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Folding and Membrane Insertion of the Pore-Forming Peptide Gramicidin Occur as a Concerted Process

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Many antibiotic peptides function by binding and inserting into membranes. Understanding this process provides an insight into the fundamentals of both membrane protein folding and antibiotic peptide function. For the first time, in this work, flow-aligned linear dichroism (LD) is used to study the folding of the antibiotic peptide gramicidin. LD provides insight into the combined processes of peptide folding and insertion and has the advantage over other similar techniques of being insensitive to off-membrane aggregation events. By combining LD data with conventional measurements of protein fluorescence and circular dichroism, the mechanism of gramicidin insertion is elucidated. The mechanism consists of five separately assignable steps that include formation of a water-insoluble gramicidin aggregate, dissociation from the aggregate, partitioning of peptide to the membrane surface, oligomerisation on the surface and concerted insertion and folding of the peptide to the double-helical form of gramicidin. Measurement of the rates of each step shows that although changes in the fluorescence signal cease 10 s after the initiation of the process, the insertion of the peptide into the membrane is actually not complete for a further 60 min. This last membrane insertion phase is only apparent by measurement of LD and circular dichroism signal changes. In summary, this study demonstrates the importance of multi-technique approaches, including LD, in studies of membrane protein folding.

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Introduction

Understanding the kinetics of spontaneous insertion of polypeptides into membranes is of fundamental importance to the design of new antimicrobial peptides and to the understanding of membrane protein folding. Research into the folding mechanism of membrane proteins has progressed at a slower rate compared with soluble proteins. This is a result of the inherent difficulties of handling membrane proteins, which are often insoluble in aqueous solution and prone to aggregation. In addition, techniques developed for studying soluble protein folding are unable to probe the orientation of peptide chains relative to the membrane, arguably the most important parameter of membrane protein folding and insertion. This presents a problem in the interpretation of mechanistic processes. The purpose of this study was to show that by using combined techniques over different concentrations, one can gain insight into folding and insertion of peptides into membranes. This is with a view to using these techniques to design better antibiotic peptides: gramicidin was chosen to demonstrate this as it is currently used in the clinic and has interesting properties of dimerisation and different configurations.

Research into the folding of membrane proteins that had been carried out has, in most cases, revealed a multi-step process.[1] These steps include a number in which the polypeptide is associated with the membrane but is visiting various parts of orientational and/or conformational space. This search by the membrane protein represents a key difference from the folding of soluble proteins that is difficult to probe using currently used techniques. Here, we demonstrate that UV linear dichroism [LD, not to be confused with circular dichroism (CD)] can be used to give real-

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Abbreviations used: LD, linear dichroism; DH, double-helical; PC, phosphatidylcholine.

time orientation information during the process of folding of the antimicrobial membrane peptide gramicidin. We have developed the use of LD information, in combination with that from CD and stopped-flow fluorescence techniques, to build a model of gramicidin insertion and folding in membranes.

In order to provide some commonality with other larger membrane protein systems, we examine the folding of the intertwined double-helical (DH, or pore) form as this form spans both leaflets of the membrane and has extensive inter-molecular lateral associations.

Linear dichroism

LD spectroscopy[2–8] is the difference in absorbance, by an aligned sample, of light polarised parallel with and perpendicular to the alignment axis. [For a rigorous treatment of the theory, see Ref. 9.] LD spectroscopy requires that the sample be aligned; this can be carried out in various ways: for example, by use of magnetic fields, stretching a film containing the chromophore or compressing a gel. However, we are generally interested in what happens in solution, so we have developed alignment systems that use shear flow. By placing the sample in the annular gap between a cylinder and a rod (orientated coaxially) and rotating either the cylinder or the rod, molecules that have a sufficiently large axial ratio and stiffness then orient. In this alignment regimen, molecules are induced to align without any appreciable damage to their structures. Recently, technical advances have enabled the use of small sample volumes (<50 μL),[6,10] thus facilitating the use of LD for biological samples where it was not previously practicable.

