Structure, function, and membrane integration of defensins

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Defensins comprise a structural class of small cationic peptides that exert broad-spectrum antimicrobial activities through membrane permeabilization. Their predominantly β-sheet structure, stabilized by three disulfide bonds, distinguishes them from other antimicrobial peptides which typically form amphiphilic helices. Defensins bind to membranes electrostatically and subsequently form apparently multimeric pores. Recent structural and biophysical studies are beginning to provide insights into the process of permeabilization.

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Introduction

Small antimicrobial peptides secure the front lines of host defense through direct physicochemical attack on the surface membranes of invading microorganisms. Invariably positively charged, they are perfectly suited to interact with negatively charged membranes to cause disruptive changes in membrane permeability. The large number of peptides now recognized to play a host-defense role has recently been reviewed by Maloy and Kari [1•]. The majority of the peptides are thought to form amphiphilic helices that facilitate membrane incorporation and disruption.

The defensins are unusual because they are stabilized by three disulfide bonds and have a β hairpin as their principal structural feature. This motif is the defining and unifying feature of the defensins which are otherwise diverse in terms of biological occurrence and evolutionary origin. Unlike amphiphilic α-helical peptides such as the magainins, a structural basis for membrane disruption is not so apparent. Fortunately, several defensin structures have now been solved by X-ray and NMR methods, and the fundamental principles of their interactions with model membranes are beginning to be understood.

These structural and biophysical studies are the subject of this review. We will emphasize the literature of the past three years but will call upon the literature of the past eight years, as necessary, in order to present as complete a picture as possible. The biological activity, distribution, isolation, and purification of defensins have been described in several recent reviews [1•,2–6,7•] and will not be discussed, except to say that there are three categories of defensins: the α-defensins, the β-defensins, and the insect defensins. Their antimicrobial activities and phylogenetic and tissue distributions are summarized in Table 1, which includes references to pertinent literature [2,3,7•,8,10,12,13,15•,17•,18,21•,22••].

Structure of defensins

The primary structures of the defensins have been discussed thoroughly in several recent reviews [2,6,7•]. All are cationic with arginine as the predominant cationic residue, and all are stabilized by three disulfide bridges. The mature α-defensins ('classic' defensins) contain 29–35 amino acids, the β-defensins 38–42, and the insect defensins 29–34. Typical sequences and the characteristic cysteine connectivities for the three families are shown in Figure 1. In the insect defensins, the six cysteine residues involved in the disulfide bonds tend to be evenly distributed along the chain except for the fifth and sixth cysteines (C_5 and C_6 respectively), which are separated from one another by only one amino acid near the carboxyl terminus. C_5 and C_6 have similar positions in the α- and β-defensins except that they are adjacent to one another in the sequence. The α-defensins have a pair of cysteine residues (C_1 and C_2) near the N terminus which are separated by a single residue.

The solution structures of three α-defensins (rabbit NP-2 [23,24], rabbit NP-5 [25,26], and human HNP-1 [23,24]) and one insect defensin (A) [27] (see Note added in proof) have been determined by NMR methods. A high-resolution crystal structure for the human α-defensin HNP-3 has been determined by Hill *et al.* [28]. The solution of structure of the β-defensin BNBD-12 has very recently been determined and found to have a fold identical to that of the α-defensins [29]. Examples of the structures of five defensins are shown in Figure 2. The most obvious common structural

Abbreviations

CD—circular dichroism; **DPPC**—dipalmitoylphosphocholine; **HNP**—human neutrophil defensin; **NP**—neutrophil peptide.

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Table 1. Antimicrobial spectrum and the phylogenetic tissue distributions of the defensins.

