Lipid membrane binding of NK-lysin

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Abstract The membrane-binding properties and pore-forming potential of the tumor-lysing and antibacterial polypeptide NK-lysin were investigated. Fluorescence quenching experiments show a drastic change of accessibility to Trp^{58} in solution and in association with a lipid membrane. Calcein release from large unilamellar vesicles and fluctuating conductivity observed across a planar lipid bilayer of asolectin show that NK-lysin renders lipid bilayers permeable in a transient fashion, indicating a non-specific lipid interaction as the mechanism underlying the biological activity. FTIR experiments show the same amount and type of regular secondary structure of NK-lysin in the membrane as in aqueous solution and exclude a structural rearrangement into a set of parallel or antiparallel α-helices as the predominant conformation. The molecular mechanism of the membrane-destabilizing effect of NK-lysin is discussed.

Key words: NK-lysin; Membrane binding; Pore formation; Fourier transform infrared spectroscopy

1. Introduction

NK-lysin is a basic polypeptide of 78 amino acid residues with antibacterial activity and the capability to lyse tumor but not red blood cells [1]. Its expression in $CD2^+$, $CD4^+$ and $CD8^+$ cells suggests that NK-lysin functions as an effector peptide of cytotoxic T cells and natural killer (NK) cells [1]. On T cell receptor-mediated binding of a target cell, perforin, granzymes and other molecules are released by cytoplasmic granules into the intercellular space. Thus, relatively high concentrations of NK-lysin may occur locally, assisting the cytotoxic effect of perforin and other membrane-destabilizing polypeptides [2].

Membrane-binding activity of NK-lysin is suggested by amino acid sequence homology to saposins, surfactant protein B (SP-B) and amoebapore peptides from *Entamoeba histolytica* [3,4], all of which have been demonstrated to associate with membranes [5–7].

The three-dimensional structure of NK-lysin in aqueous solution has recently been solved by NMR spectroscopy [8]. The structure comprises five amphipathic α-helices folded into a single globular domain with a hydrophobic core and a hydrophilic surface. A non-uniform charge distribution in NK-lysin suggests a particular orientation for its initial approach to a negatively charged lipid bilayer [8], but the structure does not suggest an obvious mechanism of membrane destabilization or pore formation. In particular, none of the helices is sufficiently long to span a lipid bilayer. NK-lysin is a member of the family of saposin-like proteins which are thought to have a conserved three-dimensional structure, based on the high conservation of buried residues and the presence of three conserved disulfide bridges. Furthermore, the circular dichroism (CD) spectrum of NK-lysin is very similar to that of SP-B and does not change in the presence of a membrane [3]. NK-lysin, SP-B, saposins and amoebapore are extremely stable against heat denaturation [3]. Thus, all available data seem to indicate that the saposin-like proteins assume the same fold which may be conserved not only in aqueous solution, but perhaps also in a membrane environment.

The present study was performed to verify the membrane-binding activity of NK-lysin, assess its pore-forming activity in receptor protein-free model systems and search for structural changes between membrane-bound and free NK-lysin.

2. Materials and methods

NK-lysin was isolated and purified as described [1]. Asolectin (Sigma Chemical) was purified using the procedure of Kagawa et al. [9]. Calcein (Sigma Chemical) was purified according to the protocol described by Kochi et al. [12].

2.1. Liposome preparation

Large unilamellar vesicles (LUV) used in calcein release and spectrofluorometric studies were prepared by extrusion according to the procedure described by Hope et al. [10]. Dry lipid films were rehydrated to a concentration of 20 mg/ml in 10 mM HEPES pH 7.2 buffer containing 62 mM calcein and subjected to five freeze/thaw cycles. The resulting preparation was extruded 10 times through two stacked 0.1 μm pore size polycarbonate filters (Nucleopore, Pleasanton, CA) with dry nitrogen gas (100–200 lbs/in^2). Unencapsulated dye was removed by passing the liposome preparation over a Sephadex G50 gel filtration column, equilibrated with 10 mM HEPES, 150 mM NaCl, 1 mM EDTA buffer, pH 7.2. Liposome concentration was estimated by measuring the lipid phosphorus content [11].

