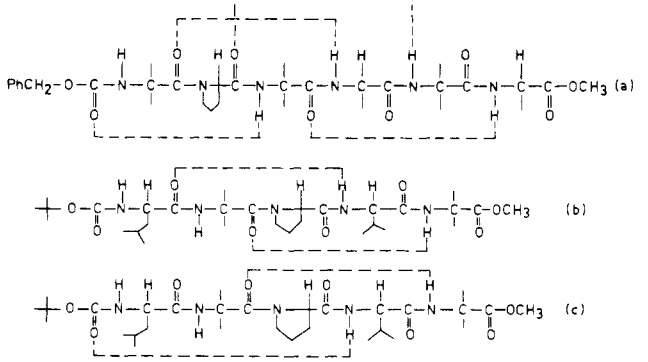

Figure 5. (a) Hydrogen-bonding scheme proposed for the alamethicin amino-terminal hexapeptide (1-6). (b and c) Possible hydrogen bonding patterns in Boc-Leu-Aib-Pro-Val-Aib-OMe. (b) Two 4→1 hydrogen bonds; (c) 5→1 and 4→1 hydrogen bonds.

would be the use of Aib residues in generating stereochemically constrained analogues of biologically active peptides in order to establish the conformational requirements for receptor interactions. This approach has been exemplified in studies on enkephalins[61,62] and other active peptides.[19]

Conformation of Alamethicin Fragments

Intramolecularly hydrogen-bonded NH groups have been delineated by means of rates of H–D exchange, solvent dependence of NH chemical shifts in $CDCl_3$–$(CD_3)_2SO$ mixtures, and temperature dependence of NH chemical shifts in $(CD_3)_2SO$.[63] Table II summarizes NMR data for various alamethicin fragments.

In the amino-terminal hexapeptide Z-Aib-Pro-Aib-Ala-Aib-Ala-OMe,[50] except for Aib(1) NH all the other amide hydrogens are intramolecularly hydrogen bonded in $CDCl_3$ and $(CD_3)_2SO$. The incipient 3_{10} helix formed by the first four residues thus appears to continue in the hexapeptide, leading to a structure involving four 10-atom (C_{10}) hydrogen bonds (Figure 5a). The vicinal coupling constants ($J_{HNC^\alpha H}$) for both Ala(4) and Ala(6) NH groups are ~8 Hz, compatible with the $\phi_{Ala} \sim -75°$, which is acceptable for a 3_{10} helical structure. In these studies the delineation of intramolecular bonds appears to be a more reliable index of conformation than $J_{HNC^\alpha H}$ since many residues lack the C^α hydrogen. Furthermore the Karplus–Bystrov curve is very steep in the region $\phi \sim -60 \pm 20°$, resulting in a wide range of J values for a very narrow region of ϕ.[64] The NMR parameters also support a highly folded conformation for the 7-13 fragment, Boc-Gln-Aib-Val-Aib-Gly-Leu-Aib-OMe in $CDCl_3$ and $(CD_3)_2SO$. The Val(3) and Leu(6) NH groups are strongly solvent shielded while the Aib(4), Gly(5), and Aib(7) protons have higher $d\delta/dT$ values, suggestive of a degree of flexibility. The NMR results are consistent with a 3_{10} helical conformation stabilized by five 4→1 hydrogen bonds. The $J_{HNC^\alpha H}$ values for Val(3) and Leu(6) are reasonably compatible with the requirements for a 3_{10} or α-helical fold of the peptide chain, but contributions from dynamic averaging cannot be ruled out. In general, in many of the peptides studied, there appears to be a tendency for the folded structures to be favored in $CDCl_3$, with some "loosening" in $(CD_3)_2SO$, which can hydrogen bond with peptide NH groups.