Defensin type and designation	Phylogenetic distribution	Tissue distribution	Antimicrobial spectrum	References
α-defensins				
MCP 1 and 2	Rabbit	Lung macrophages	G+, G–, fungi, enveloped viruses	[3,4,6,7•]
NP 1–5	Rabbit	Neutrophils	G+, G–, fungi, enveloped viruses	[3,4,6,7•]
GNCP 1 and 2	Guinea pig	Neutrophils	G+, G–, fungi, enveloped viruses	[3,4,6,7•]
RatNP 1–4	Rat	Neutrophils	G+, G–, fungi	[3,4,6,7•]
HNP 1–4	Human	Neutrophils	G+, G–, fungi, enveloped viruses	[3,4,6,7•]
Cryptdins	Mouse, rat	Intestinal Paneth cells	G+, G–, fungi, protozoans	[7•,12,15•,16]
HD 5 and 6	Human	Intestinal Paneth cells	Unknown	[9,10]
β-defensins				
BNBD 1–13	Cattle	Neutrophils	G+, G–, fungi	[11,13]
TAP	Cattle	Trachea	G+, G–, fungi	[8]
LAP	Cattle	Tongue	G+, G–, fungi	[22••]
Gallinacins; CHPs	Chickens	Heterophils	G+, G–, fungi	[17•,21•]
THPs	Turkeys	Heterophils	G+, G–	[21•]
Insect defensins				
Fly defensins (e.g. sapecin, phormia A and B)	Blowflies, fleshflies, fruit flies	Fat bodies, hemolymph, thrombocytoids	Primarily G+	[2,5,20]
Scorpion defensin	Scorpion	Hemolymph	Primarily G+	[14]
Royalisin	Bees	Hemolymph	Primarily G+	[19]

BNBD, bovine neutrophil β-defensin; CHP, chicken heterophil peptide; GNCP, guinea pig neutrophil cationic peptide; G+, Gram-positive; HD, human defensin; HNP, human neutrophil peptide; LAP, lingual antimicrobial peptide; MCP, macrophage cationic peptide; NP, neutrophil peptide; RatNP, rat neutrophil peptide; TAP, tracheal antimicrobial peptide; THP, turkey heterophil peptide.

feature is a hydrogen-bonded pair of antiparallel β strands connected by a short turn to form a β hairpin comprising the last 15 or so residues of the sequence. The C_3-C_5 disulfide bridge 'closes' the open end of the hairpin in the α-defensins. The insect defensin does not have this closure.

Two disulfide bridges invariably connect additional secondary-structure elements to the characteristic β hairpin. In the case of insect defensin A, this element is an 11 amino acid α helix in the middle of the sequence (residues 14–24; see Note added in proof) that runs roughly parallel to the hairpin. In the α-defensins, the additional element is a β strand near the amino terminus that hydrogen bonds to the β hairpin to create a three-stranded β sheet. These disulfide- and hydrogen-bond-stabilized elements are the best defined portions of the molecules in the NMR solution structures and presumably the most rigid. The most ill-defined portions of the solution structures, and presumably the most flexible, are a loop in the insect defensins formed by the first 13 amino-terminal residues and a loop in the α-defensins formed from 7 residues (residues 7–13; see arrows in Fig. 2) that separate the amino-terminal β strand from the C-terminal β hairpin.

An important result to have emerged from comparisons of the structural measurements is that the human neutrophil defensins (HNPs 1–3) apparently exist in solution as dimers whereas the rabbit neutrophil peptides (NPs 1–5) exist as monomers. The crystal structure of HNP-3 [28] demonstrated dimers formed from monomers that come into close contact along the edges of their β hairpins to form a local twofold rotation axis. The result is a six-stranded β sheet within the dimer stabilized by hydrogen bonds and hydrophobic contacts (Figs 2,3). HNP-3 also appears to exist as a dimer in solution, based upon equilibrium sedimentation measurements and attempts to dissociate the dimer under strongly denaturing conditions (9 M urea, pH 2.3) [24,30]. A large number of slowly exchanging amide protons are observed in HNP-1 compared with NP-2 and NP-5, which suggests that HNP-1 forms aggregates of some sort in solution [24,30]. Taken together, these data suggest that the rabbit neutrophil defensins are monomers in solution whereas the human neutrophil defensins are dimers.