Previous work by Ardhammar *et al.* showed that LD can be measured for small molecules bound to membranes.[11,12] We have subsequently shown that this can be extended to membrane peptides and proteins[8] and to peptides and proteins with small molecules[7] (Fig. 1). The physical basis for the alignment that underlies these measurements is centred on the observation that spherical liposomes become ellipsoidal in shear flow, thus orienting with their long axes arranged circumferentially around the cell.

Gramicidin

Gramicidin A is a linear pentadecapeptide antibiotic produced by the soil bacterium *Bacillus brevis* and contains alternating L- and D-amino acids.[13] The mode of action of gramicidin involves its insertion into the membrane, leading to ionic leakage from the bacterium and disruption of membrane structure. Gramicidin is known to form different structures in the membrane: a tail-to-tail helical dimer (or channel form)[14] and several intertwined DH (pore) forms, which can be distinguished using CD spectroscopy.[15,16] Each form has specific spectroscopic features, the most obvious difference being in the sign of the signal at around 230 nm: in the helical dimer form, the CD is positive at 230 nm, whereas in the DH form, there is a strong negative band.

Factors that affect the conformation of the peptide include the composition of the lipid membrane in which it is inserted, the concentration of gramicidin, the temperature and the peptide-to-lipid ratio[17,18] and the solvent in which it is dissolved prior to insertion.[19]

In this study, we chose conditions that gave the pore form (see below) and then used a multi-

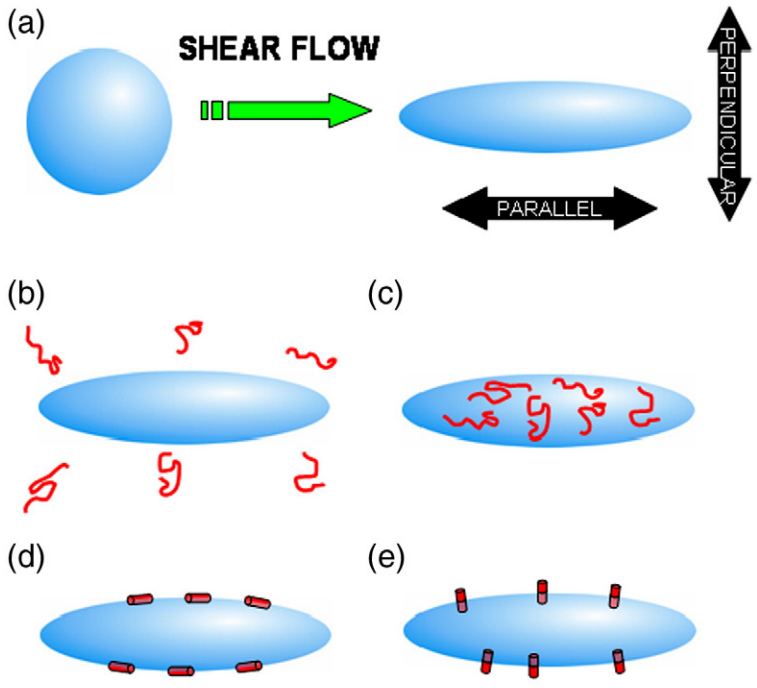

Fig. 1. (a) Liposomes can be distorted by shear flow and align with their long axis parallel with the flow direction. (b) Peptides in solution are isotropically oriented in space and absorb light polarised parallel with and perpendicular to the orientation axis of the liposome equally. This results in zero LD. (c) Unfolded peptides stuck to the surface of the liposome will give very little LD signal above 210 nm as the *n*-to-π* peptide transitions will cancel out. (d) Peptides folded on the surface of the liposomes absorb light polarised parallel with the orientation axis to a different extent to light polarised perpendicular to this. For example, an α-helical peptide in this orientation will absorb light at ~225 nm (the *n*-to-π* peptide bond transition) in the parallel polarisation direction less than in the perpendicular direction. This results in a negative LD at this wavelength. (e) Conversely, if the same peptide were inserted into the membrane, the LD signal would be positive around 225 nm. This is because the transition moments in the peptide that result in the absorbance at 225 nm are oriented perpendicular to the case illustrated in (d).