2.2. Calcein fluorescence

Fluorescence spectra were recorded on a SLM Aminco 8000 fluorimeter (SLM Instruments Inc., Urbana, IL). Release of the fluorescent dye calcein from preloaded LUV was monitored. The experiments were conducted in a 1 ml stirred cuvette at 37°C, with right angle illumination [12]. Excitation and emission wavelengths were set at 430 and 520 nm, respectively, employing a slit width of 4 nm. NK-lysin was added to liposomes (1 mg/ml) preequilibrated at the desired pH value. The addition of Triton X-100 to a final concentration of 0.1% (v/v) was used to determine maximal calcein release. The percentage of total fluorescence was defined as

$$\%F_t = \frac{I_t - I_o}{I_f - I_o} \times 100$$

where I_o is the initial fluorescence, I_f the total fluorescence observed

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Abbreviations: ATR, attenuated total reflection; CD, circular dichroism; EDTA, ethylene-diamine-tetraacetate; FTIR spectroscopy, Fourier transform infrared spectroscopy; LUV, large unilamellar vesicle; NK, natural killer; SP-B, surfactant protein B

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after addition of Triton X-100, and I_t the fluorescence after addition of NK-lysin.

2.3. Tryptophan fluorescence and fluorescence quenching experiments

Tryptophan fluorescence spectra were recorded as a function of the emission wavelength, using an excitation wavelength of 280 nm. Large unilamellar asolectin vesicles were prepared as described above. Fluorescence quenching experiments were performed by adding increasing amounts of potassium iodide from a 4 M stock solution to aqueous solutions of free NK-lysin or to NK-lysin associated with asolectin vesicles. The KI stock solution contained 1 mM $Na_2S_2O_3$ to prevent the formation of I_2 and I_3^- [13]. Fluorescence intensities were read at 333 nm after addition of quencher. Measurements were carried out at 25°C.

2.4. Electrical measurements in planer lipid bilayers

Planar lipid bilayers were formed using the Mueller-Rudin technique [14] over a 200 μm hole drilled in styrene co-polymer cups. Lipid bilayers were formed from asolectin dissolved in *n*-decane (1% w/v). All experiments were performed at room temperature. The solutions were prepared with triple-distilled water and filtered through filters with 0.2 μm pore size (Millipore). The two chambers were filled with 150 mM NaCl, 10 mM MES, pH 5.5, and connected to the electronic equipment through Ag/AgCl electrodes and a bridge of 3 M KCl/2% agar [15]. The current was measured using a Biologic RK-300 amplifier (Claix, France). The electrical potential was defined as *cis* versus *trans* which was connected to earth [15].

2.5. FTIR spectroscopy

Attenuated total reflection (ATR) Fourier transform infrared (FTIR) spectra [16] were recorded on a Bruker IFS-50 infrared spectrophotometer equipped with a liquid nitrogen-cooled MCT detector. The internal reflection element was a germanium ATR plate of dimensions 50×20×2 mm. The incident beam is tilted at 45° with respect to the germanium plate yielding 25 internal reflections. 256 scans were averaged for each spectrum. The spectra were recorded at a nominal resolution of 2 cm^{-1}. The spectrophotometer was continuously purged with air dried on a silica gel column of dimensions 5×130 cm using a flow rate of 7 l/min.

The samples were prepared as described by Fringeli and Günthard [17] by slowly evaporating the protein solution or the liposome suspension under a N_2 stream to deposit a thin film on one side of the germanium plate. When starting from a vesicle suspension, this procedure results in the formation of oriented multilayers at the surface of the plate [16]. The ATR plate was then sealed in a universal sample holder.

The amount of lipid-bound NK-lysin was determined by comparison of the relative intensities of the amide I and lipid carbonyl bands [18]. The spectrum of lipid-bound NK-lysin was recorded after two times pelleting the lipid-NK-lysin mixture at 14 000×*g* for 45 min and resuspending in water.

