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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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Keywords: membrane protein; folding; kinetics; gramicidin; linear dichroism

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-

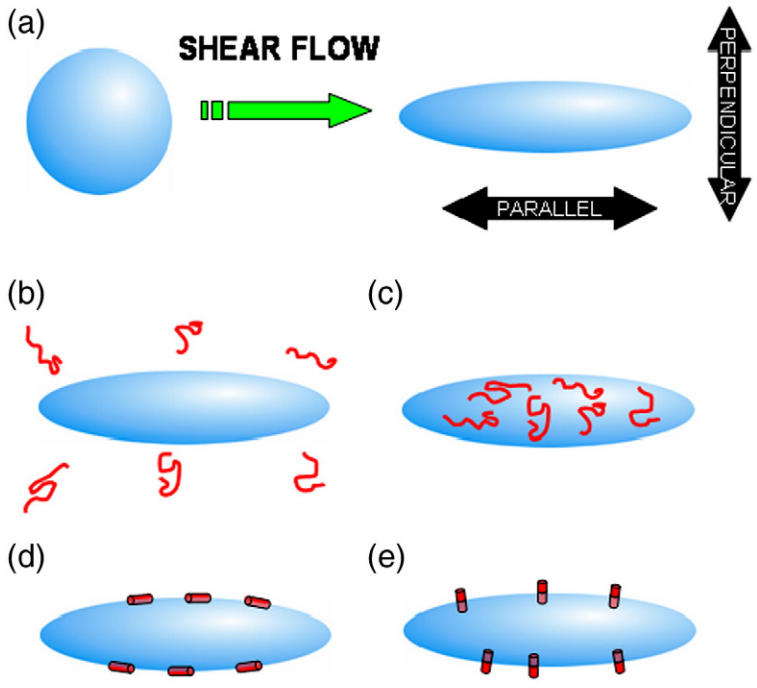

Fig. 1. (a) Liposomes can be distorted by shear flow and align with their long axis parallel with the flow direction. (b) Peptides in solution are isotropically oriented in space and absorb light polarised parallel with and perpendicular to the orientation axis of the liposome equally. This results in 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).

technique approach to probe separately the lipid association (stopped-flow fluorescence), backbone conformation (CD) and insertion/alignment (LD) of the peptide. This reveals the mechanism of insertion of the DH form of gramicidin, which has to date remained unclear.

Results

Measurement of LD and CD spectra of the pore form of gramicidin

The gramicidin peptide was dissolved in TFE and added to small unilamellar phosphatidylcholine (PC) vesicles in water to produce the pore form. Adding gramicidin to vesicles from TFE often gives the channel form at low concentrations. However, there are other factors that favour formation of the DH pore form in these experiments: (1) the relatively high concentrations used here[17] and (2) the fact that the natural soybean lipid used here favours the pore form.[15,20] In Fig. 2, we see CD spectra characteristic of the pore form with a negative maximum at around 230 nm. The LD spectra of gramicidin in soybean lipids (Fig. 3) as a function of time show that the LD spectrum develops over tens of minutes post-injection into the lipid-containing solution. The spectrum has features in both the backbone (<260 nm) and aromatic (270–300 nm) regions of the spectrum, indicating that the peptide has inserted into the lipid and formed a regular folded structure. These features can be used to provide information on the orientation of the chromophores within the protein with respect to the membrane.

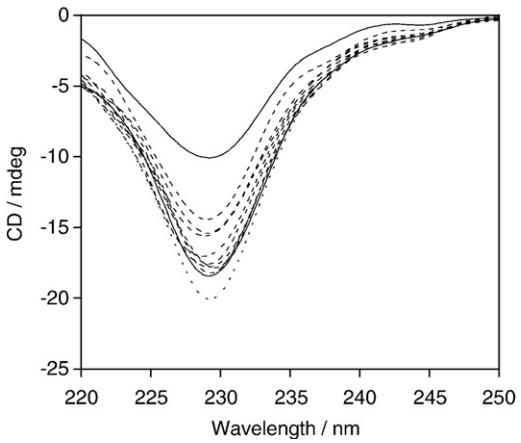

Fig. 2. CD spectra of gramicidin insertion into PC liposomes: the spectra shown here are 0–12 min (continuous line, low signal) and 118–120 min (continuous line, high signal) with spectra every 12 min in between (dashed lines). The same sample after 24 h is also shown (dotted line). These spectra are for 100 $\mu g.mL^{-1}$ of gramicidin in 10% v/v TFE/water, with 1.8 $mg.mL^{-1}$ of PC and using a 1-mm cuvette. These concentrations give a molar ratio of lipid to peptide of 44:1.

