Interaction of indolicidin, a 13-residue peptide rich in tryptophan and proline and its analogues with model membranes

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Indolicidin is a 13-residue broad-spectrum antibacterial peptide isolated from bovine neutrophils. The primary structure of the peptide ILPWKWPWWPWRR-amide (IL) reveals an unusually high percentage of tryptophan residues. IL and its analogues where proline residues have been replaced by alanine (ILA) and trp replaced by phe (ILF) show comparable antibacterial activities. While IL and ILA are haemolytic, ILF does not have this property. Since aromatic residues would strongly favour partitioning of the peptide into the lipid bilayer interface, the biological activities of IL and its analogues could conceivably arise due perturbation of the lipid bilayer of membranes. We have therefore investigated the interaction of IL and its analogues with lipid vesicles. Peptides IL and ILA bind to lipid vesicles composed of phosphatidylcholine and phosphatidylethanol amine : phosphatidyl glycerol : cardiolipin. The position of λ_{max} and I^- quenching experiments suggest that the trp residues are localized at the membrane interface and not associated with the hydrophobic core of the lipid bilayer in both the peptides. Hence, membrane permeabilization is likely to occur due to deformation of the membrane surface rather than formation of transmembrane channels by indolicidin and its analogues. Peptides ILA, IL and ILF cause the release of entrapped carboxyfluorescein from phosphatidyl choline vesicles. The peptide–lipid ratios indicate that ILF is less effective than IL and ILA in permeabilizing lipid vesicles, correlating with their haemolytic activities.

1. Introduction

Indolicidin is a 13-residue peptide isolated from cytoplasmic granules of bovine neutrophils and possessing broad spectrum antibacterial activity (Selsted *et al* 1992; Aley *et al* 1994; Ahmad *et al* 1995). The peptide also exhibits cytotoxic activity against eukaryotic cells like rat and human T lymphocytes and erythrocytes (Schluesner *et al* 1993). Indolicidin which has the primary structure ILPWKWPWWPWRR-amide is characterized by an unusually high percentage of tryptophan, which is unique among endogenous antibacterial peptides. The trp residues appear to be essential for both the biological activities; an analogue where W has been replaced by F has only selective antimicrobial activity and no haemolytic activity (Subbalakshmi *et al* 1996). Since trp residues would strongly favour partitioning of the peptide to the lipid bilayer interface (Wimley and White 1996) the cytolytic activity of indolicidin could be due to its partitioning into the lipid bilayer of membranes. This could lead to permeabilization, either by forming pores or introducing defects in the bilayer like several other endogenous cytolytic peptides. The five trp residues in indolicidin would permit the study of its interaction with membranes by fluorescence spectroscopy as fluorescence of trp is sensitive to environment and has been used extensively to study lipid-peptide interactions (Lakowicz 1983). Even in peptides containing more than one trp, fluorescence studies permit inferences on the orientation in the lipid bilayer as with gramicidin A (Killian 1992; Mukherjee and Chattopadhya 1994). Thus, indolicidin is an attractive candidate for studying lipid protein interaction especially with respect to the orientation of trp residues in the lipid bilayer. We have shown that an

Keywords. Antibacterial peptide; alanine analogue; indolicidin; model membranes; peptide-membrane interactions; phenylalanine analogue

analogue where proline residues have been replaced by alanine has antibacterial and haemolytic activities very similar to that of indolicidin, whereas an analogue where W has been replaced by F exhibits only antibacterial activity (Subbalakshmi *et al* 1996). In this report, the interaction of indolicidin and its two analogues with lipid vesicles are described.

2. Materials and methods

The synthesis and characterization of the three peptides indolicidin (IL) and its two analogues ILAWKWAWWAWRR-amide (ILA) and ILPFKFPFFPFRR-amide (ILF) have been reported earlier (Subbalakshmi *et al* 1996). The lipids bovine heart cardiolipin (CL), dioleoylphosphotidyl glycerol (DOPG), *Escherichia coli* phosphatidyl ethanolamine (PE) and palmitoyl oleoyl phosphatidylcholine (POPC) were from Avanti polar lipids (Birmingham, AL, USA).

