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Equilibria Governing the Membrane Insertion of Polypeptides and Their Interactions with Other Biomacromolecules

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1. Introduction

Many biological processes are governed by chemical equilibria and the conventional thermodynamic formalisms apply. In particular the association of ligands to macromolecules, such as enzymes to large supramolecular structures, to large assemblies of proteins and/or nucleic acids (e.g. the ribosome) or to membranes has important consequences for the regulation of many biological activities and a quantitative understanding of these processes is of utmost importance for basic biological sciences and even more so for applied biomedical research. As the macromolecules involved can participate in many such regulatory interactions and these interactions are interconnected it is often necessary to describe such interaction networks by systems biology approaches. Here we illustrate the underlying thermodynamic principles by taking as an example a family of peptides that exhibit antimicrobial and cell penetrating activities which are potentially at the center for the transport of nucleic acids into cells, a property that is useful for some of state-of-the art medical treatments using highly-specific small interfering RNA or DNA molecules. The thermodynamic concepts will be illustrated by focusing on the interactions of these peptides, namely their partitioning into lipid membranes and the association of such peptides with nucleic acids, and we conclude with more detailed presentations of some of the methods that have been used for their investigation.

2. Observations on the membrane interactions of amphipathic helical peptides

Amphipathic helical peptides adopt a variety of alignments in lipid membranes. Membrane-active peptides are produced in nature, such as alamethicin and melittin (Bechinger, 1997), or are created by design (Lear et al., 1988; Bechinger, 1996; Killian et al., 1996) and their study by biophysical approaches has provided valuable insight into their lipid interactions as well as their mechanisms of membrane permeabilization. Of the peptides occurring in nature the dodecameric peptide alamethicin causes well-characterized voltage-dependent conductance changes of the membrane and therefore these peptides have early on served as a paradigm for large voltage- or ligand-gated channel proteins (reviewed e.g. in (Sansom, 1993; Bechinger, 1997; Leitgeb et al., 2007)). The open alamethicin pore is thought to consist

of a 'transmembrane helical bundle' in which the individual helices are grouped with their more hydrophilic side facing the water-filled pore (Sansom, 1993). Such barrel shaped pores may be less regular and more asymmetric than the first models suggested (Unwin, 2005). Similar transmembrane helical bundle arrangements have been observed for a variety of membrane proteins (Long et al., 2007; Traaseth et al., 2007; Cady et al., 2010).

Although a strong propensity for transmembrane alignments (TM) of the very hydrophobic alamethicin is indeed observed (Salnikov et al., 2009) a variety of biophysical approaches including oriented solid-state NMR and CD spectroscopies (cf. 4.5) indicate that alamethicin can also adopt in-planar (IP) alignments (Salnikov et al., 2010). Possibly, the in-planar state of alamethicin is an intermediate during membrane association and channel gating (Sansom, 1993; Bechinger, 1997) and a series of subsequent equilibria govern the polypeptide interactions within the membrane (in-plane ↔ transmembrane ↔ TM oligomers, cf. Figure 1). Various models for the molecular mechanism of alamethicin pore-formation are based on interactions of its helix dipole with the TM electric field, where reorientation of the dipole, enhanced partitioning of alamethicin into the bilayer and/or membrane insertion of the N-terminus results in the voltage-gating of the channel structure (reviewed in Sansom, 1993; Bechinger, 1997). As other shorter peptaibols, i.e. peptides of amino acid composition, hydrophobicity and amphipathicity closely related to alamethicin, are predominantly found oriented parallel to the membrane surface the hydrophobic matching between the membrane thickness and the peptide helix has been found to be an important parameter governing this equilibrium (Bechinger et al., 2001; Salnikov et al., 2009; Salnikov & Bechinger, 2011).

Another class of amphipathic peptides that are found in nature protects the producing host organism from pathogenic infections. The best studied of these sequences are the cationic amphipathic polypeptides magainin, cecropin and melittin. These antimicrobial compounds were first isolated from frogs and insects and when compared to alamethicin and related peptides it is noteworthy that they carry several positive charges on their hydrophilic faces. As a consequence magainins and ceropins stably intercalate into the membrane with the helix axis oriented parallel to the membrane surface (Bechinger, 1999). By interacting with the membranes of bacteria and/or fungi these peptides are capable to disturb their bilayer integrity and/or enter the cell interior and thereby develop their antimicrobial activities (Bechinger, 1999; Brogden, 2005).

The alignment of the magainin helices parallel to the membrane surface places their hydrophobic region about 10 Å above the bilayer centre in agreement with the amphipathic distribution of polar-charged and hydrophobic residues (Matsuzaki et al., 1994). Notably, in this manner the peptides have pronounced effects on the membrane structure including the disruption of the fatty acyl chain packing (Salnikov et al., 2009), membrane thinning (Ludtke et al., 1995), pore formation (Gregory et al., 2008) and macroscopic phase transitions of the peptide-lipid assemblies (Bechinger, 2009). All of these modifications are associated with changes in the enthalpy and entropy of the system and merit consideration in a thermodynamic analysis.

When added to pre-formed bilayers magainins and melittin have been shown to partition into the membranes within tens of seconds (Mozsolits et al., 2001; Papo & Shai, 2003). The partitioning of magainin 2 into the membrane interface is characterized by a coefficient of about 1000 M^{-1} in the presence of zwitterionic but overall neutral membranes (Wieprecht et al., 1999), a value that apparently increases by orders of magnitude for negatively charged surfaces (Wenk & Seelig, 1998). This is explained by an augmentation of the local

concentration of positively charged peptides next to the anionic membrane surface (illustrated in Bechinger, 2004). Furthermore, these cationic peptides have been shown to preferentially interact with the negatively charged phospholipids in mixed model membranes resulting in their segregation into domains rich in anionic phospholipids and cationic peptides (Mason et al., 2006). Therefore, electrostatic interactions not only control the membrane association of cationic amphiphiles, but they also have a pronounced effect on the lateral distribution of the lipids within mixed bilayers.