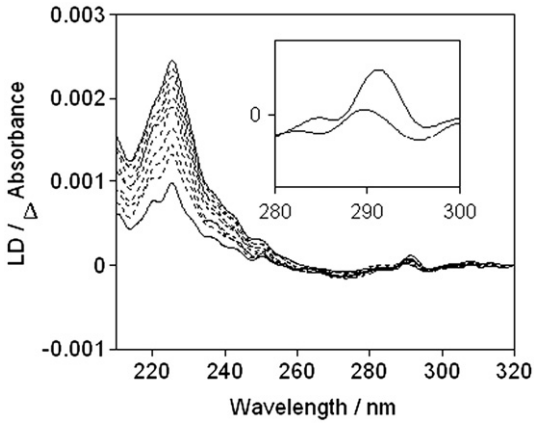

Fig. 3. LD spectra of gramicidin insertion into PC liposomes: the spectra shown here are 0–12 min (continuous line, low signal) and 118–120 min (continuous line, high signal) with spectra every 12 min in between (dashed lines). The aromatic region is shown in the inset for the start (small signal) and end (large signal) points. These spectra are for 100 $\mu g.mL^{-1}$ of gramicidin in 10% v/v TFE/water, with 1.8 $mg.mL^{-1}$ of PC and using a 0.5-mm microvolume Couette cell. These concentrations give a molar ratio of lipid to peptide of 44:1.

Measurement of the kinetics of the late events of gramicidin insertion using LD and CD

Knowing that under the conditions described gramicidin was able to fold, dimerise and insert into the membrane in a few hours, we decided to look into the kinetics of the events that occur during the process. LD-based methods are particularly attractive for this since an LD signal will only appear *if* a chromophore becomes uniformly aligned; unaligned isotropic material is invisible in LD. In the case of this study of membrane protein insertion, the membrane itself is the aligned element. This means that any change in the LD signal that occurs during the folding experiment can only be the result of events taking place on or in the membrane. Importantly, this prevents LD signals from being distorted by off-membrane events, such as protein aggregation, that interfere with other biophysical measurements, such as fluorescence and CD.

Current LD instrumentation limits the dead time of any experiment to approximately 30 s. In addition, although fast CD measurements can be made in stopped-flow experiments, sensitivity and signal to noise are often poor. Therefore, experiments carried out using both LD and CD were begun with a consistent dead time of 60 s post-mixing. This restricted LD and CD observations to those that occur between 60 s and 2 h (or longer had this been required).

Upon initiation of folding by addition of gramicidin in TFE to PC liposomes, both CD and LD spectra tended toward those recorded for the system at equilibrium. It should be noted that there was no significant change in the size of the liposomes (diameter around 100 nm) after addition of gramicidin as assessed by dynamic light-scattering measurements

(data not shown). Spectra are shown in Figs. 1 and 2 for CD and LD measurements, respectively. After 2 h, it was concluded that the reaction had proceeded to completion. Further measurements were made of these samples over days, and no change to the spectra was observed (data not shown). This confirmed that over 2 h, the system had reached equilibrium. Kinetic parameters for the processes were determined by monitoring CD and LD signals and analysing the mean signal between 228 and 230 nm (CD) and that between 226 and 228 nm (LD) as a function of time (Fig. 4a). These signals reflect alterations in the backbone conformation and configuration/orientation. Analysis of the CD data showed that between 60 s and 2 h, the CD signal at 229 nm increases with a profile that fits to a single exponential, indicating the presence of a single rate-determining process. The CD spectra at 60 s post-mixing showed only a weak (pore form) signal, suggesting that the process occurring over this period was that of peptide-chain folding. The LD signal showed a change in the backbone region that also fitted to a single exponential. The sign of LD signal for the backbone region shows that at the end of the process, the peptide is inserted in the membrane, as expected for the pore form. These data suggest that the alignment (insertion into the membrane) of the peptide backbone relative to the membrane occurred in a single concerted step. The LD rate constants ($7.4\pm 0.8\times10^{-4}$ s^{-1}) equal those observed for the CD ($6.3\pm 0.9\times10^{-4}$ s^{-1}) within experimental error, providing strong evidence that folding (detected by CD) and insertion (detected by LD) are simultaneous processes. The rates are mean values over three concentrations, and the errors are the standard deviation of the mean.