2.1 *Binding of peptides to lipid vesicles*

The interaction of IL and ILA with lipid vesicles composed of either POPC or PE : PG : CL (7 : 1·5 : 1·5) was characterized by measuring changes in the fluorescence intensity of trp on titration with small unilamellar vesicles (SUV) (prepared by sonication) in 5 mM Hepes buffer pH 7·4, 150 mM NaCl. Lipid vesicles were added to a fixed amount of peptide and the emission spectra recorded with excitation monochromator set at 280 nm and using a slit width of 5 nm. The increase in fluorescence intensity was calculated at a fixed wavelength after correction for volume change. Binding isotherms were obtained as described recently (Rizzo *et al* 1987; Schwarz *et al* 1986; Beschiaschvili and Seelig 1990; Rapoport and Shai 1991) using the formula

$$X_b^* = K_p^* C_f.$$

When X_b^* is defined as the molar ratio of bound peptide (C_b) per 60% of total lipid assuming that the peptide gets partitioned only into the outer leaflet of SUV (Beschiaschvili and Seelig 1990). K_p^* corresponds to the experimental partition coefficient and C_f represents the equilibrium concentration of the free peptide in solution. Binding isotherms were obtained by plotting X_b^* vs C_f.

2.2 *Quenching of tryptophan fluorescence*

The influence of quenchers on the tryptophan fluorescence of IL and ILA was studied by addition of increasing amount of 4 M KI solution and monitoring quenching of fluorescence in the absence and presence of lipid vesicles. The KI solution also contained 1 mM $Na_2S_2O_3$ to prevent the formation of I^{-3}. The solution was allowed to equilibrate after each addition before the measurements. The quenching data were analysed by Stern-Volmer plots using the equation

$$I_o/I = 1 + K_{SV}[Q],$$

where I_o and I are the fluorescence intensities at a constant wavelength in the absence and presence of the quencher [Q], the molar concentration of the quencher and K_{SV} the Stern-Volmer quenching constant.

2.3 *Membrane-permeabilization induced by peptides*

The peptide induced release of carboxy fluorescein (CF) from lipid vesicles was monitored as previously described (Blumenthal *et al* 1977; Sitaram and Nagaraj 1993). In brief, CF entrapped SUV were prepared by sonication of a dispersion of lipid film (POPC) in 5 mM Hepes (pH 7·4) containing 50 mM NaCl and 100 mM CF to clarity, in a Branson sonifier. Liposomes were separated from non-encapsulated CF by gel filtration on Sephadex G-75 using 5 mM Hepes, 150 mM NaCl, 1 mM EDTA pH 7·4 for elution. The excitation and emission monochromators were set at 493 nm and 520 nm respectively and fluorescence was continuously monitored after addition of different aliquots of peptides from an aqueous stock to lipid vesicles with stirring. The increase in fluorescence intensity which occur due to dilution of CF represents vesicle permeabilization. Complete release of CF was obtained by addition of Triton X-100 (0·1% v/v).