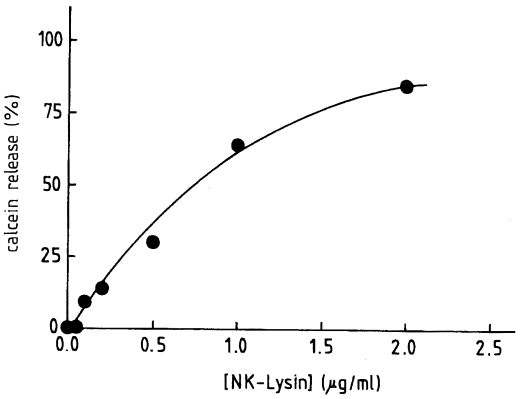

Fig. 1. Calcein release from LUV made of asolectin (200 μg/ml) as a function of NK-lysin concentration. Calcein release was monitored by fluorescence measured 10 min after the addition of NK-lysin.

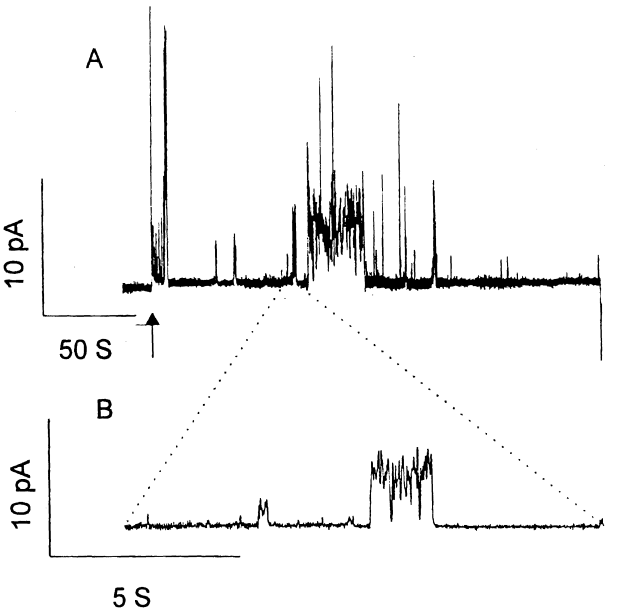

Fig. 2. Electric current fluctuations across a planar asolectin bilayer induced by 2.5 μg/ml NK-lysin. A: A voltage of 50 mV was applied across the membrane at the time point indicated by the arrow. B: Ten-fold expansion of part of the trace in A, showing details of the current fluctuations.

3. Results

3.1. Pore formation

Release of calcein was observed after addition of NK-lysin to LUV of asolectin (Fig. 1). For all NK-lysin concentrations used, leakage was almost complete within 10 min. These measurements indicate that NK-lysin destabilizes the lipid bilayer but do not provide information about the mechanism of this destabilization. In an attempt to distinguish between unspecific destabilization and specific channel formation in the lipid bilayer we measured the flow of electric current through a planar lipid bilayer.

Addition of 2.5 μg/ml NK-lysin to the *cis* compartment of a planar asolectin lipid bilayer induced random current fluctuations through the membrane when 50 mV voltage difference was applied across the lipid membrane (Fig. 2). Positive and negative voltages produced similar results (data not shown). The amplitude of the current varied as well as the durations of the current fluctuations which were observed for time periods of a few tens of milliseconds up to several tens of seconds (Fig. 2). The largest current fluctuations would correspond to a pore with a conductance of about 400 pS.

3.2. Membrane binding mode

Trp^{58} is the only tryptophan residue in NK-lysin. Its side chain is largely solvent-exposed in the NMR structure determined in aqueous solution [8]. The fluorescence emission spectrum of free NK-lysin shows a maximum at 350 nm. After incubation with asolectin LUVs, this maximum is shifted to 325 nm (data not shown), indicating the insertion of Trp^{58} into a more hydrophobic environment.