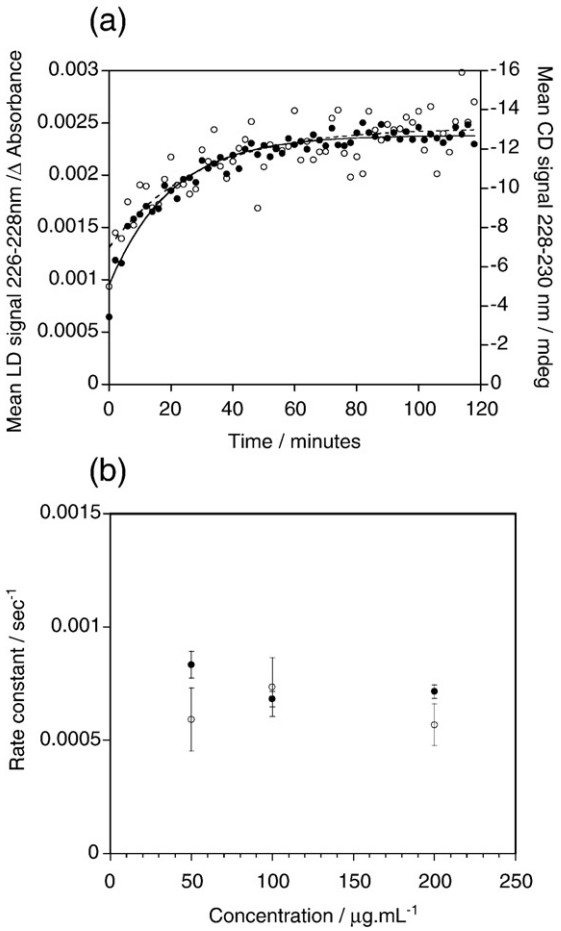

Fig. 4. Rates of folding and insertion of gramicidin into liposomes. (a) Example of fits to a single-exponential process for backbone signals from LD and CD. Filled circles (continuous line): LD of 50 $\mu g.mL^{-1}$ of gramicidin in 1800 $\mu g.mL^{-1}$ of PC, 10% v/v TFE and 0.5-mm path length. Open circles (dashed line): CD of 50 $\mu g.mL^{-1}$ of gramicidin in 1800 $\mu g.mL^{-1}$ of PC, 10% v/v TFE and 1-mm path length. (b) Rate constants and change in signal over 2 h determined by CD and LD. The rate constants measured by CD and LD over a range of peptide concentrations were relatively concentration independent and were similar. Error bars are the standard error in the fit of the rate constants.

Concentration dependence of the late events of gramicidin insertion

The pore form of gramicidin is thought to be dimeric in nature. It is therefore possible that the rate-limiting process of gramicidin insertion under these conditions may be the dimerisation of the peptide. Kinetic measurements using LD and CD were made at different starting concentrations of gramicidin but constant lipid concentrations to determine whether this was the case. The rate constants for different peptide concentrations (determined as described above) were found to be independent of peptide concentration, suggesting that the folding of peptide, rather than its dimerisation, is the rate-limiting process under these conditions (Fig. 4b).

Measurement of the kinetics of the early events of gramicidin insertion

In order to probe events that happen on a faster time scale than those available to LD measurements, we used stopped-flow fluorescence on rapidly mixed peptide and lipid solutions with a dead time of 10 ms. Measurements were made by exciting the tryptophan residues in gramicidin at 292 nm and measuring emitted light above 320 nm (Fig. 5a). Analysis of the kinetic traces from these experiments showed that they did not conform to a simple single exponential and instead fitted a double exponential. This is suggestive of two sequential processes, both of which are characterised by a decrease in total fluorescence. The decrease in fluorescence observed in the stopped-flow experiments was confirmed by measuring the fluorescence emission of gramicidin in 10% TFE in the absence and in the presence of liposomes (Fig. 5b). In addition to the decrease in total fluorescence, there is also a blue shift in the wavelength of the emission maximum; this is consistent with the (partial) burial of tryptophan residues in a membrane.

Rapid kinetic measurements were carried out at a range of peptide concentrations to ascertain whether either of the steps was peptide concentration

dependent. Analysis of these data shows that the first step decreased in rate with increasing concentration, while the second step increased in rate (Fig. 5c). The decrease in rate with increasing concentration for the first step is indicative of a dissociation event, while the increase in the second rate suggests a subsequent association, which is discussed further below.