The different examples mentioned above show that membrane-active peptides can occur in many different alignments and the predominant topology depends on a number of factors such as lipid composition, peptide-to-lipid ratio, hydration, temperature, pH and the presence of additional membrane components (Bechinger, 1996; Huang, 2000; Vogt et al., 2000; Tremouilhac et al., 2006; Salnikov & Bechinger, 2011). The membrane alignment of several peptides has been investigated by biophysical approaches such as circular dichroism or ^{15}N solid-state NMR spectroscopy (cf. 4.5), where the peptides have been reconstituted into oriented and more recently also non-oriented membranes (Wu et al., 1990; Bechinger & Sizun, 2003; Prongidi-Fix et al., 2007). The transitions between such states have been characterized by equilibria such as those shown in Figure 1.

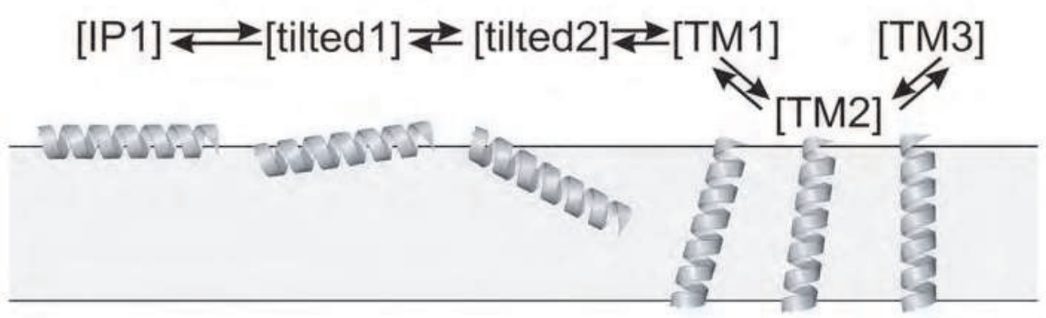

Fig. 1. Shows a selection of the equilibria that interconnect the many different membrane topologies.

The different TM states could represent, for example, different tilt angles such as those observed for the antimicrobial peptide PGLa (Salnikov & Bechinger, 2011) and/or oligomerisation states, and the possibility exists that the IP topology is also to be diversified into additional sub-states (Figure 1). The thermodynamic treatment of the in-plane to transmembrane transition will be presented in section 3.2.

When added to lipid bilayers amphipathic peptides induce alterations in the lipid macroscopic phase properties that in many ways resemble changes observed in the presence of detergents (Bechinger, 1999; Bechinger, 2005). Indeed, a decrease in the order parameter of lipid bilayers due the presence of magainins and other amphipathic peptides has been monitored in solid-state NMR measurements (Dvinskikh et al., 2006; Mason et al., 2007; Salnikov et al., 2009). As a consequence the bilayer packing is disturbed within an estimated radius of approximately 50 Å (Chen et al., 2003; Mecke et al., 2005). These effects have been explained by taking into consideration the molecular shapes of the lipids and peptides. Whereas cylindrical molecules, which equally fill the membrane interior and the interface, form stable bilayer structures amphipathic molecules that occupy a much larger space at the interface than in the hydrophobic part of the membranes cause positive curvature strain,

and lipids with very small head groups have the opposite effect (Israelachvili et al., 1980; Bechinger, 2009). Therefore the consequences of peptide association with the membrane are dependent on the ensemble of peptide molecular properties but also the size and shape of the lipid head groups, i.e. the membrane lipid composition (Bechinger, 2009). Notably, when added at high enough concentrations the peptides can even cause membrane disintegration (Dufourc et al., 1986; Hallock et al., 2002; Bechinger, 2005). The many possible outcomes of this plasticity of phospholipid membranes when interacting with peptides is best described by phase diagrams where the wide range of structures, configurations and morphologies are shown as a function of peptide-to-lipid ratio, the detailed membrane composition, temperature, hydration and buffer composition (Bechinger, 1999; Bechinger & Lohner, 2006). Within such representations regions exists where the bilayers are only slightly perturbed or even stabilized due to the presence of polypeptide, whereas at the other extreme the membrane undergoes lysis. Furthermore, conditions where membrane openings form in a more regular manner can be found and these are probably the ones that are most interesting for a formal thermodynamic treatment. The peptide-induced phase alterations can be transient and local, or they can affect the supramolecular assembly as a whole (Bechinger, 2009).