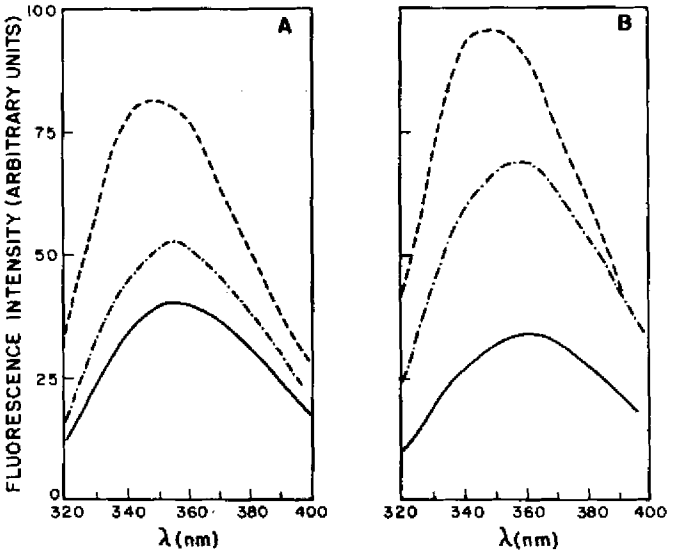
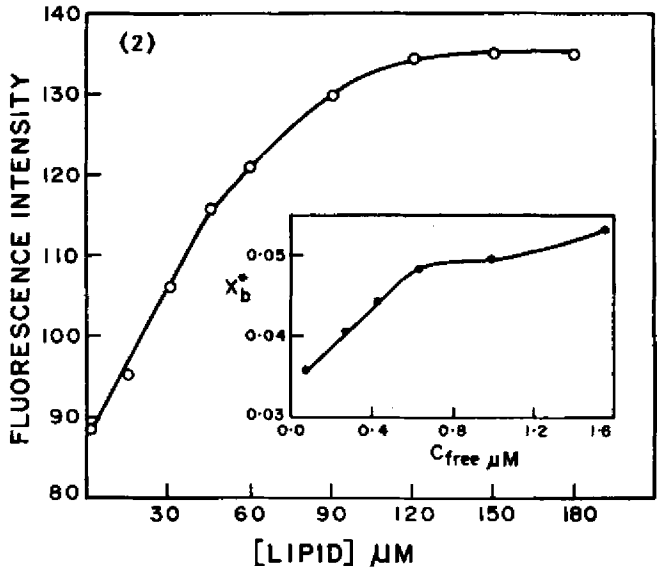


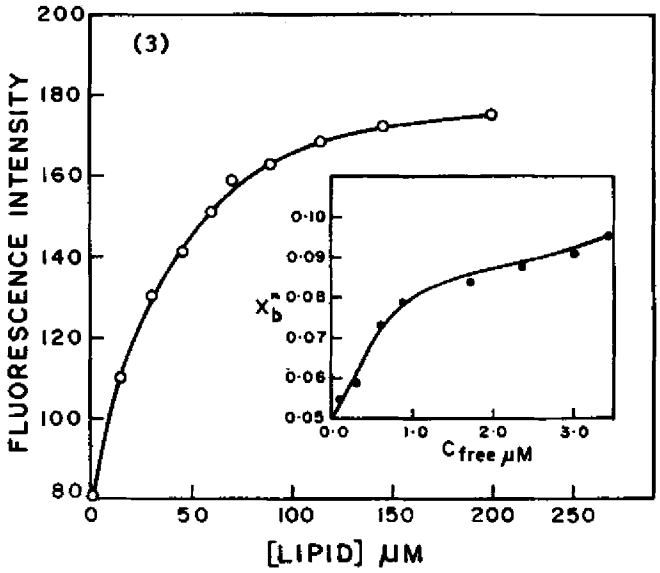
Figure 1. Fluorescence spectra of IL and ILA in buffer and in the presence of lipid vesicles. (**A**), IL; (**B**), ILA. (– – –), buffer (5 mM Hepes pH 7·4, 150 mM NaCl); (– · – · –), PE : PG : CL (7 : 1·5 : 1·5) lipid vesicles, lipid = 100 μM; (—), POPC vesicles, lipid = 230 μM. Peptide = 3 μM.

All fluorescence experiments were carried out on a Hitachi 650-10S Spectrofluorimeter at 25°C.