Fluorescence quenching experiments by iodide performed in the presence of asolectin LUVs showed no change in tryptophan fluorescence at iodide concentrations of up to 50 mM, indicating the protection of Trp^{58} from iodide access in the

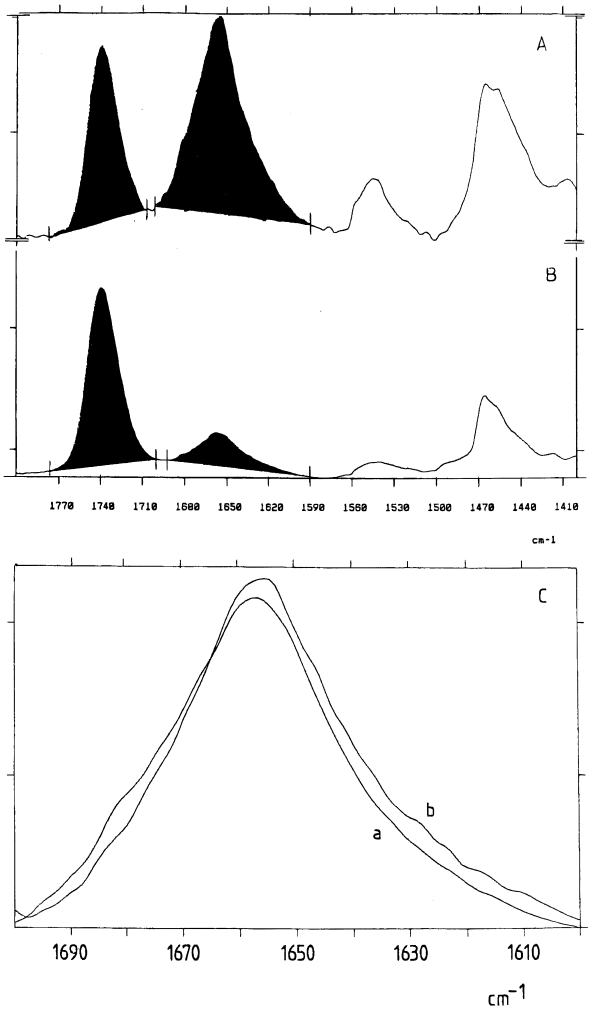

Fig. 3. FTIR spectra of free and asolectin-associated NK-lysin. A: 1800–1400 cm^{-1} range of a spectrum recorded with 100 μg of a 7:1 (w/w) mixture of asolectin/NK-lysin. B: Same spectral range as in A, recorded with 100 μg of the pellet obtained after centrifugation of the sample of A and resuspension in water. The protein amide I and lipid carbonyl bands are hatched in A and B. C: Comparison of the shape of the amide I band of (a) free NK-lysin and (b) NK-lysin bound to asolectin vesicles obtained after two-fold pelleting of the asolectin/NK-lysin mixture and resuspension in pure water.

membrane-bound form. In the absence of asolectin LUVs, the fluorescence intensity decreased linearly with iodide concentration, confirming the solvent accessibility of Trp^{58} in solution [8] (data not shown).

The binding of NK-lysin to bilayers of asolectin and its secondary structure in a lipid environment were investigated by FTIR spectroscopy. The spectrum of Fig. 3A was recorded after mixing aqueous solutions of asolectin LUVs and NK-lysin. The bands at 1740 and 1656 cm^{-1} are the carbonyl band of the lipid and the amide I band of NK-lysin, respectively. The spectrum of Fig. 3B was obtained after pelleting the vesicles by centrifugation and resuspension in water to remove unbound NK-lysin. The relative intensities of the amide I band in Fig. 3A,B show that about 10% of the NK-lysin was tightly associated with the lipid. A second cycle of pelleting and resuspension in water yielded the spectrum shown in Fig. 3B which was indistinguishable from that obtained after the first pelleting (data not shown). The shape of the amide I band at 1656 cm^{-1} is characteristic of a highly helical peptide [16]. Remarkably, the shape of the amide I band did not significantly change after lipid binding (Fig. 3C), showing that the composition and content of regular secondary structure in NK-lysin was not changed by binding to the lipid bilayer.

In order to assess the orientation of the helices with respect to the lipid bilayer, spectra were recorded with the incident light beam tilted by 45° with respect to the germanium plate and polarized parallel or perpendicular to the incidence plane. No significant difference of intensity could be measured in the amide I band for either polarization (data not shown), clearly indicating the absence of any major fractions of helix bundles with parallel or antiparallel helices running perpendicular or parallel to the membrane surface [16]. The data are in agreement with a globular conformation similar to that observed in aqueous solution [8].