Comparison of the emission spectra for gramicidin in 100% TFE and that in 10% TFE in the absence of lipid (Fig. 5b) shows a large increase in fluorescence and a blue shift in the wavelength of the emission maximum. These are consistent with the formation of an aggregate with partially buried tryptophan residues on mixing the gramicidin in 100% TFE with water to give 10% TFE. Indeed, it was observed that gramicidin samples in 10% TFE were visibly turbid and could be sedimented at low centrifugal forces (data not shown).

The increase in fluorescence upon mixing also demonstrates that this initial phase occurs within the dead time of the stopped-flow measurements (recall that both processes observed in the stopped-flow experiments resulted in a decrease in fluorescence). The fluorescence decreases observed (Fig. 5a) occur only in the presence of lipid (data not shown), indicating that it is the presence of lipid that induces the dissociation of the gramicidin aggregate and subsequent folding. Consistent with this is the observation that CD spectra measured at equilibrium reveal that the signal at 229 nm for gramicidin is slightly positive when dissolved in TFE, is near zero in 10% TFE (data not shown) and only becomes strongly negative in the presence of lipid (Fig. 2).

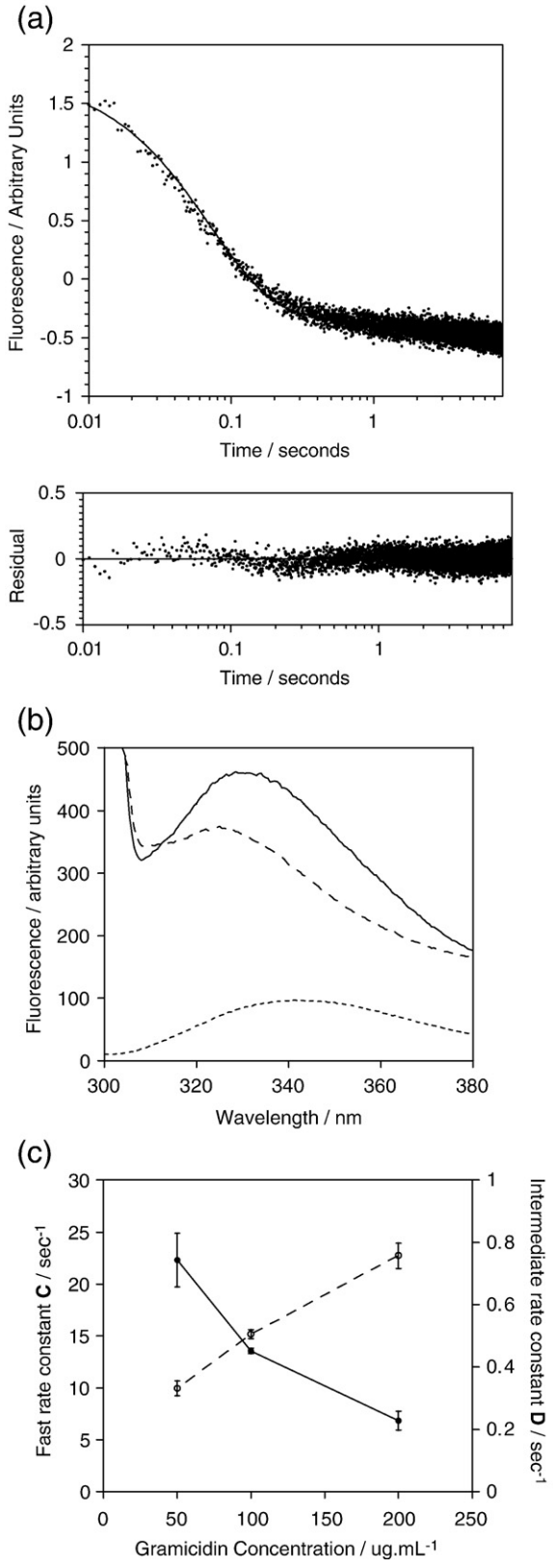

Fig. 5. Fast kinetics and fluorescence of gramicidin. (a) Change in fluorescence upon mixing gramicidin with liposomes. The fluorescence signal is shown as dots with data every 1 ms. A fit of a function consisting of two exponential decays is shown as a continuous black line. The lower panel shows the residual between the fit and the data for the same data set. (b) Fluorescence of gramicidin in TFE (short dashes), in 10% TFE (continuous line) and in 10% TFE in the presence of PC liposomes (long dashes). (c) Stopped-flow measurements of gramicidin fluorescence in the time range 0–8 s revealed two processes. The rate constants for these processes were ascertained by fitting the data to a sum of two exponential functions. The fast rate constant **C** (closed circles, continuous line) decreases with gramicidin concentration, and the intermediate rate constant **D** (open circles, dashed line) increases with gramicidin concentration. The samples were in 10% v/v TFE and 1.8 mg.mL^{-1} of PC with a path length of 2 mm. Error bars are the standard error of the mean of triplicate measurements.

Discussion

Studies of membrane protein folding pose a number of complex technical problems that cannot be resolved by methods developed to study the folding of soluble globular proteins. In this work, we demonstrate, for the first time, the use of flow-aligned LD spectroscopy to study the kinetics of the folding of a peptide on/in a membrane. LD is ideally suited to this type of study as it senses only the events that occur in contact with the flow-aligned membrane, providing, in addition,

information on the secondary structure, side-chain arrangement and novel information on the orientation of the protein.