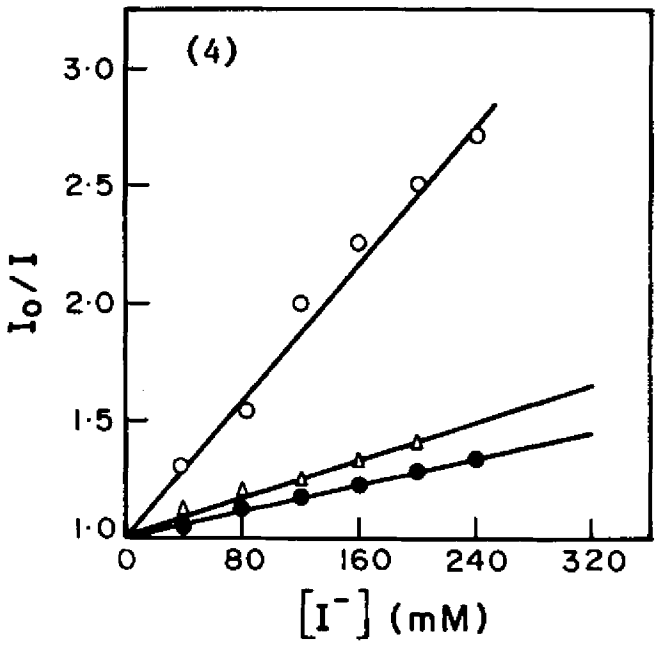
3. Results and discussion

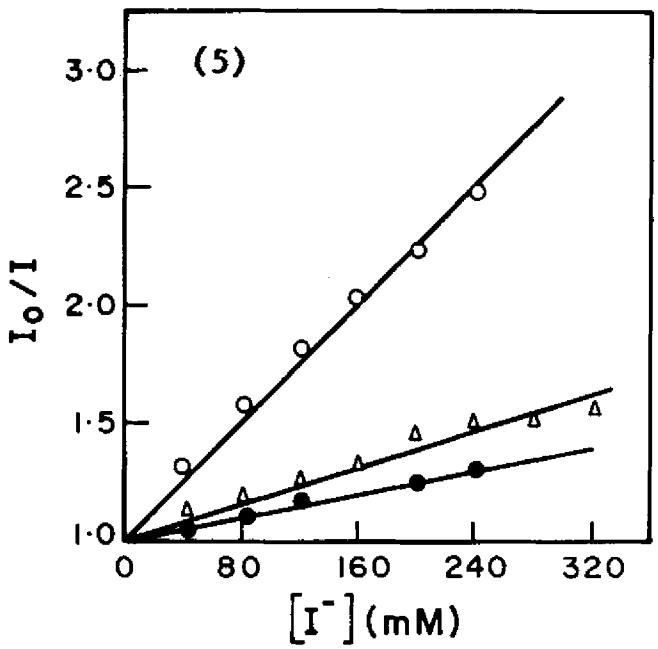
IL and ILA, unlike ILF, have trp residues which would facilitate studying the interaction of these peptides with lipid vesicles by fluorescence spectroscopy. The emission characteristics of trp is sensitive to its environment and has been extensively used to study peptide-lipid interactions (Lakowicz 1983). Hence, binding of IL and ILA to lipid vesicles and their location in the bilayer was assessed by monitoring trp fluorescence. The emission spectra of IL and ILA in buffer and in the presence of PC and PE : PG : CL vesicles are shown in figure 1. The λ_{max} is centered ~ 355 nm in buffer indicating that trp residues are exposed to aqueous environment. No concentration dependent blue shift or non linear increase in fluorescence intensity, characteristic of peptide aggregation, was observed in the range 1–15 μM indicating that the peptides did not have the ability to self aggregate.





Figures 2 and 3. Increase in fluorescence of IL (2) and ILA (3) on titration with POPC vesicles. Peptide = 3 μM. *Insets*: Binding isotherms derived from the titration curves.