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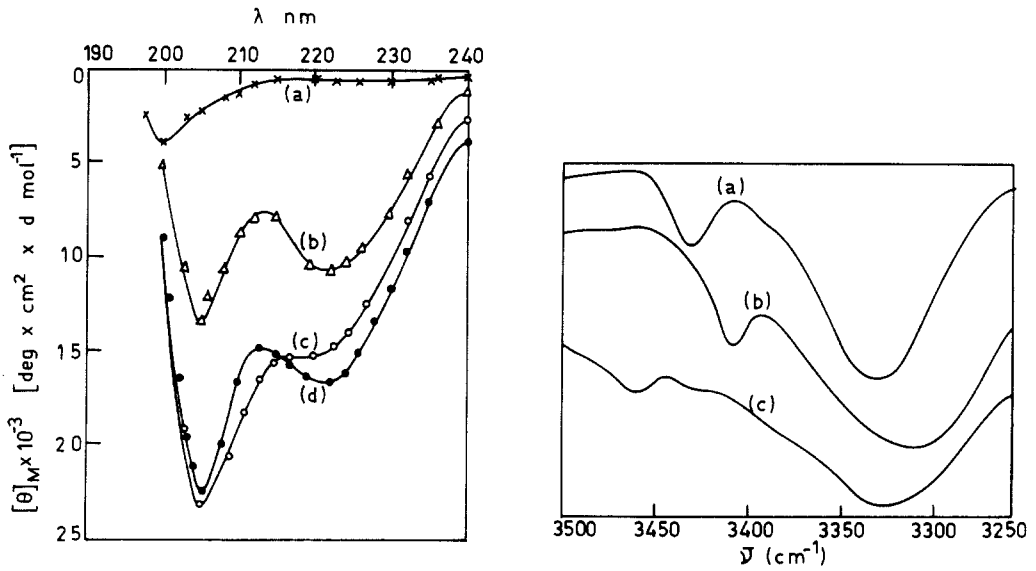

Figure 6. (Left) CD spectra of alamethicin fragments in trifluoroethanol: (a) acetyl-1-6-OMe; (b) Z-1-13-OMe; (c) Z-1-17-OMe; (d) synthetic alamethicin. (Right) NH stretching bonds in IR spectra of alamethicin fragment in $CHCl_3$: (a) Z-1-6-OMe; (b) Boc-7-13-OMe; (c) Z-1-13-OMe.

In the central fragments Boc-Leu-Aib-Pro-Val-Aib-OMe (12–16) and Boc-Gly-Leu-Aib-Pro-Val-Aib-OMe (11–16), NMR results suggest that the Val(4) and Aib(5) NH groups are hydrogen bonded. In both penta- and hexapeptides, the Pro residue occupies a central position, preventing the formation of the 4→1 hydrogen-bond-stabilized Leu-Aib β turn. It is therefore necessary to consider the possible formation of 5→1 (C_{13}) hydrogen bonds involving the Boc CO in the pentapeptide and Gly CO in the hexapeptide. Two possible hydrogen-bonding schemes are illustrated in Figure 5b,c. The $J_{HNC^{\alpha}H}$ of Val in these peptides is ~9.0 Hz, yielding $\phi \sim -100°$, which is moderately close to the values expected for C_{10} (3_{10} helix) and C_{13} (α-helix) conformations. A distinction between these structures is not possible only on the basis of ^{1}H NMR studies.[50]

The NMR data strongly support our contention that the 1–6, 7–13, 12–16, and 11–16 fragments of alamethicin adopt highly folded structures in solution, largely stabilized by 4→1 (C_{10}) hydrogen bonds. Thus a 3_{10} helical conformation appears to be strongly favored over a major portion of the alamethicin sequence and is modified only by the presence of Pro(14), with expansion to an α-helical conformation possible in segment 11–16.

Infrared and Circular Dichroism

Inferences about peptide conformation in dilute solution may be derived by examination of the NH and CO stretching bands. The integrated intensity of the hydrogen-bonded NH stretching band at 3330–3370 cm^{-1} (ν_{NH} (bonded)) has been used to quantitate the number of intramolecular C_{10} hydrogen bonds in dilute $CHCl_3$ solution. Assuming a value of 2 for the tetrapeptide Z-Aib-Pro-Aib-Ala-OMe, the number of hydrogen bonds calculated, from the integrated intensity for the alamethicin fragments, are summarized in Table II.[41,42] An excellent correlation between the NMR and IR data is clearly seen. IR studies of model Aib peptides have also yielded agreement between the number of intramolecular hydrogen bonds determined in dilute solution and in the solid state. Such quantitative correlations from the intensities of ν_{NH} (bonded) peaks are possible within a given series of similar peptides differing in chain length and appear to be useful in studies of conformationally restricted peptides. Analysis of the urethane ν_{CO} peaks in the 11–16 and 12–16 fragments appears to favor the 5→1 (C_{13}) hydrogen-bonded structures in these fragments.[42] However, a recent crystal structure analysis of Boc-Leu-Aib-Pro-Val-Aib-OMe has established the presence of two 4→1 (C_{10}) hydrogen bonds (Ch. Pulla Rao, unpublished).