3. The LAH4 family of peptides

The so called LAH4 sequences were designed to test if peptides in a predominantly in-planar configuration can indeed cause pore formation and the antimicrobial activities which have been described for many amphipathic sequences. The polar face of the helical LAH4 peptides consists of histidines and the hydrophobic region of alanines and leucines (Figure 2). Two lysines at each terminus have been added to increase the solubility of the peptides in aqueous environments. The histidines exhibit pK_a values around 6 and these residues can therefore be used to tune the hydrophobic moment of these sequences merely by changing the pH (Georgescu & Bechinger, 2010). The LAH4 peptides interact with membranes (Bechinger, 1996) and they exhibit antimicrobial activities (Vogt & Bechinger, 1999; Mason et al., 2009; Bechinger, 2011). Interestingly, more recent investigations show that derivatives of LAH4 exhibit potent antimicrobial action against a number of pathogens found in clinical environments, and these activities are quite pronounced when the peptides occur in orientations parallel to the membrane surface (Mason et al., 2006). Furthermore the LAH4 sequences are also capable to transport big hydrophilic cargo such as DNA and RNA across the hydrophobic interior of membranes and thereby exhibit potent transfection capacities (Kichler et al., 2003; Langlet-Bertin et al., 2010; Bechinger et al., 2011). Because of these important biological activities the thermodynamics of the peptides' interactions with membranes and nucleic acids were investigated in considerable detail and these investigations shall be presented in the following.

3.1 Membrane topology of LAH4 peptides

The interactions of LAH4 peptides with oriented phospholipid bilayers were analysed using solid-state NMR and ATR-FTIR spectroscopies (cf. 4.5). Whereas an alignment parallel to the bilayer surface was observed at pH < 6 when the histidines are cationic, transmembrane orientations are found when the histidines are discharged (Bechinger, 1996; Bechinger et al., 1999). Furthermore CD- and multidimensional solution NMR spectroscopies are indicative

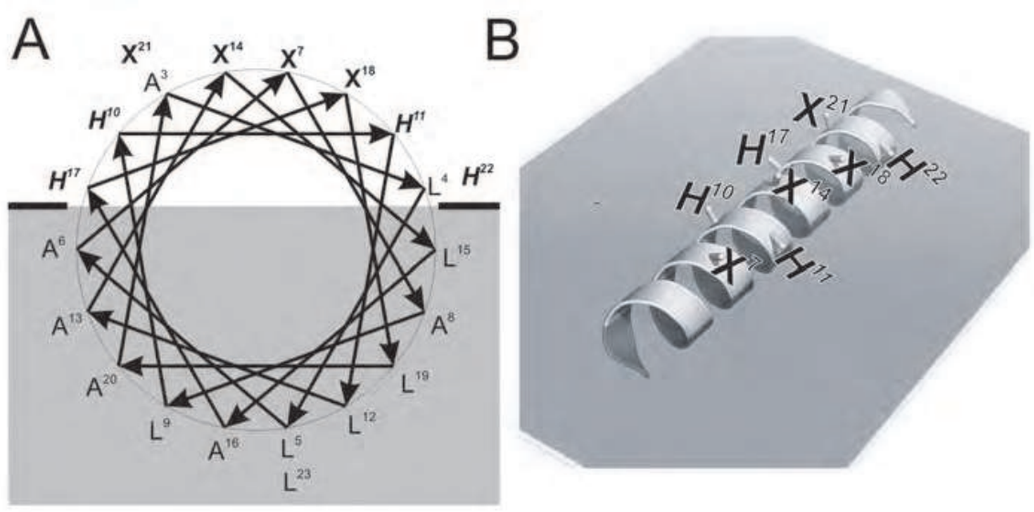

Fig. 2. Helical wheel representation of LAH4X4 (Aisenbrey et al., 2006), a sequence of the LAH4 family, obtained by viewing the arrangement of residues as a projection along the helix long axis (left) and top view onto the structure (right). An amphipathic distribution of residues becomes apparent when considering that the leucine (L) and alanine (A) residues exhibit a hydrophobic character (exposed to the membrane interior shaded in grey). In contrast, the histidines (H), are polar or charged, depending on the pH of the solvent. Variation of the character of the residues labelled X allows one to modulate the driving forces for membrane insertion and thereby the transmembrane alignment of the helical peptide. The N- and C-terminal lysines, two at each end, have been omitted for clarity.

that these histidine-rich peptides exhibit a high degree of α-helical conformations in micellar environments (Vogt & Bechinger, 1999; Georgescu & Bechinger, 2010). Remarkably, the outlines of the α-helical structures are pH dependent and shift from a C-terminal (encompassing residues 9-24 at pH 4.1) to a more N-terminal position (residues 4-21 at pH 7.8). At intermediate pH two short helices are interconnected by a hinge region of residues 10-13 (Georgescu & Bechinger, 2010). This flexible domain probably facilitates the transition from in-plane to transmembrane alignments. Notably, although aqueous solutions of the peptide appear transparent by eye dynamic light scattering measurements indicate that the peptides form small α-helical aggregates at neutral pH (Marquette et al., 2008). In contrast the hydrodynamic radii measured at acidic pH agree with an extended monomer. On a functional level the members of the LAH4 family exhibit membrane pore-formation in model membranes (Marquette et al., 2008) and antimicrobial action at both neutral and acidic pH (Vogt & Bechinger, 1999). These observations indicate that a well-defined transmembrane channel structure is not required for biological action.

Notably the transition between in-planar and transmembrane alignments is reversible, a requirement to evaluate the transfer energy of amino acid side chains from the membrane interface to the membrane interior using equilibrium thermodynamics. A direct measurement of these transition energies has been achieved to our knowledge for the first time by modulating the composition of the polar face through amino acid replacements (Figure 2) and by analysing the resulting shifts in the transition pH (Aisenbrey et al., 2006;

Aisenbrey et al., 2006). In order to achieve this goal it was necessary to develop the thermodynamic formalism presented in the next section.