Figures 4 and 5. Stern-Volmer plots of the quenching of IL (4) and ILA (5) fluorescence by iodide. (○), buffer; (△), PE : PG : CL vesicles (100 μM); (●), POPC vesicles, 230 μM. Peptide = 3 μM.

In the presence of PC vesicles, a small blue shift and enhancement in intensity is observed, suggesting a less polar environment for the trp residues. The changes in fluorescence intensity is less in the presence of PE : PG : CL vesicles. The lipid concentrations at which the spectra were recorded correspond to maximal peptide fluorescence. Hence, it appears that the peptides have lower affinity for lipid vesicles having the composition of *E. coli* plasma membrane in spite of pronounced antibacterial activity. Alternately, the mode of association with PE : PG : CL vesicles could be different from that of PC vesicles. The variation in fluorescence intensity as a function of lipid concentration and the binding isotherms derived from them are shown in figures 2 and 3. The shape of the binding isotherms suggests that after a certain fraction of peptide is bound to lipid vesicles, further binding is affected. A similar negative co-operative binding has been observed with melittin analogues (Oren and Shai 1997). The partition coefficients derived from the binding isotherms for IL and ILA are $3{\cdot}0 \times 10^4\ M^{-1}$ and $2{\cdot}3 \times 10^4\ M^{-1}$ respectively indicating that both peptides have similar affinities for zwitterionic vesicles. Since fluorescence changes in the presence of PE : PG : CL vesicles were relatively small, binding isotherms were not generated.

In order to determine the location of trp in the lipid bilayer the quenching of trp fluorescence by I^- in IL and ILA was monitored. The quenching curves in the presence of I^- in PC and PE : PG : CL vesicles for IL is shown in figure 4. While quenching is discernible in buffer, it is considerably less in the presence of lipid vesicles indicating that the trp residues are not completely accessible to the water soluble quencher KI. In spite of the relatively small changes in fluorescence in the presence of PE : PG : CL vesicles, the trp residues do not appear to be accessible to I^- indicating that the peptide also associates with PE : PG : CL lipid vesicles. The linear nature of the plots suggests the absence of gross differences in the environment of the various trp residues and they are equally accessible to the quencher. The K_{SV} values in the presence of zwitterionic and anionic lipid vesicles are $1{\cdot}43\ M^{-1}$ and $3{\cdot}0\ M^{-1}$ respectively. The KI quenching data for ILA is shown in figure 5. The values of K_{SV} in the presence of PC and PE : PG : CL lipid vesicles are $1{\cdot}3\ M^{-1}$ and $1{\cdot}95\ M^{-1}$ respectively suggesting that the trp residues are slightly less accessible as compared to IL. The linear nature of the plots again indicate that all the trp residues are in equivalent environment.

The ability of IL, ILA and ILF to release entrapped CF from POPC vesicles is shown in figure 6. The release profiles are characterized by an initial rapid efflux followed by slow release. It appears that the initial association of the peptides with lipid vesicles results in rapid permeabilization. The peptide-lipid ratios indicate that ILA is more effective than IL or ILF in permeabilizing lipid vesicles. The relatively high peptide-lipid ratios at which ILF causes release of CF as compared to ILA or IL correlates with its lack of haemolytic activity.

Our investigations provide insights into the role of trp in peptide aggregation and interaction with lipids. In spite of the presence of 5 trp residues out of 13 residues there is no evidence for aggregation over the concentration range 1–15 μM although IL does aggregate at concentration greater than 30 μM (Ahmad *et al* 1995). Thus, the presence of a large number of trp residues does not favour self association at low concentrations. The quenching data indicate that the trp residues are not exposed to the aqueous surface as there is no effective quenching by I^-.