The CD spectra of the 1–6, 1–13, and 1–17 fragments and synthetic alamethicin are shown in Figure 6a. Two negative bands at 205 nm (π–π^*) and 220 nm (n–π^*) are clearly discernible. The appearance of the CD spectra of these peptides is reminiscent of patterns obtained for α-helical polypeptides.[65,66] When the $[\theta]_M$ at 220 nm for the 1–13 and 1–17 peptides and alamethicin are compared with a value of −38 825 deg cm^2 dmol^{-1} for poly(Glu) in 100% α-helical form, a helix content of 27, 39, and 40%, respectively, is obtained.[50] The presence of secondary structure is also evident in the CD spectra of the central 7–13, 11–16, and 12–16 alamethicin fragments. A calculation of percentage helix is probably not very meaningful in these relatively small peptides. ^{1}H NMR and IR studies favor highly folded conformations in solution for alamethicin fragments, whereas the helix content estimated by CD studies is likely to be an underestimation. A knowledge of the effects of α alkylation on the CD properties of peptides and the chiroptical distinction of 3_{10} and α-helical structures is necessary before a quantitative interpretation of the CD spectra of Aib containing peptides can be attempted.[67-70]

Detailed ^{1}H NMR studies on the conformation of the 1–13 and 1–17 alamethicin fragments are rendered

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difficult by the considerable spectral overlap of amide NH and $C^{\alpha}H$ resonances of different residues even at 270 MHz. But a comparison of the CD spectra of the 1–6 and 1–13 fragments suggests that helical folding is maintained in the larger peptide. A comparison of the IR spectra of the 1–6, 7–13, and 1–13 fragments (Figure 6b) shows a dramatic increase in ν_{NH} (bonded) in the 1–13 peptide, suggesting a folded conformation stabilized by a large number of intramolecular hydrogen bonds. Thus the 3_{10} helical structures suggested from NMR data for the 1–6 and 7–13 fragments appear to be maintained in the 1–13 fragment. Recent studies of large fragments of the related polypeptide suzukacillin (see Figure 2) have yielded extremely well-resolved amide NH resonances for the 1–10, 11–21, and 5–21 fragments. Detailed analysis of hydrogen bonding in $CDCl_3$ and $(CD_3)_2SO$ suggests that these peptides also adopt highly folded 3_{10} helical conformations.[71,72]

Conformation of Alamethicin

The steric constraints introduced by Aib residues appear to dictate the folding of short segments, which are largely unperturbed by further chain elongation. Conclusions about the conformation of alamethicin itself may be drawn on the basis of studies on fragments. The studies reviewed in this account are compatible with a structure for the alamethicin backbone involving a 3_{10} helical segment from residues 1–10 followed by an expansion to a turn of an α-helix at residues 11–14 which is then tightened to a 3_{10} helix from residues 14 to 17. The flexibility noted for the Gly-Leu segment could provide the necessary "structural hinge" allowing the molecule to interconvert between two conformational states, involving different spatial orientations of the rigid helical segments 1–10 and 13–17. The possibility of limited conformational flexibility may be useful in explaining the voltage-dependent conductance characteristics of alamethicin channels in artificial membranes. ^1H NMR studies on Boc-Glu-Gln-Phol suggest that no particular conformation is favored in this fragment. The polar charged tail, Glu-Gln-Phol, is likely to be flexible and should facilitate proper orientation of alamethicin in the lipid bilayer.[50]