An expected feature of antimicrobial peptides is amphiphilicity, which is assumed to be a prerequisite for membrane disruption and pore formation. The solution structure of the HNP-1 monomer reported by Pardi *et al.* [24] reveals charged residues that face away from a distinctive hydrophobic surface so that the monomer has amphiphilic character. Sequence similarities of the α-defensins indicates that this will be a general feature of the monomers. However, the amphiphilicity of the dimer may be of greater importance, at least for the human defensins which form dimers in solution. Hill *et al.* [28] described the HNP-3 dimer as being shaped like a basket (Fig. 3) that has a hydrophobic bottom (exposed surfaces of the β hairpins) and a polar top (containing the N- and C-termini), and suggested several ways that this

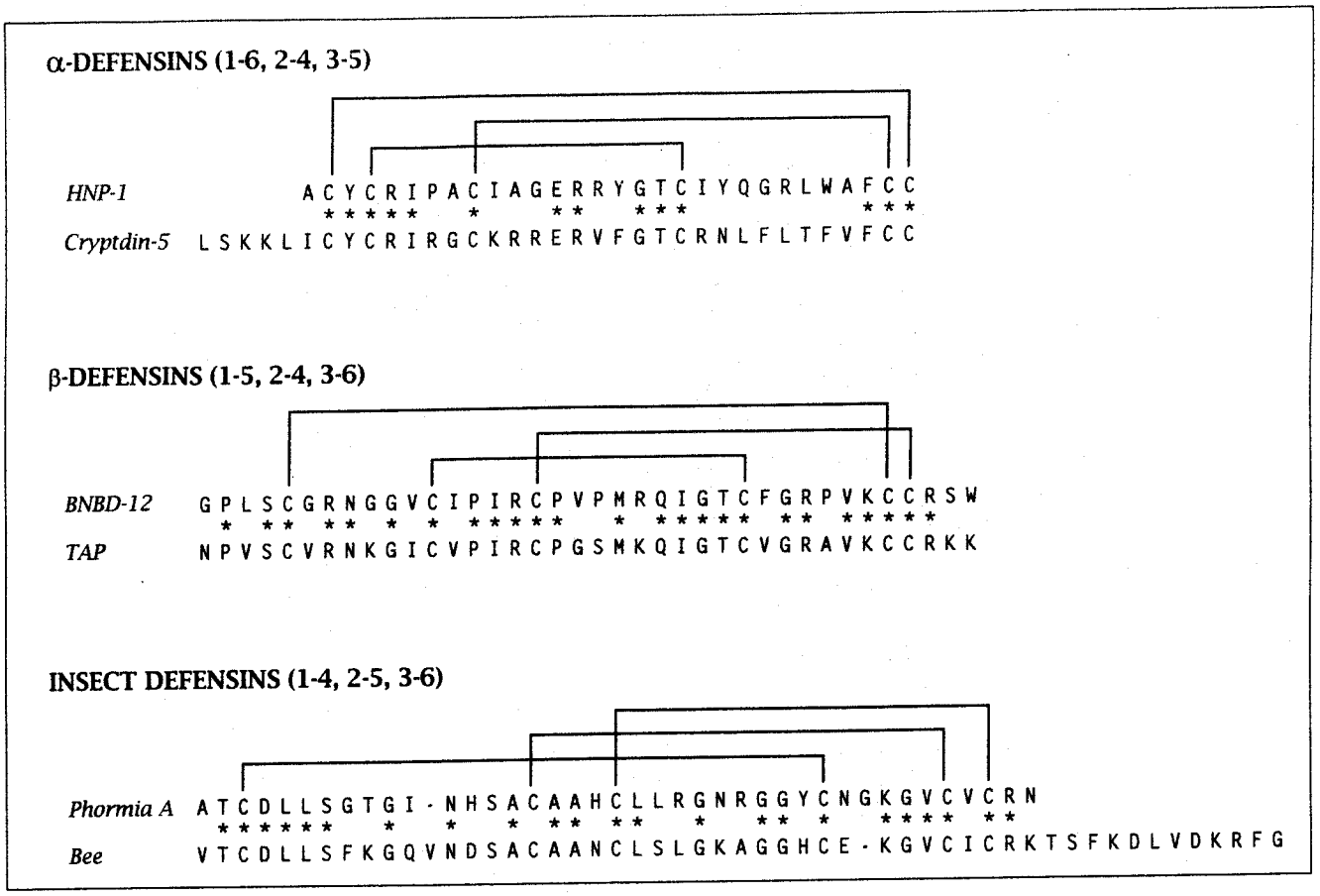

Fig. 1. Typical covalent structures of α-, β- and insect defensins. Examples of each class of defensin are shown, using the single-letter amino acid code. The cysteine connectivities are shown schematically as solid lines and the numbering of the cysteine pairs is listed in parentheses for each defensin class. Identities between the sequences are indicated with asterisks.

amphiphilic structure could interact with membranes to form channels or pores (see below). The apparent lack of dimerization of the non-human defensins in aqueous solutions creates a fundamental uncertainty about how these defensins assemble into permeabilizing structures.