3.2 Formalism to describe the membrane topological transitions of amphipathic peptides

In order to describe the in-plane to transmembrane transition of membrane-associated LAH4 peptides a series of consecutive equilibria connecting subsequent states is considered: $IP^{ch} \leftrightarrow IP^{o} \leftrightarrow TM$ (Aisenbrey et al., 2006; Aisenbrey et al., 2006). The in plane configuration (IP) of the helices, can occur with the histidines charged (ch) or neutral (o) and TM represents the transmembrane inserted state (Figure 3). This model can be extended by also considering peptides that are dissolved in the aqueous buffer and which are in exchange with the surface-associated state IP^{ch}.

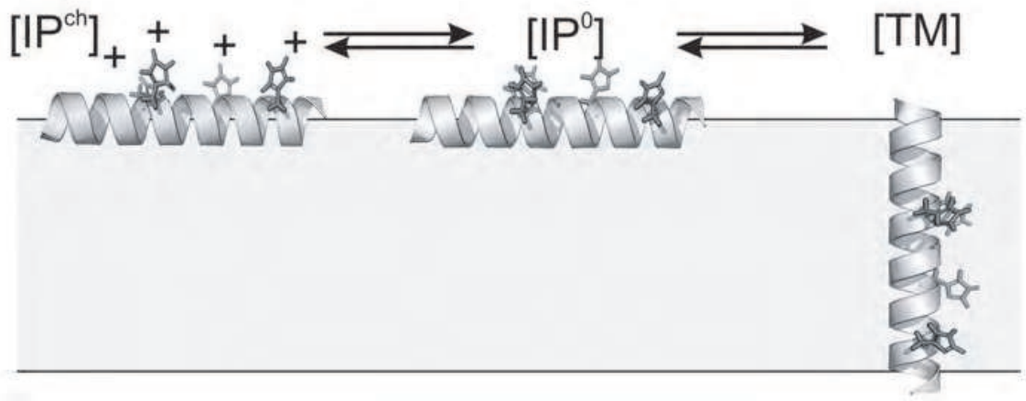

Fig. 3. Model of LAH4 in the lipid membrane. The transmembrane state is in thermodynamic equilibrium with in-planar alignments and uncharged histidines. Notably, two in-planar states are considered namely one where the histidines are neutral and another one where these side chains are protonated. The charges carried by the histidines strongly depend on the pH of the surrounding medium.

The energy contributions that govern the in-plane → transmembrane reorientation process include the interactions of hydrophobic and polar amino acids in different environments but also contributions such as van der Waals, steric or entropic contributions that change upon disruption of the lipid packing and are different when peptides partition to the membrane interface or when they insert in a transmembrane fashion (reviewed in Bechinger, 1996). Furthermore, according to the model presented in Figure 3 only uncharged histidines are capable to insert into the membrane interior and their discharge is a prerequisite for LAH4 transmembrane alignments. Therefore, peptides carrying charged histidine side chains remain at the membrane surface and adopt in-plane orientations.

However, even at pH values smaller or around the pK_a the histidine side chains can be neutralized (Bechinger, 1996; White & Wimley, 1999) thereby permitting membrane insertion at lower energetic cost than when placing a charged residue into the hydrophobic membrane interior (Israelachvili et al., 1980). The required energy of discharge must derive from other driving forces that are associated with changes in the peptide topology including for example favourable 'hydrophobic' contributions. As a consequence the transmembrane-insertion, conformational changes that are associated with membrane-association and the

discharge of side chains are often tightly connected processes (Liu et al., 1996; Aisenbrey et al., 2006) and if this is the case a direct equilibrium between the transmembrane uncharged peptide and the in-planar charged peptide can be established ($IP^{ch} \leftrightarrow TM$).
On the other hand, when additional polar amino acids (other than the histidines) favourably interact with the water phase even the complete neutralisation of the histidines is insufficient to promote the transmembrane insertion, therefore the in-planar state remains strongly populated even when the histidines are uncharged (IP^{o}). When the pH of the sample is augmented the four histidines of LAH4 and related sequences become neutral in a gradual manner and close to the pK_a values of the histidines, only a fraction of these side chains are cationic. Therefore, under such conditions a transmembrane uncharged state (TM), an uncharged in-planar state (IP^{o}) and in-planar states carrying a variable number of charges have all to be taken into consideration (IP^{1+}, IP^{2+}, IP^{3+}, IP^{4+}).
Standard thermodynamic relationships allow one to calculate the equilibrium constant for the IP to TM transition from the Gibb's free energy ΔG associated with the transition of the peptide from the interface into the membrane interior (or vice versa). Whereas the in-plane alignment occurs on both sides of the lipid bilayer two possible orientations exist for the transmembrane peptide. Therefore, the equilibrium constant is calculated according to

$$k_{TM} = \frac{[TM]}{[IP^{o}]} = \mathrm{e}^{\frac{-\Delta G}{RT}} \tag{1}$$

Furthermore, the uncharged in-planar configuration (IP^{o}) is in equilibrium exchange with the series of charged in-planar states (IP^{ch}). Assuming that none of these charged states inserts into the membrane in a transmembrane fashion, they combine to $[IP^{ch}] = [IP^{1+}]+[IP^{2+}]+[IP^{3+}]+[IP^{4+}]$. The reciprocal of the constant (k_{ch}) for the transition $IP^{o} \leftrightarrow IP^{ch}$ is expressed as a sum of the reciprocals of the individual constants:

$$1/k_{Ch} = \frac{[IP^{ch}]}{[IP^{o}]} = \sum 1/k_j \text{, with } k_j = \frac{[IP^{o}]}{[IP^{j+}]} \tag{2}$$