4. Discussion

The release of calcein encapsulated in asolectin vesicles demonstrates that lipid bilayers can become permeable by the direct action of NK-lysin. Furthermore, the ion current experiments across a planar asolectin membrane show that NK-lysin leads to transient pore formation. The non-specific interaction with lipid bilayers could explain the broad antibacterial and tumorolytic activity of NK-lysin [1].

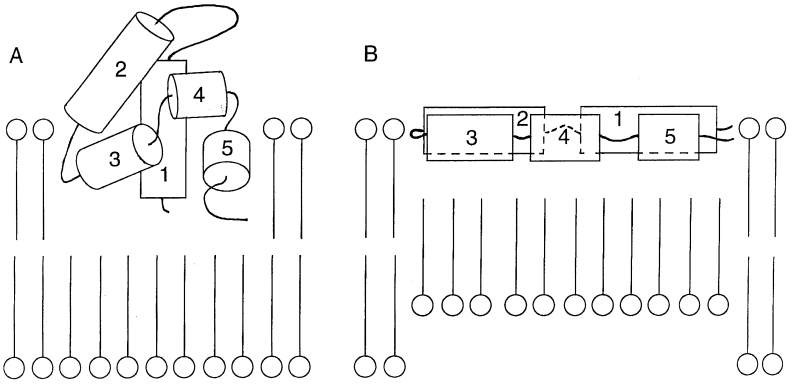

Fig. 4. Models of NK-lysin binding to a lipid bilayer. The α-helices of NK-lysin are represented as cylinders. A: Solution structure of NK-lysin [8] embedded in the membrane. B: Hairpin arrangement of the amphiphilic α-helices of NK-lysin at the lipid-water interface.

The ion current observed across a membrane of asolectin fluctuated randomly between high conductivity indicative of ion pores and low conductivity characteristic of an intact membrane (Fig. 2). Pore formation appeared to be both transient and reversible, with variable amplitudes of the electric current. It thus appears that NK-lysin does not refold to form specific ion channels of well-defined structure. Rather, all available data seem to indicate that the solution conformation of NK-lysin is essentially preserved after binding to a membrane. In particular, the FTIR data (Fig. 3C) show no evidence for any difference in the amount and type of regular secondary structure in NK-lysin between the water and membrane phases. The apparent conservation of regular secondary structure has also been reported previously based on CD data recorded of NK-lysin in the presence and absence of lipid bilayers [3]. However, the amount of lipid-bound NK-lysin had not been assessed in those experiments.

Neither CD nor IR data report on the conservation of the tertiary fold of NK-lysin in a membrane environment. In fact, it has been pointed out that the three disulfide bridges present in NK-lysin would not preclude a simple hairpin-like structure, where the amphiphilic helices would be embedded in the surface of the membrane with their hydrophobic and hydrophilic faces directed towards the interior of the lipid bilayer and the solvent, respectively (Fig. 4B) [3]. FTIR experiments with planarly polarized light on oriented lipid bilayers clearly indicate that a parallel arrangement of the helices parallel to the membrane surface as in Fig. 4B can be excluded as the equilibrium structure of membrane-bound NK-lysin. Likewise, a permanent transmembrane arrangement of the helix hairpin modelled in Fig. 4B is equally excluded by the same experiments.

The globular solution structure of NK-lysin contains most of the positively charged side chains of Arg and Lys residues in an equatorial belt around the 'waist' of the protein, while the negatively charged side chains of Asp and Glu residues are located in the upper half of the molecule and the base of the molecule contains an extended surface area composed of hydrophobic amino acid side chains [8]. It was thus proposed that NK-lysin could bind to membranes with the bottom dipping into the membrane, the ring of lysine and arginine residues interacting with the negatively charged lipid head groups and the negatively charged top half of NK-lysin remaining solvent-exposed (Fig. 4A). This model appears plausible at least as a picture of the initial approach of NK-lysin to a lipid bilayer before it unfolds and inserts more deeply into the membrane. It is, however, also possible that this model represents the predominant conformation of membrane-bound NK-lysin. It would account for the observed protection of the side chain of Trp^{58} from iodide access and for the apparent absence of a preferential orientation of the total helix dipole moment of membrane-bound NK-lysin with respect to the membrane surface or the lipid side chains. It would further account for the conserved secondary structure and agree with the unusual thermostability of the NK-lysin structure in aqueous solution, which does not denature even near boiling temperatures [3]. The importance of the correct tertiary structure of NK-lysin has been demonstrated by the fact that the biological activity of NK-lysin is lost after reduction of one or several of the disulfide bridges [19]. The question is whether a 'superficial' membrane association like that shown in Fig. 4A could be sufficient to cause the transient formation of ion channels in lipid membranes.