Far-UV CD can be used to identify the fold that the gramicidin adopts in the membrane. It is clear that the peptide folds to the pore or DH form under the conditions used here (Fig. 2). The far-UV LD spectra recorded at equilibrium contain peaks for backbone electronic transitions that, in agreement with the CD, confirmed the presence of a transmembrane gramicidin peptide in the pore conformation. More specifically, the positive transition in the LD around 226 nm is assigned to the n-to-π* backbone transition.[21] This transition moment is oriented perpendicular to the helical axis in gramicidin. Since LD of aligned membrane systems is defined as the absorbance of light polarised parallel with the membrane surface minus the absorbance of light polarised perpendicular to the membrane surface, this transition moment must be more parallel with than perpendicular to the membrane surface. Thus, the helical axis is more perpendicular to than parallel with the membrane surface (i.e., the helix traverses the membrane). The LD signal and absorbance are related to the angle of the moment for the transition observed in the spectrum by Eq. (1).[8,11]

$$\mathrm{LD}^{\mathrm{r}} = \frac{\mathrm{LD}}{A_{\mathrm{iso}}} = \frac{3S}{4}\left(1 - 3\cos^2\beta_i\right) \qquad (1)$$

where LD^{r} is the reduced LD, A_{iso} is the absorbance of an unoriented sample in the same path length, S is the orientation factor and β_i is the angle that the ith transition moment makes with the membrane normal to the membrane surface. S is 1 for a perfectly aligned sample and is 0 for an unoriented sample. Since $(1-3\cos^2 54.7°)=0$, a positive LD signal means that $\beta_i > 54.7°$; it should be noted that one does not need to know S or A_{iso} in order to interpret the data at this level. The aromatic signals observed in gramicidin are produced by the tryptophans (four per chain; so, >25% of the peptide); the L_{a} and L_{b} transitions for tryptophan are around 280 and 290 nm, respectively.[22,23] From examination of a crystal structure of the pore form of gramicidin (Protein Data Bank code 1c4d), one can calculate the average direction of the transition moments for the L_{a} and L_{b} transitions of tryptophan using the coordinates from the Protein Data Bank file for a geometry where it is inserted into the membrane. For this structure, the L_{a} transition gives the vector (−3.8,7.8,0.78) and the L_{b} transition gives the vector (7.6,−3.8,9.7) (x,y,z), where the membrane surface lies in the x,z plane (i.e., the membrane normal, and the long axis of the gramicidin helix when inserted, is in the y-direction). From this, the angle that the vector makes with the y-axis (β in Eq. (1)) can be calculated. The angle is 26° for L_{a} and is 73° for L_{b}. Thus, when inserted as a pore, gramicidin is expected to have a negative LD signal at ~280 nm ($\beta < 55°$) and a positive LD signal at ~290 nm ($\beta > 55°$). The 290-nm signal is indeed positive in the data presented here; however, there is no negative peak around 280 nm. This requires that the angle that the L_{a} transition moment makes with the membrane normal is, in reality, larger than the one calculated by simply inserting the crystal structure into the membrane parallel with the membrane normal. In other words, the indole ring lies more in plane with the membrane surface than suggested by the crystal structure (the structure was determined in methanol/CsCl). This could be due to tilting of either the whole pore structure or the tryptophans.