Where k_j depends on the free energy difference by charging j histidines, which is statistically possible in $\varepsilon_j = \binom{4}{j}$ different ways. The constants are given by:

$$k_j = \frac{[IP^{o}]}{[IP^{j}]} = \frac{1}{\varepsilon_j}\mathrm{e}^{-2.3\ j\left(pH - pK_a\right)} \tag{3}$$

Combining equations [2] and [3] gives

$$1/k_{Ch} = \frac{[IP^{ch}]}{[IP^{o}]} = \sum_{j=1}^{4}\binom{4}{j}\left(\mathrm{e}^{2.3\left(pH - pK_a\right)}\right)^{j} = \left(1+\mathrm{e}^{2.3\left(pK_a - pH\right)}\right)^{4} - 1 \tag{4}$$

The ratio of the transmembrane over total peptide concentration, p_{TM}, which can be determined by various experimental methods (cf. 4.5), is:

$$p_{TM} = \frac{[TM]}{[TM] + [IP^{o}] + [IP^{ch}]} = \frac{1}{1 + e^{\frac{\Delta G}{RT}} \left(1 + e^{2.3(pK_a - pH)}\right)^4} \tag{5}$$

Equation [5] is graphically represented in Figure 4. The simulations show that the steepness of the transition depends on ΔG, being steeper for the more favoured transitions (i.e. negative ΔG). In contrast, if the overall driving force approaches zero or for positive ΔG the transmembrane state is only partly populated regardless of pH. Figure 4 also illustrates that for ΔG near zero or at positive values, the transition mid-point is shifted towards pH values higher than the pK_a of the histidines. This shift arises from the degeneracy of the in-planar states where statistically the configurations carrying one to three charges is possible in multiple (ε_j) ways.

Through addition of hydrophobic residues at the X-positions (Fig. 2) ΔG << 0 and the transition takes place at pH < pK_a. Under such conditions an approximation for the fraction of transmembrane peptide is obtained by

$$p_{TM} \approx \frac{1}{1 + e^{4 \cdot 2.3\left(pK_a + \frac{\Delta G}{2.3 \cdot 4\,RT} - pH\right)}} \tag{6}$$

The resulting curve is sigmoidal with the transition at $pK_a - \frac{\Delta G}{4 \cdot 2.3RT}$, a result also obtained by considering a simple model where the transition is described by the equilibrium $IP^{4+} \leftrightarrow TM$ as described before (Bechinger, 1996). In this simpler case the midpoint of transition occurs at pH_{50}, the pH value where 50% of the molecules are in transmembrane state (p_{TM}=1/2). Using equation [5] one obtains:

$$p_{TM} = \frac{[TM]}{[TM] + [IP^{o}] + [IP^{ch}]} = \frac{1}{1 + e^{\frac{\Delta G}{RT}} \left(1 + e^{2.3(pK_a - pH)}\right)^4} \tag{7}$$

When peptides of moderate or low hydrophobicity are studied a better description of the transition is obtained when measuring $p_{1/2}$, which indicates the pH where 50% of the maximal p_{TM} is reached ($p_{1/2}$=1/2 $p_{TM}^{\max}$ cf. Figure 4). With

$$p_{TM}^{max} = \frac{1}{1 + e^{\frac{\Delta G}{RT}}} \tag{8}$$

$pH_{1/2}$ can be expressed as

$$pH_{1/2} = pK_a - \frac{1}{2.3} \ln\left(\left(e^{\frac{\Delta G}{4RT}} + 2\right)^{\frac{1}{4}} - 1\right) \tag{9}$$

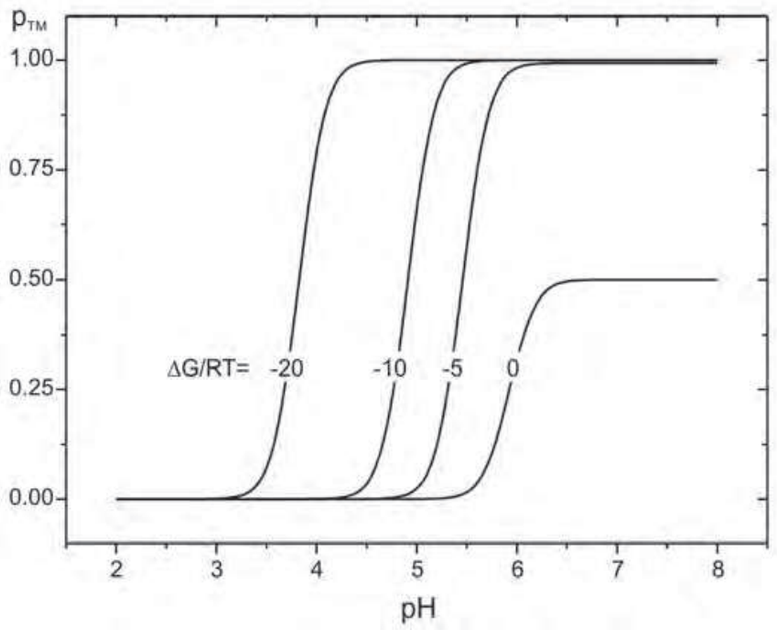

Fig. 4. Graphical representations of equation [5]. The pH-dependent transmembrane fraction of histidine-rich amphipathic peptides (LAH4 family) is shown as a function of the Gibb's free energy of the IP → TM transition.