All the suggested helical structural possibilities (like 3_{10} and α-helical conformations) have very small internal diameters that cannot account for the passage of ions through the helix interior, as postulated for gramicidin A channels. Membrane channels of alamethicin must therefore be built up of aggregates, and there is considerable evidence for an oligomeric aggregate as the functional channel. Studies on the modes of aggregation and membrane activity of alamethicin and related peptides would therefore be relevant. While general agreement exists that peptide aggregates constitute the channel, the site of peptide association remains to be established. Both aqueous phase[14] and membrane phase[73] aggregation have been proposed, with the aggregate in the former case inserting into the lipid membrane, in response to an electric field.[14]

Peptide Association in Aqueous and Nonaqueous Solvents

An approach to the study of aqueous-phase aggregation of Aib peptides uses synthetic, fluorescent, 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) labeled peptides. Inferences on aggregation behavior and interactions with lipids have been drawn by noting changes in the emission parameters of the fluorophore. Blue shifts and enhancements of fluorescence intensities accompany aggregation. This approach has been exemplified in a study of the amino-terminal fragments of emerimicin.[44] The nonapeptide ester DNS-Phe-$(Aib)_3$-Val-Gly-Leu-$(Aib)_2$-OMe aggregates strongly at concentrations around 8 μM whereas the corresponding acid does not. The nonapeptide ester interacts strongly with lipids as compared to the acid. Short fragments do not aggregate or interact strongly with lipids.[44]

Studies on labeled peptides of alamethicin have established that the 1–10, 1–13, and 1–17 ester fragments aggregate at very low concentrations (2–10 μM). Critical micellar concentrations may be determined, which establish that aggregation is facilitated by increasing peptide chain length and is inhibited by the presence of a negative charge in the peptide acids. The free acids, however, aggregate very effectively at high ionic strength (>2 M NaCl). The peptide aggregates are stabilized by increasing salt concentration and dissociated by urea, suggestive of hydrophobic stabilization of the aggregates. The enthalpy of association for the 10- and 17-residue ester fragments of alamethicin has been estimated to be between −1 and −3 kcal mol^{-1} of monomer (M. K. Mathew, R. Nagaraj, and P. Balaram, submitted). The aqueous phase aggregation of these hydrophobic peptides at low concentration suggests that preformed aggregates of channel-forming peptides may exist at the membrane interface. Insertion of a peptide aggregate into the lipid bilayer may be the initial step in the constitution of a functional channel. While these studies have not yet led to a clear determination of the aggregation number in the "peptide micelles", early studies of alamethicin by ultracentrifugation suggest as many as 12–16 molecules in the aggregate.[74]

Aggregation of apolar Aib peptides in organic solvents can serve as models for association in the lipid phase. Detailed studies of the 1–10 and 11–21 suzukacillin fragments using concentration dependences of NH chemical shifts and their temperature coefficients have been carried out. Both fragments associate in $CDCl_3$ at concentrations of ~0.009 M, while in $(CD_3)_2SO$ aggregation is less favorable. Peptide association is without effect on the helical conformations of these fragments, and the results suggest that rodlike peptides aggregate by intermolecular hydrogen-bond formation involving NH and CO groups that are not bonded intramolecularly. A detailed comparison of the peptides Boc-Gln-Aib-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OMe and Boc-Ala-Aib-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OMe suggests that the Gln side-chain carboxamide group plays an important role in the association of this fragment in nonpolar solvents (M. Iqbal and P. Balaram, submitted). It is pertinent that a Gln residue is found approximately in the middle of the hydrophobic segment of the channel formers alamethicin, suzukacillin, hypelcin, and trichotoxin A-40 (Figure 2). A possible mode of stabilization of the aggregate of peptide helices, in nonpolar media, is schematically