Dissociation of gramicidin oligomers occurs rapidly prior to folding

The rate of the first process as measured by a change in fluorescence is proportional to the inverse of gramicidin concentration. This indicates that some kind of disassociation reaction takes place prior to any folding. Mixing and aggregation (**A**) happen within the dead time of the measurement (~10 ms or a rate of 100 s^{-1}) with a small population of monomeric gramicidin being present in solution (**B**). In the next step, we propose that a small population of soluble gramicidin monomer binds to the membrane, leading to the measured decrease in fluorescence (**C**), with a rate of 22 to 7 s^{-1}. This has the effect of depleting the solution concentration of the monomeric species, inducing dissociation of the larger aggregate. This dissociation step (**B**) becomes the rate-determining step for the early stages of gramicidin insertion, hence resulting in the observed concentration dependency. We have summarised these stages in Eq. (2):

$$\begin{aligned} \mathrm{G}^{\mathrm{TFE}} + \mathrm{water} &\xrightarrow{A} \mathrm{GGGGG}^{\mathrm{TFE:water}} \\ + \mathrm{lipid} &\overset{B}{\leftrightarrow} \mathrm{G}^{\mathrm{TFE:water}} \overset{C}{\leftrightarrow} \mathrm{G}^{\mathrm{lipid}}, \end{aligned} \qquad (2)$$

where $\mathrm{G}^{\mathrm{TFE}}$ denotes gramicidin dissolved in TFE, $\mathrm{GGGGG}^{\mathrm{TFE:water}}$ is an oligomer of gramicidin in 10% TFE/water, lipid is the liposome, $\mathrm{G}^{\mathrm{TFE:water}}$ is a monomer of gramicidin in 10% TFE/water and $\mathrm{G}^{\mathrm{lipid}}$ is a monomer of gramicidin associated with lipid.

After about 1−3 s, we observe a second change in tryptophan fluorescence, the rate of which is proportional to gramicidin concentration (**D**). This step is not observed in the absence of membrane, so it can be concluded to be an event that is occurring on membrane. It should also be noted that by this time little or no secondary structure has been detected by CD. The concentration dependence of this step suggests an association reaction, which is consistent with the pore form of gramicidin being dimer, as summarised in Eq. (3):

$$\mathrm{G}^{\mathrm{lipid}} \overset{D}{\leftrightarrow} \mathrm{GG}^{\mathrm{lipid}} \qquad (3)$$

However, the lack of signals for CD or LD at this stage suggests that this form has yet to fold or insert transversely into the membrane. After this phase in the reaction, no further change in fluorescence is

observed. This highlights the advantage of the use of dichroic techniques (particularly LD) in the study of membrane protein folding. A study investigating gramicidin insertion using fluorescence only would conclude that the pore is now fully formed. However, the processes of folding and insertion have actually not begun in earnest. In the final phase of folding, which takes 25 min and is insensitive to protein concentration under these conditions, we observe a concerted increase in CD and LD signals (**E**). This indicates that the unfolded dimer of gramicidin undergoes folding and insertion into the membrane in a single process, as summarised in Eq. (4):

$$GG^{lipid}(\text{unfolded, surface}) \overset{E}{\leftrightarrow} GG^{lipid}(\text{folded, inserted}) \quad (4)$$

This model is summarised overall in Fig. 6, where we see that the peptide folds and inserts into the membrane as a dimeric species. For α-helical membrane proteins, the prevailing model is the two-stage model with an updated three-stage model suggested in the work of Engelman *et al.*,[1] where α-helices are folded in the membrane and then associate (two-stage model) with subsequent ligand binding, loop folding, peripheral domain insertion and/or quaternary structure formation (three-stage model). This model is not operative in this case. The underlying reasons why the DH form of gramicidin does not dimerise after insertion probably relate to the fact that a monomeric DH form cannot exist since this fold requires inter-chain hydrogen bonding as opposed to the intra-chain hydrogen bonding in α-helices.

Since the membrane protein sequence has evolved to be folded when it is inserted into the membrane leaflet, this sequence of events makes mechanistic sense. It is often overlooked that the membrane leaflet is not a simple lipid continuum but is made up of a number of lateral zones of differing physical properties. The most obvious classification of these zones is the head groups of the lipids on inner and outer leaflets and the internal hydrophobic core. It is likely that the membrane protein has evolved to optimise the interactions with each of these zones once folded and inserted. Thus, only when these interactions are available will the folded form of a membrane protein be fully stabilised. This observation would also suggest that insertion for such sequences may be enhanced by ensuring that the membrane protein inserts as a disordered chain. In this form, the inherent flexibility of the chain is likely the aid in the insertion between the phospholipids of the membrane when compared with insertion of the relatively rigid fully folded form.