An analysis of a large non-redundant database of protein sequences reveals that small proteins (fewer than 100 amino acids) have greatly elevated frequencies of cysteine, arginine, and lysine relative to large proteins [31]. In addition, membrane-associating small proteins are found to be particularly rich is cysteine (mean of seven per sequence) compared with DNA-associating small proteins. The defensins therefore appear to be 'typical' small proteins. An intriguing finding is that the scorpion venom charybdotoxin, which blocks K^+ channels, and several other toxins have folds that are very similar to that of the insect defensin ([32]; see Note added in proof). This suggests that many small membrane-active proteins have defensin-like folds but diverse functions. Small proteins present special stability problems [31] and it may be that the β hairpin linked by disulfide bridges to other structural elements is a common stabilizing element.

Mode of action of defensins in the killing of microorganisms

Early studies of defensins [33,34] revealed the general mode of action as being permeabilization of the plasma membrane and consequent leakage of cell contents. A study by Cociancich *et al.* [35] of the interaction of insect defensin A with *Micrococcus luteus* represents the most comprehensive examination of the mechanism of action. These authors demonstrated that defensin A perturbs the cytoplasmic membrane of *M. luteus* to cause loss of cytoplasmic K^+, membrane depolarization, a decrease in cytoplasmic ATP, and inhibition of respiration. Permeabilization of energized *M. luteus* was reported to cause the transmembrane potential ($\Delta\Psi$) to drop from −200 mV to −110 mV (potential inside the cell with respect to that outside the cell). They concluded that a $\Delta\Psi$ of at least −110 mV is required for defensin action, which would explain the lack of an effect on host-organism cells which have a much smaller potential.

However, possible inconsistencies in their data suggest that $\Delta\Psi$ was 0 mV rather than −110 mV. First, they reported that defensin caused the cells to lose all of their