Finally we should note that the local pH close to the membrane surface or in the proximity of other charges and dipoles can exhibit significant differences when compared to the proton activity in bulk solution. As a consequence when the the pK_a values of LAH4 and related peptides associated to DPC detergent micelles were determined using NMR spectroscopy the titration curves appeared less steep than expected from the Henderson-Hasselbach equation (Bechinger, 1996; Georgescu & Bechinger, 2010). Therefore, in order to take into account attractive or repulsive interactions at the biomolecular surfaces a correction parameter c was introduced and equation [5] modified:

$$p_{TM} = \frac{1}{1 + e^{\frac{\Delta G}{RT}\left(1 + e^{2.3\ c\left(pK_a - pH\right)}\right)^4}} \tag{10}$$

By fitting the pH-dependent ^{1}H NMR chemical shifts of the indol 4H positions of LAH4 (and related peptides) average values of c=0.8 and pK_a=6.0 are obtained (Aisenbrey et al., 2006; Aisenbrey et al., 2006).

The formalism introduced in this section was used in previous publications to follow the membrane topological changes of LAH4 and other amphipathic peptides using solid-state NMR and ATR-FTIR spectroscopies on oriented phospholipid bilayers (Bechinger, 1996; Bechinger et al., 1999; Vogt et al., 2000; Bechinger, 2001). Notably this has allowed to directly measure the energies that are associated with the transition of amino acid residues from a membrane interfacial location to the membrane interior (Aisenbrey et al., 2006; Aisenbrey et al., 2006) and to compare these values with calculations where this transition has been part of a thermodynamic cycle (White & Wimley, 1999). Interestingly when amino acid residues

are compared to each other the preferences are somewhat different when the transitions from the interface → membrane interior are compared to those from the aqueous phase → membrane interior. In particular the alanine residues that are considered 'hydrophobic' on the latter scale have an equal preference for interfacial and inner positions (Bechinger, 2001; Aisenbrey et al., 2006). Furthermore, the measurements indicate that tyrosine, tryptophan and valine exhibit a tendency to remain associated with the membrane interface (Aisenbrey et al., 2006).

When considering the interaction contributions that govern IP to TM transitions of helical peptides as a whole, $\Delta G_{IP \Leftrightarrow TM}$, changes in the environment of amino acid side chains have to be considered as discussed in the previous paragraph (White & Wimley, 1999; Bechinger, 2000; Aisenbrey et al., 2006). In addition the ensemble of contributions that arise from changes in the packing of the phospholipid membrane, hydrophobic mismatch and peptide-peptide interactions are relevant to dissect the energy contributions governing these equilibria (Bechinger, 1996; Harzer & Bechinger, 2000). The hydrophobic mismatch contributions have been found particularly important when the topologies of the antimicrobial peptide PGLa (Salnikov & Bechinger, 2011) or that of peptaibols, such as the 15 residue zervamicin II or ampullosporin A were studied (Bechinger et al., 2001; Salnikov et al., 2009). Although the data indicate that hydrophobic mismatch has a pronounced influence on the peptide topology, it was also found that the peptaibols adopt transmembrane alignments only within bilayers that are thinner than one would expect from their hydrophobic length alone. Therefore, in order to explain such differences other interactions need to be taken into consideration as well. Furthermore, more recent studies indicate an interfacial localisation of alamethicin, a hydrophobic peptide generally found to adopt transmembrane alignments, is stabilized in the presence of phosphatidylethanolamine. This lipid carries a relatively small head group and it is thought that the alamethicin helix relieves some of the packing frustration in a bilayer environment by occupying space at the bilayer interface (Salnikov et al., 2010).

Finally it should be mentioned that amphipathic sequences such as cationic antimicrobial peptides or LAH4 also dissolve in the aqueous phase surrounding the membranes. Therefore an additional reversible equilibrium merits thermodynamic analysis namely the transition from the water phase to the membrane-associated state. The membrane-association of these peptides has been quantitatively analysed by centrifugation assays where the residual amount of peptide in the aqueous phase in the presence of increasing amounts of lipids is determined by following the changes in spectroscopic signals such as for example the tryptophan fluorescence (cf. 4.1), or by performing a full thermodynamic analysis using isothermal titration calorimetry (Wenk & Seelig, 1998; Seelig, 2004). Notably, many of these methodologies do not differentiate between various membrane associated states and therefore include all of the topological possibilities discussed in the previous sections.

3.3 Interactions of LAH4 peptides with DNA

In addition to the pronounced antimicrobial properties that were discussed in section 3.1 (Vogt & Bechinger, 1999; Mason et al., 2006; Mason et al., 2007; Mason et al., 2009) the LAH4 peptides also exhibit potent DNA transfection activities (Kichler et al., 2003). Furthermore, it was recently shown that they are potent siRNA delivery vehicles with efficiencies that are higher than those of several well-established compounds such as Lipofectamine, DOTAP and polyethylenimine (Langlet-Bertin et al., 2010). In our laboratory a number of biophysical

investigations have been performed, supported by many biochemical studies by our long-standing collaborators, which aimed at understanding the mechanisms of these transfection activities. Indeed, the ensemble of data has allowed to modify the initial LAH4 sequence in such a manner to obtain considerably improved transfection activities (Kichler et al., 2006; Mason et al., 2006; Mason et al., 2007; Mason et al., 2007). Interestingly, in some of the hereditary diseases, such as cystic fibrosis, the patients suffer from increased infection rates and augmented sensibility to some highly pathogenic species. Therefore, the simultaneous antimicrobial and DNA transfection activities of the LAH4-transfection complexes are of potential interest for multimodal applications (Kichler et al., 2003).