With all data taken together, we have shown that the insertion of gramicidin into a phospholipid membrane is a multi-step process. This process culminates in a concerted insertion and folding step that is fluorescently silent and can only be detected by the novel combination of two dichroic spectroscopies.

Conclusions

For gramicidin under the conditions used in this work (gramicidin in TFE diluted to 10% v/v TFE/water, to give a final concentration of 50–200 μg.

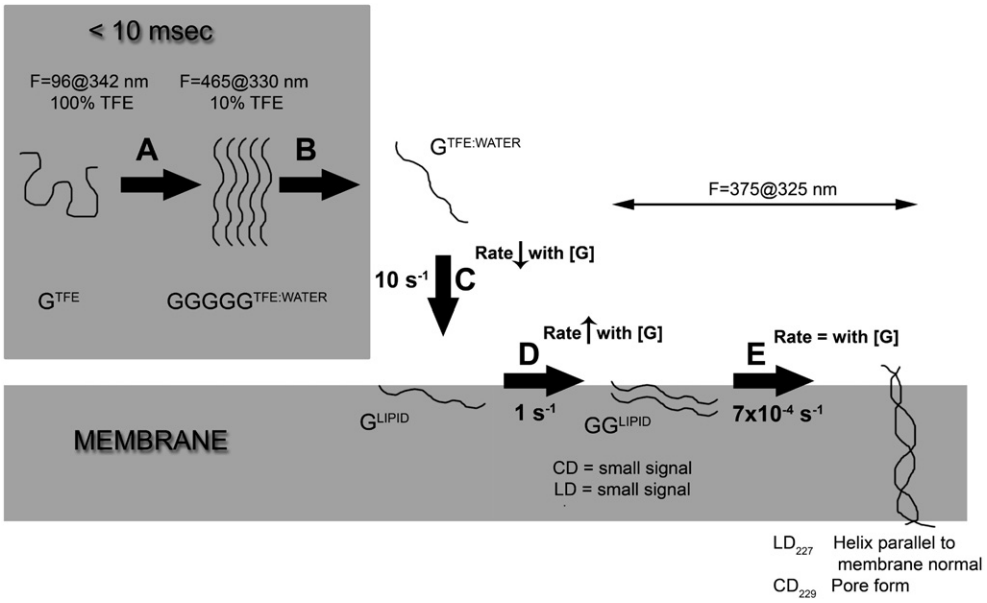

Fig. 6. The steps in the process are denoted by capital letters A to E in boldface. The processes in the light gray box occur in the dead time of the stopped-flow instrument. Fluorescence emission values are of the format *F* = intensity@maximum emission wavelength, and the reaction rates are given for the measurable rates in per-second units. [G] represents gramicidin concentration, and the ways in which the rates are affected by [G] are shown by ↑ (an increase), ↓ (a decrease) and = (little change).

mL^{-1} of gramicidin in the presence of liposomes of soybean PC at 1.8 $mg.mL^{-1}$), the processes of insertion and folding cannot be separated. For gramicidin, it is clear that folding proceeds in a concerted manner, at all structural levels from side-chain and backbone conformations, to form the final inserted conformation in the membrane (Fig. 6).

In our system, there is no evidence for a folded form of gramicidin that is not in a transmembrane orientation. It should be noted that the structural plasticity of gramicidin means that under different conditions, it is possible that other folding pathways might exist. Overall, these data show that with the use of a combination of techniques (both steady-state and kinetic), including tryptophan fluorescence and CD together with LD, a description of the binding, folding and orientation of membrane proteins can be obtained. Fluorescence is predominantly sensitive to environment; CD, to secondary structure; and LD, to orientation. All three are needed for a clear picture. The application of this approach to other membrane protein systems is anticipated to produce some exciting mechanistic insights that will further our understanding of the folding of membrane proteins.

Methods

All measurements were performed at least in triplicate.