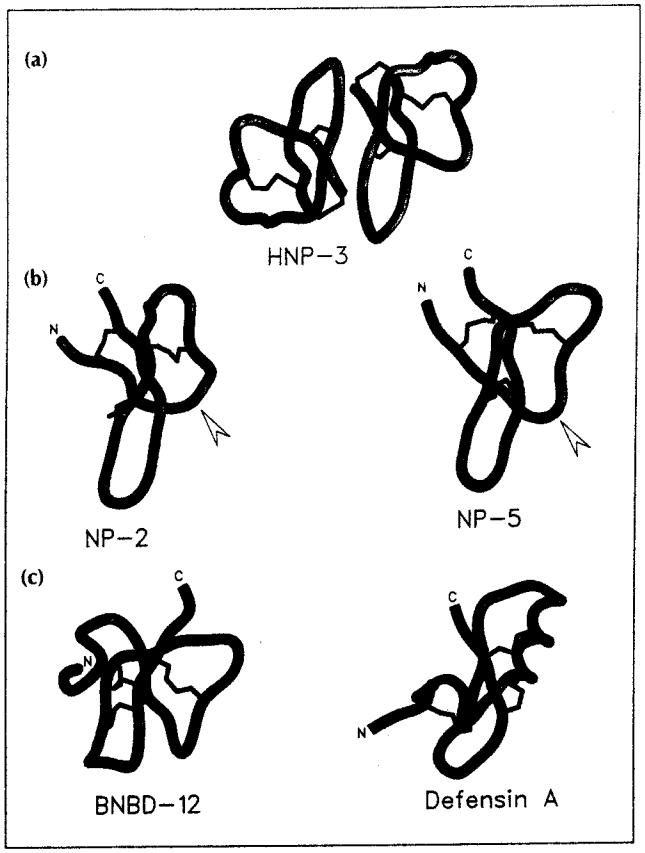

Fig. 2. The backbone conformations of the three classes of defensins. The backbone structures are shown as tubes with the disulfides indicated as narrow rods. The structures have been arranged so that the β hairpins have similar orientations. **(a)** Dimeric α-defensin (Brookhaven Protein Data Bank [PDB] identifier 1DFN). **(b)** Two monomeric α-defensins. Note that the two structures differ primarily in the disordered loop region (arrows). **(c)** β-defensin (left structure, PDB identifier 1BNB) and insect defensin (right structure, PDB Identifier 1ICA). A significant shared difference between the β- and insect defensins and the α-defensins is that the N and C termini tend to be much farther apart. Note, however, that the fold of the β-defensin is otherwise identical to the α-defensin fold. The insect defensin differs from all of the others in two respects: it has an α helix and its topology is such that its N-terminal domain passes in front of the β hairpin rather than behind. The images were produced using the software packages SETOR and SETORPLOT [42].

internal K^+ so that the internal concentration equaled the external concentration (0.5 mM). If that were the case, the membrane potential, judged by the distribution of K^+, would be 0 mV. Second, the application of the protonophore tetrachlorosalicylanilide (TCS), which should dissipate the proton motive force (PMF), seemed to cause $\Delta\Psi$ to fall to only −110 mV, rather than the expected value of 0 mV. The only value of $\Delta\Psi$ consistent with equal internal and external concentrations of K^+ and a dissipated PMF is 0 mV. It is therefore possible that a potential of 0 mV rather than −110 mV prevails after permeabilization by defensin.

Gálvez *et al.* [36] reported measurements very similar to those of Cociancich *et al.* [35], for the permeabilization of the membranes of *Enterococcus faecalis* by the peptide antibiotic AS-48. They observed a potential of −60 mV that collapsed to 0 mV in the presence of AS-48 or

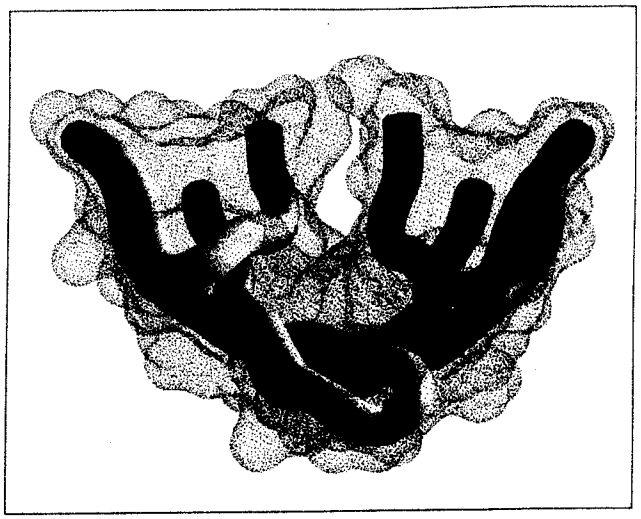

Fig. 3. Backbone and surface contours of an α-defensin dimer. The backbone conformation of dimeric HNP-3 is shown overlaid with the calculated van der Waals surface. The base of the dimer (β hairpins) is populated with hydrophobic side chains, whereas the 'top' is composed of the polar N-terminus, C-terminus and arginine side chains which are distributed equatorially. The resulting amphiphilic topology is thought to facilitate the interactions with biological membranes. The image was produced using the software package GRASP [41].

dicyclohexylcarbodiimide. If a similar scenario holds for the defensin A interaction with *M. luteus*, doubt is cast on the idea of a threshold membrane potential for defensin action.

Several additional conclusions about the interaction of defensin A with *M. luteus* [35] are important. First, increasing ionic strength decreased defensin-induced K^+ efflux, which suggests an electrostatic interaction. Second, denaturation of defensin A with 8.5 M urea abolished activity. Third, K^+ efflux did not occur unless the number of defensin molecules/cell exceeded a critical value. Cociancich *et al.* [35] proposed that monomers adsorb to the membrane and assemble via lateral diffusion into oligomers. Fourth, even if the critical number of defensins/cell were exceeded, K^+ efflux was only partial. Cociancich *et al.* argued that this would occur under two circumstances: either all of the cells are 'hit' and lose some of their K^+ (graded loss), or only some of the cells are hit and those that are lose all of their K^+ (all-or-none loss). Evidence and arguments for the latter case were presented.