Indolicidin and its Ala analogue bind to PC and PE : PG : CL lipid vesicles although they do not have high propensity for helical conformation or possess amphipathic character. Free energies of transfer indicate that IL binds strongly, though reversible, to POPC and POPG vesicles (Ladokhin *et al* 1997). Since trp residues do have the propensity to partition into membrane interface (Wimley and White 1996). The presence of 5 trps provide the driving force for IL and ILA to bind to lipid vesicles. The position of λ_{max} suggests that the trp residues are not associated with the hydrophobic core

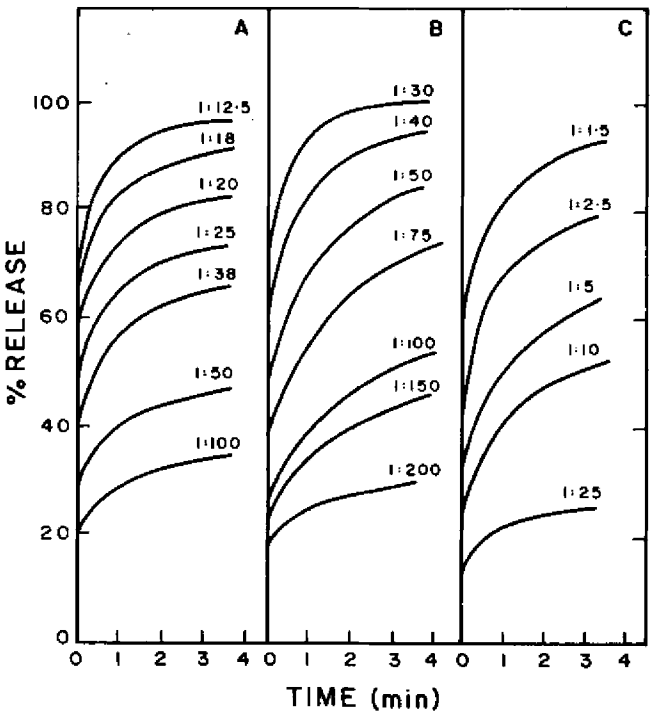


Figure 6. Release of CF from POPC vesicles at different peptide-lipid molar ratios. (**A**), IL; (**b**), ILA; (**C**), ILF. Release obtained with Triton X-100 was taken as 100%. Lipid = 100 μM.

of the lipid bilayer and are probably located below the head group region. Current voltage characteristics of black lipid membranes in the presence of an analogue of indolicidin with C-terminal as –COOH have been attributed to the formation of channels (Falla *et al* 1996). It is not clear if indolicidin with the C-terminal as $-CONH_2$ also modulates current voltage characteristics similar to indolicidin acid. The presence of a large number of aromatic residues do not permit unequivocal assignment of secondary structure in IL ILA and ILF. However, the spectra of the peptides in trifluoroethanol (Subbalakshmi *et al* 1996) and IL in lipid vesicles (Ladokhin *et al* 1997) suggest low propensity for folded structures. Hence, it is unlikely that transmembrane channels are formed like Gramicidin A (Killian 1992). Presence of peptide on the bilayer surface could result in the deformation of the membrane surface resulting in the formation of toroidal pores as proposed for explaining the channel activity of magainins (Matsuzaki *et al* 1995; Ludtke *et al* 1996).

It is unlikely that the inability of IL to adopt folded structures in structure promoting solvents and lipid vesicles is due to the presence of three proline residues as the analogue ILA where all the proline residues in IL have been replaced by alanine also does not show high propensity for ordered structure in solvents like trifluoroethanol. It appears that multiple trp residues act as 'structure breakers'. Since partition of trp residues into membranes is an energetically favourable process, IL and ILA bind to lipid vesicles in spite of their inability to adopt folded structures. Judiciously chosen analogues of indolicidin should help in not only generating molecules with improved and selective biological activity but should also help in evaluating the structural roles of multiple tryptophan residues in host peptides as well as binding to membranes.

Acknowledgement

Financial support from the Department of Science and Technology, New Delhi is gratefully acknowledged.

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MS received 22 July 1997; accepted 23 December 1997

Corresponding editor: R JAYARAMAN