Gel shift and biochemical experiments indicate that large peptide-DNA complexes form when the peptide and the nucleic acids are mixed at neutral conditions and this complexation is required for the entry into cells via an endosomal pathway (Kichler et al., 2003; Kichler et al., 2007). However, efficient transfection also requires that the information carried by the nucleic acids is made available at their cytoplasmic or nuclear destination, respectively. It is therefore essential that at their destination these macromolecules are liberated from their carrier, which requires that the interactions are reversible and not too tight. Therefore, the association/dissociation equilibria of the LAH4-DNA transfection complexes have been investigated as a function of pH using isothermal titration calorimetry (ITC), a method that provides a full thermodynamic analysis of binding constant, number of binding sites, ΔG, ΔH and ΔS (cf. 4.4).

The ITC analysis of the LAH4-DNA interactions indicate that under saturating conditions and at pH 7.5 one peptide is associated with about 2 base pairs of DNA. Association occurs in the μM range and the thermodynamic signature suggests that under these conditions is driven by electrostatic interactions. As the histidine side chains are probably uncharged at neutral conditions (Bechinger, 1996) the data have been suggestive that the cationic lysine termini of LAH4 interact with the negatively charged phosphates of the DNA, a conclusion that was recently confirmed by solid-state NMR distance measurements (Bechinger et al., 2011). Indeed only the lysine side chain nitrogens show close-by contacts with the DNA phosphates (< 8 Å). Condensation of the DNA strands into compact globular structures can therefore be explained by interactions of the anionic phosphates with two lysine side chains at each terminus of the peptide thereby enabling the cross-linking of distant parts of the extended DNA molecule, as well as of different DNA strands.

At pH < 6, such as it occurs in the endosomal compartment, the four histidine side chains become positively charged thereby doubling the overall charge of the peptide. As a consequence about half of the peptides are released from the transfection complex (Prongidi-Fix et al., 2007). At the same time the thermodynamic signature of the ITC experiments are indicative of structural arrangements as hydrophobic and van der Waals contacts also become important and complement the electrostatic interactions. Importantly the peptides liberated from the complex at low pH are readily available to interact with the endosomal membranes.

4. Methods for the quantitative measurement of thermodynamic quantities

4.1 Peptide-association with lipid vesicles using tryptophan fluorescence spectroscopy

The adsorption of peptides to lipid membranes can be measured by any physical property that changes upon the process and following the fluorescence signal of tryptophan (Musse

et al., 2006) or circular dichroism spectroscopy (cf. 4.2) are widely used methods. A mayor advantage of tryptophan fluorescence spectroscopy is the natural occurrence of this amino acid residue within the sequence of many proteins and peptides (Lakey et al., 1993). As the diameter of the vesicles is of similar size as the wave length of the emitted light recording of such spectra has to be done with care and precautions need to be taken during the processing of the experimental data (Ladokhin et al., 2000). The tryptophan fluorescence signal is highly dependent on its environment (Fig. 5A-C). Whereas the spectral intensities are quenched (reduced) and red shifted in polar environments, an increased and blue-shifted emission spectrum is observed in more apolar solvents (Sun et al., 2010). This effect is used, for example, in the stopped-flow kinetic analysis of protein folding, where tryptophan moieties tend to move from the polar aqueous buffer into the hydrophobic interior of the protein. In the presence of lipid vesicles an increased fluorescence signal is an indication for the insertion of this amino acid side chain into the less polar interface or into the apolar interior of the membrane (Musse et al., 2006).

An essay for the membrane-association of polypeptides has been established where an increasing amount of small unilamellar phospholipid vesicles is titrated in a step-wise manner into a peptide solution of given concentration. This leads to a series of continuously changing spectra, and the alterations are used to follow polypeptide association and to calculate the association parameters. The use of small vesicles in such experiments (ca. 100 nm in diameter) avoids extensive scattering artefacts in the UV spectral range, which is typically used to monitor the tryptophan fluorescence.

Provided that during membrane-association the changes in the environment of the tryptophan side chains can be approximated by a transition between two distinct states, each being characterized by a sufficiently different fluorescence signal, a relatively simple analysis of the titration experiment is possible. In such a case the peptide simply transfers between 'free in solution' and 'associated with the membrane' (Fig. 5C). Notably, the two-state approximation is also possible if different configurations of the polypeptide, such as different aggregation states, result in equivalent fluorescence signals. For example, together the monomeric and the dimeric state of a polypeptide in solution would be considered as the 'water soluble protein fraction' as long as the tryptophan fluorescence is not affected by the association.

In such cases the fluorescence intensities of the polypeptide in aqueous buffer (0% bound) and in the presence of an excess of lipid membranes (100% bound) serve as reference for the analysis of the titration experiment. Provided that complete adsorption for the highest lipid concentration is reached the normalized change in signal intensity (or peak maximum) correspond directly to the fraction of bound peptide since all spectra are linear combinations of the two reference spectra. In order to obtain the binding curve with the highest accuracy possible the frequency with the biggest difference between the free and adsorbed state is generally used. However the normalized intensity change at any other frequency should lead to the same result except if additional equilibria need to be taken into consideration. In the latter case the data are analysed by a more sophisticated approach (Aisenbrey et al., 2008).