Interactions of defensins with model membranes

Only three studies devoted entirely to the interaction of defensins with model membranes have been reported [37,38,39••]. Kagan *et al.* [37] examined the interaction of rabbit NP-1 and HNP-1 with planar bilayer membranes formed from lipid mixtures containing various proportions of phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine. They found that the

defensins form weakly anion-selective voltage-dependent channels independent of the lipid mixture used, including pure phosphatidylcholine. With the addition of defensins (50 μg/ml), the current through the membrane increased rapidly when the side of the membrane opposite (trans) to the defensin addition was negative (typically −90 mV). When the voltage on the trans side was switched to positive polarity, the current immediately reversed direction but then decayed back to very low values. This suggests that a membrane potential is required for defensin permeabilization. They also observed single-channel activity which could not be characterized by any particular unitary conductance value. A similar observation was made for insect defensin A by Cociancich *et al.* [35], who patch-clamped giant liposomes formed from asolectin. Measurements of the dependence of the logarithm of membrane conductance on the logarithm of NP-1 concentration resulted in a straight line with a slope of 2–4. This indicates that the NP-1 channel is formed by multimers of 2–4 molecules. A similar observation obtained for HNP-1 is interesting because this molecule, unlike the rabbit neutrophil defensins, forms multimers in solution [24]. A final observation of importance is that reduced and carboxymethylated NP-1 had no apparent effect on bilayer conductance, indicating that an intact tertiary structure maintained by the disulfide bonds is required for this activity.

The various defensins carry a net charge of between +2 and +9, which is primarily attributable to arginine. Because biological membranes are invariably negatively charged, the interactions of defensins with charged lipids is of considerable importance. Fujii *et al.* [38] used small unilamellar vesicles formed from 3:1 (mol/mol) mixtures of dipalmitoylphosphocholine (DPPC; electrically neutral) and dioleoylphosphatidylserine (negatively charged) to study the lipid interactions of several α-defensins. They found that all of the defensins caused fusion and lysis of vesicles and that the interactions are primarily electrostatic and secondarily hydrophobic. Fluorescence and circular dichroism (CD) measurements showed that the interactions had no effect on the secondary structure of native defensins. The defensins tended not to interact strongly with vesicles formed from DPPC and cholesterol although NP-1, reduced NP-1, and reduced HNP-1 did cause modest fusion, but not lysis, in the presence of phosphate buffer.

The lack of strong interactions with neutral lipids is contrary to the finding of Kagan *et al.* [37] that only the transmembrane potential is important in determining channel formation. The role of membrane potential cannot be easily examined in vesicle experiments. Although reduced defensins had no apparent effect on planar bilayers in the study of Kagan *et al.* [37], Fujii *et al.* [38] found reduced defensins to be about equally effective in causing fusion and lysis of vesicles formed with negatively charged lipids. In addition, CD measurements indicated that the reduced defensins in the presence of lipid formed secondary structure: β sheet in the case of reduced HNP-1 and α helix (20%) in the case of NP-1 and NP-5.