Theoretical calculations of membrane-associated annexin V have shown that the electrostatic field generated by this protein can lead to an electrostatic potential difference of more than 0.2 V across the lipid bilayer which is sufficient for permeabilization of the membrane by an electroporation mechanism [20,21]. Like NK-lysin, annexin V is thought to bind only superficially to the surface of a membrane. Electroporation triggered by the association of highly charged NK-lysin would be consistent with the observed variations in conductivity (Fig. 2). Further experiments will be required to verify this model.

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References

[1] Anderson, M., Gunne, H., Agerberth, B., Boman, A., Bergman, T., Sillard, R., Jörnvall, H., Mutt, V., Olsson, B., Wigzell, H., Dagerlind, A., Boman, H.G. and Gudmundsson, G.H. (1995) EMBO J. 14, 1615–1625.
[2] Tschopp, J. and Hofmann, K. (1996) Trends Microbiol. 4, 91–94.
[3] Andersson, M., Curstedt, T., Jörnvall, H. and Johansson, J. (1995) FEBS Lett. 362, 328–332.
[4] Leippe, M. (1995) Cell 83, 17–18.
[5] Vaccaro, A.M., Salvioli, R., Barca, A., Tatti, M., Ciaffoni, F., Maras, B., Siciliano, R., Zappacosta, F., Amoresano, A. and Pucci, P. (1995) J. Biol. Chem. 270, 9953–9960.
[6] Longo, M.L., Bisagno, A.M., Zasadzinski, J.A., Bruni, R. and Waring, A.J. (1993) Science 261, 453–456.
[7] Leippe, M., Ebel, S., Schoenberger, O.L., Horstmann, R.D. and Müller-Eberhard, H.J. (1991) Proc. Natl. Acad. Sci. USA 88, 7659–7663.
[8] Liepinsh, E., Andersson, M., Ruysschaert, J.-M. and Otting, G. (1997) Nature Struct. Biol. 4, 793–795.
[9] Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487.
[10] Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65.
[11] Mrsny, R.J., Volwerk, J.J. and Griffith, O.H. (1986) Chem. Phys. Lipids 39, 185–191.
[12] Kochi, S.K., Martin, I., Schiavo, G., Mock, M. and Cabiaux, V. (1994) Biochemistry 33, 2604–2609.
[13] Sonveaux, N., Shapiro, A.B., Goormaghtigh, E., Ling, V. and Ruysschaert, J.-M. (1996) J. Biol. Chem. 271, 24617–24624.
[14] Mueller, P., Rudin, D.O., Tien, H. and Wescott, W.C. (1962) Nature 194, 979–980.
[15] Fuks, B. and Homble, F. (1994) Biophys. J. 66, 1404–1414.
[16] Goormaghtigh, E., Cabiaux, V. and Ruysschaert, J.-M. (1995) Subcell. Biochem. 23, 329–450.
[17] Fringeli, U.P. and Günthard, H.H. (1981) in: Membrane Spectroscopy (Grell, E., Ed.), pp. 270–332, Springer Verlag, Heidelberg.
[18] Goormaghtigh, E., Cabiaux, V. and Ruysschaert, J.-M. (1990) Eur. J. Biochem. 193, 409–420.
[19] Andersson, M., Holmgren, A. and Spyrou, G. (1996) J. Biol. Chem. 271, 10116–10120.
[20] Karshikov, A., Berendes, R., Burger, A., Cavalié, A., Lux, H.-D. and Huber, R. (1992) Eur. Biophys. J. 20, 337–344.
[21] Neumann, E. (1988) Ferroelectrics 86, 325–333.