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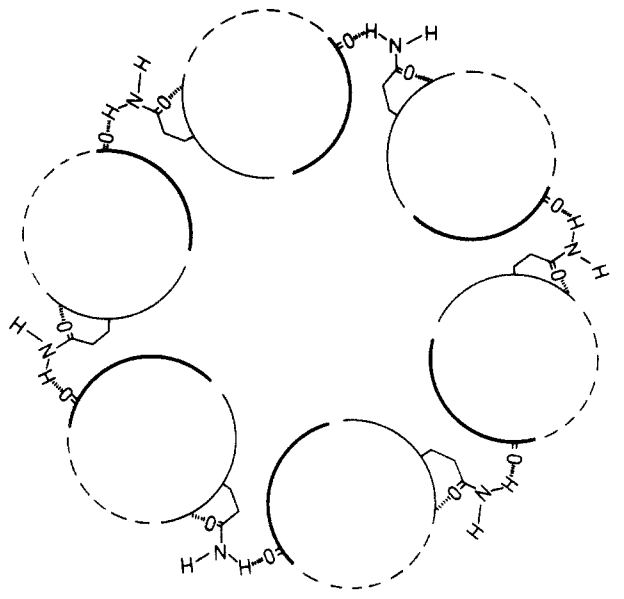

Figure 7. Proposed model for a hexameric aggregate of channel-forming polypeptides like alamethicin or suzukacillin. Adjacent helices are held together by hydrogen bonding between a Gln side-chain carboxamide NH group and a backbone CO group.

illustrated for a hexameric aggregate in Figure 7. The central core is lined with hydrophobic side chains and would accommodate an ordered water network, a medium that has been suggested to be effective in cation transport across membranes.[75]

Membrane-Modifying Activity of Alamethicin Fragments

The ionophoretic activity of alamethicin and its fragments have been examined by a fluorescence technique using small, unilamellar liposomes and the probe, chlortetracycline.[45] These studies suggest that a minimum length of 13 residues is required for activity and that charge effects diminish with size, the negative charge being inhibitory, in small fragments. Both synthetic and "natural" alamethicin are efficient cation translocators in liposomes. Alamethicin derivatives where the Glu(18) γ-carboxyl group is blocked are also very active, suggesting that the C-terminal negative charge may be unimportant in certain aspects of membrane modification. In the family of Aib-containing membrane-active peptides, alamethicin, suzukacillin, and trichlotoxin A-40 have a negative charge whereas the others are neutral. The protected 1–17 alamethicin fragment is an efficient cation translocator, suggesting that the predominantly hydrophobic segment is sufficient for channel formation.

Recent studies in this laboratory have shown that synthetic alamethicin and its fragments are very effective uncouplers of oxidative phosphorylation in mitochondria, a property noted also for hypelcin and "natural" alamethicin.[76] The uncoupling activity of the synthetic peptides show chain length and charge dependences similar to those obtained by monitoring liposomal Ca^{2+} translocating activity.[77] Most known uncouplers are proton ionophores and probably function by breaking down the mitochondrial pH gradient.[78] An ordered water channel, formed within the matrix of a peptide aggregate,[75] would be particularly effective in proton transport across membranes. Fluorescent labeled alamethicin fragments, which are useful in monitoring aggregation behavior, have been shown to behave identically in both the assays for membrane modifying activity. For these peptides the ease of aggregation correlates very well with membrane activity (M. K. Mathew, R. Nagaraj, and P. Balaram, submitted). The differences in the activity and aggregation behavior that have been noted for various synthetic fragments of alamethicin should eventually prove useful in developing molecular models for both passive as well as gateable channels in membranes.

Conclusions

The development of secondary structure in the membrane-modifying polypeptide alamethicin has been studied by an approach involving the synthesis of fragments and conformational analysis by X-ray diffraction and spectroscopic methods. A considerable body of evidence now exists which establishes that the backbone conformation of Aib-containing peptides are extremely restricted. It has been clearly shown that Aib residues tend to promote conformations in the 3_{10} helical region. Studies of alamethicin fragments suggest that in the antibiotic a largely 3_{10} helical conformation is favored over a considerable length of the polypeptide chain. Channel formation is then likely to involve molecular aggregates, since such individual 3_{10} or α helices cannot accommodate the passage of ions. Fluorescent peptide derivatives have been used in studies of aqueous aggregation and establish that large fragments aggregate at very low concentration. Similar structural dependences have been observed for diverse properties like cation translocation in liposomes, uncoupling of oxidative phosphorylation in mitochondria, and aqueous phase aggregation. Studies over the past few years have provided some insight into the structural characteristics of the alamethicin transmembrane channel. This body of work should provide the framework for establishing a detailed structure of the channel, the molecular basis for changes in channel conductance states, and the gating phenomenon.

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