Liposome preparation

Liposomes were prepared by dissolving L-α-PC (type IV-S) from soybean (40%) (Sigma-Aldrich, UK) in chloroform (99.8%) (Sigma-Aldrich) and evaporating the chloroform under nitrogen to produce a thin film in a glass vial. After several hours under vacuum to remove residual chloroform, the lipid film was resuspended in water at 10 $mg.mL^{-1}$ of lipid. The aqueous suspension of lipid was sonicated five times (at room temperature) in an FB11021 sonicating water bath (Fisher Scientific UK Ltd., Loughborough, UK) for 30 s each time. From this stock solution, a working stock was prepared by dilution to 2 $mg.mL^{-1}$. Liposomes were stored at 4 °C in sealed glass vials.

Gramicidin

Gramicidin D (96%) (Sigma-Aldrich) was dissolved in TFE (≥99% gas chromatography) (Sigma-Aldrich) at 10 $mg.mL^{-1}$. From this stock solution, working stocks were prepared by dilution to 2, 1 and 0.5 $mg.mL^{-1}$; these were stored at 4 °C in sealed glass vials.

Equilibrium fluorescence

Measurements were carried out using a Perkin Elmer LS50B fluorimeter with emission and excitation slits set to 8 nm, an excitation wavelength of 292 nm and emission scanned, at 100 $nm.min^{-1}$, from 300 to 380 nm. Samples were mixed manually to the same composition as that used for the stopped-flow measurements. The cell used was a quartz microvolume cell with a path length of 3 mm (Starna, Optiglass Ltd., Hainault, UK).

Fast kinetics

Different concentrations of gramicidin (dissolved in TFE) were mixed rapidly with a constant concentration of PC liposomes (in water) in order to follow the kinetics of interaction of gramicidin with the membrane. Experiments were carried out using a BioLogic MOS-450 spectropolarimeter fitted with an SFM300 rapid mixing unit. All experiments were carried out at room temperature (temperature controlled, 21 °C). The change in total fluorescence emission above 320 nm was monitored for 8 s after initiation of mixing. The excitation wavelength was 292 nm, with a bandwidth of 8 nm. Data points were recorded every 1 ms. The flow rate was 10 $mL.s^{-1}$, and the mixing ratio was 9 volumes of liposomes to 1 volume of gramicidin in TFE, with a total volume of 250 μL. Runs were performed from low gramicidin concentration to high gramicidin concentration; for each new concentration, repeat shots were made until two consecutive traces overlaid, and at least three shots were used for the data presented here.

Data analysis was carried out using the program Origin (MicroCal, Northampton, MA), and data were fit to a sum of two exponential decay functions (Eq. (5)).

$$F = F_0 + A_1 e^{-t\tau_1} + A_2 e^{-t\tau_2} \quad (5)$$

Here, F is fluorescence, F_0 is the fluorescence after infinite time, A_1 and A_2 scale the amplitudes of the two exponentials, t is the time expressed in seconds and $\hat{o}_1$ and $\hat{o}_2$ are the rate constants for the two processes expressed in per-second units.

Slow kinetics

CD experiments were carried out using a Jasco J-715 spectropolarimeter (Jasco UK, Great Dunmow, UK). Samples of the same concentrations as used for the fast kinetics were mixed manually and loaded into a 1-mm path-length quartz cuvette (Starna, Optiglass Ltd.). Data were collected from 60 s after initial mixing, and spectra were measured every 2 min for 2 h. The wavelength range recorded was 320–200 nm with a data pitch of 0.2 nm, a bandwidth of 1 nm, a scanning speed of 100 $nm.min^{-1}$ and a response time of 1 s. All measurements were carried out at room temperature (temperature controlled, 21 °C).

LD experiments were carried out on the same samples as with the CD experiments with the same parameters, except that the samples were in a microvolume Couette cell with an applied voltage of 3 V (for alignment); this corresponds to a rotation speed of 3000 rpm. The Couette cell used was built in-house; equivalent models are commercially available (Kromatek, Great Dunmow, UK).

CD kinetic data were analysed by averaging the data points from 228 to 230 nm (inclusive) for each of the 60 spectra and plotting this value *versus* time. The data fit well to a single-exponential function. LD kinetic data were analysed in the same way except that the data were averaged for the range 226–228 nm; these data also fit well to a single-exponential function with respect to time. Spectra presented here have had the appropriate lipid baseline subtracted, and they have been smoothed using the binomial smoothing algorithm in the Jasco spectrum analysis program (version 1.53.01, Jasco UK). Each spectrum shown is the average of 6 spectra. The smoothed spectra had the same spectral shape as the unsmoothed data.

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