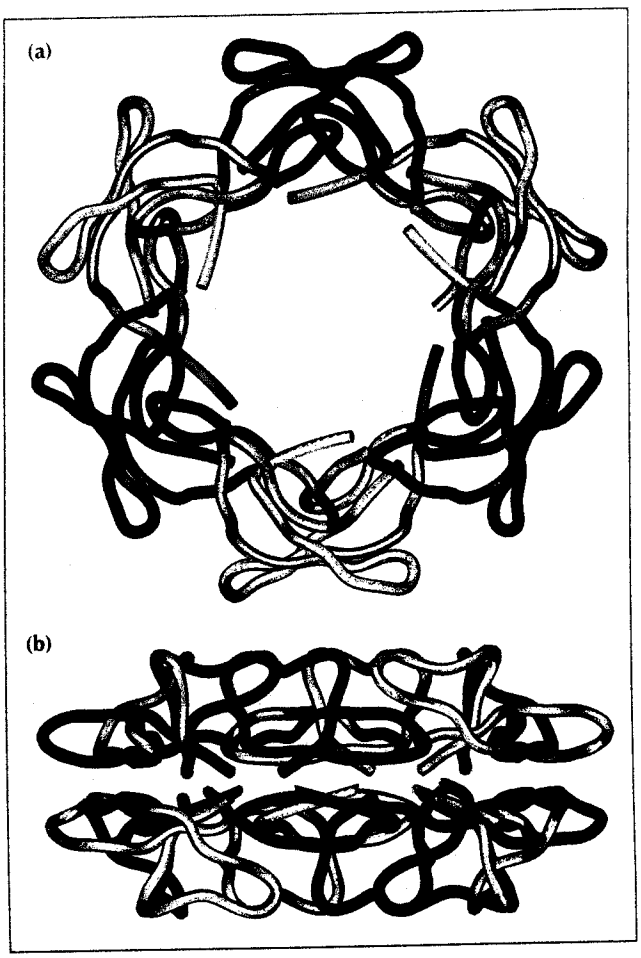

Fig. 4. Model of a transmembrane α-defensin pore. Studies on the HNP-2-induced leakage of anionic phospholipid vesicles [39••] indicate that dimeric defensins assemble to form a pore with a diameter of ~25 Å. Such a pore, viewed axially **(a)** and from the side **(b)**, can be formed by an assembly of six HNP-2 dimers. The polar 'tops' (see Figs 2,3) line the aqueous channel and the hydrophobic bases bind to the hydrocarbon phase of the bilayer. Images prepared with the software packages SETOR and SETORPLOT [42].

Hill *et al.* [28] suggested three possible mechanisms for the permeabilization of membranes by human neutrophil defensins: first, detergent-like action by monomeric dimers; second, a dimer of dimers with a solvent channel between them; or, third, an annulus of defensin dimers that forms a large pore. These possibilities were examined carefully by Wimley *et al.* [39••], who studied the interaction of HNP-2 with large unilamellar vesicles formed from the negatively charged lipid palmitoyloleoylphosphatidylglycerol. They showed conclusively by studying the release of entrapped dextrans that native HNP-2 forms pores with a diameter of ~25 Å, and suggested a speculative model in which an annular pore is formed by a hexamer of dimers (Fig. 4). By entrapping a fluorescent dye along with a fluorescence quencher, they showed that both native and reduced HNP-2 caused leakage of the dye, based upon

increases in fluorescence upon the addition of defensin. Yet the mechanisms of leakage for the two forms of defensin were shown to be different. Using a novel requenching method, they showed that reduced HNP-2 caused graded leakage in which all vesicles leaked some of their contents very quickly. Native defensin, on the other hand, caused all-or-none leakage on a slower time scale in which some of the vesicles released all of their contents while others released none. This is similar to the findings of Cociancich *et al.* [35] for the release of K^+ from *M. luteus* induced by insect defensin A. Furthermore, also in agreement with the findings of Cociancich *et al.*, HNP-2 causes all-or-none release only when a minimum number of HNP-2 dimers are bound to the vesicles.

Conclusions

Collectively, the published literature supports the view that defensins permeabilize membranes through the formation of multimeric pores. The initial step in assembly involves primarily electrostatic interactions with membranes in the form of charge–charge interactions of the cationic peptides with anionic lipids and/or defensin insertion driven by the transmembrane potential. The interplay of these two electrostatic phenomena is uncertain and requires further examination.

A particularly puzzling observation is that only the human neutrophil defensins appear to form dimers in solution. Does this mean that dimerization, either in solution or on the membrane, is a prerequisite for pore formation? In any case, the subsequent steps of assembly following membrane binding are virtually unknown. They probably involve hydrophobic interactions as well as electrostatic ones but the defensins with their disulfide-bond stabilized β sheet defy easy analysis because of our lack of fundamental understanding of peptide–bilayer interactions [40•]. Clarification of the process of assembling defensins into pores will no doubt lead to a broader understanding of such interactions.

Note added in proof

Since this manuscript was submitted, Cornet *et al.* [43•] have published the first highly refined structure of an insect defensin and a comparison of the structures of scorpion toxins with that of defensin A.

Acknowledgements

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