4.2 Titration experiments using circular dichroism spectroscopy

Circular dichroism spectroscopy is a relatively fast and inexpensive tool to estimate the secondary structure composition of proteins and peptides (Sreerama & Woody, 1993; Lees et

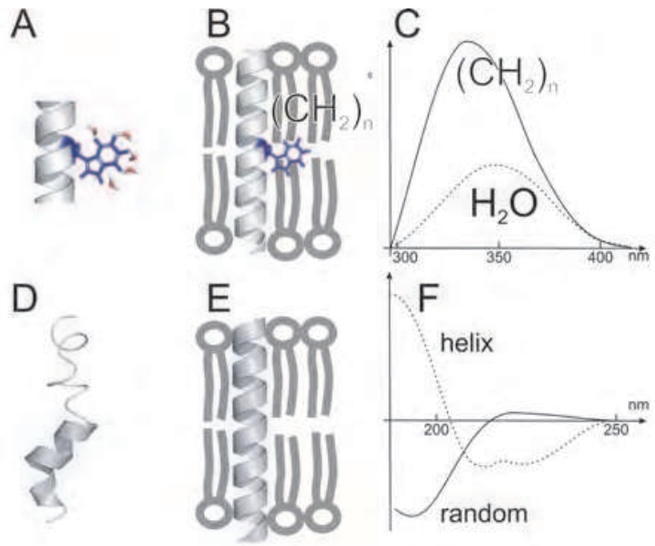

Fig. 5. Illustrates the principle of adsorption measurements by fluorescence and circular dichroism spectroscopies. Whereas the tryptophan fluorescence is quenched and red shifted in the polar aqueous environment (A) the fluorescence quantum yield increases upon transfer into the apolar environment of the membrane (B). As a consequence the fluorescence signal is increased and blue shifted in a hydrophobic environment (C solid line) when compared to those obtained in aqueous buffers (C, dotted line). Peptides that are mostly random coil in aqueous solution (D) often adopt helical structures when membrane-associated (E). Concomitant with this structural transition are CD spectral changes from random coil (F, solid line) to helical signatures (F, dashed lines).

al., 2006). It is based on the chirality of the peptide bond with its adsorption maximum in the range of 214 nm, therefore, circular dichroism spectrometers with a powerful light source in the UV spectral range are needed for these measurements. Provided that the peptide changes its secondary structure upon adsorption to lipid membranes the concomitant spectral changes can be used to follow the adsorption isotherm (Fig. 5F). Most commonly small unilamellar vesicles are titrated to a given concentration of peptide. In titration experiments using circular dichroism spectroscopy a good indication for the presence of a two-state transition is the presence of an isosbestic point. In this case similar considerations apply for the analysis of these experiments as those outlined for titrations using fluorescence spectroscopy (4.1).

4.3 Analysis of binding data in the case of more than two states

In the case of the presence of three states the relation between the concentrations (c_i of state i) and the measured signals S_{CD} and S_{fluo} is given by the following matrix, where S_{CD}^i and S_{fluo}^i are the CD and fluorescence signal intensities of state I, respectively.

$$\begin{pmatrix} S_{CD} \\ S_{fluo} \\ C_{peptide} \end{pmatrix} = \underbrace{\begin{pmatrix} S_{CD}^A & S_{CD}^B & S_{CD}^C \\ S_{fluo}^A & S_{fluo}^B & S_{fluo}^C \\ 1 & 1 & 1 \end{pmatrix}}_{SC} \begin{pmatrix} C_A \\ C_B \\ C_C \end{pmatrix} \tag{11}$$

The third line in the matrix assures the conservation of the masses. If the matrix elements of SC are known the concentrations can be calculated from the experimental fluorescence and circular dichroism signals by matrix inversion. This works only when the two optical techniques provide complementary information, i.e. the fluorescence change should not parallel exactly the CD transitions. If both methods provide redundant information the inverse matrix cannot be calculated. To determine the matrix elements it is necessary to measure the CD- and fluorescence signals of the pure states A, B and C, or to make some assumption that can resolve this issue. Such an assumption could be, that the polypeptide does not change its average secondary structure even though the tryptophans are in different chemical environments.
Even though the previous sections were dedicated to (tryptophan-)fluorescence and circular dichroism the principles of data analysis remain valid for other experimental methods as long as a change in the signal parallels the binding isotherm.

4.4 Measurement of enthalpy and free energy of binding by isothermal titration calorimetry

Isothermal titration calorimetry is based on the measurement of the reaction enthalpy when molecular interactions occur. The experiment is performed in such a manner that a ligand is added to a substrate in a step-wise manner and the heat of reaction measured with high precision during each injection of the ligand into the solution with the substrate (Fig. 6). The free energy of binding (the binding constant) is obtained by fitting a thermodynamic model to the resulting titration curve. The beginning of a titration experiment is characterized by an excess of substrate (Figure 6 A, red ¾ circles) and provided that the substrate has been made available at high enough concentrations all of the injected ligands bind to one of the substrates (Figure 6, green ¼ circles). Thus the reaction enthalpy per injected ligand is directly obtained. At the end of the experiment the binding sites of the substrate molecules are saturated and no further heat is released or consumed upon further addition of ligand (Figure 6B). If the binding constant is high with respect to the concentrations used in the experiment, the binding reaction abolishes in a sudden manner once all the binding sites are occupied (Fig. 7). For smaller binding constants this transition occurs more smoothly (Fig. 7). Therefore the shape of the titration curve is used to determine the binding constant (and thereby the free energy of binding).

4.4.1 A possible model to analyse ITC traces and its mathematical consequences

In a simple case the ligand (L) binds to the substrate (S) following the chemical equation $S + L \leftrightarrow SL$.
After correction for the dilution of the substrate concentration upon injection of the ligand the measured enthalpy per injection is directly proportional to the amount of newly formed complexes after each injection. Therefore knowing the concentration of the complex after each injection of substrate provides access to the binding constant k. With S being the total concentration of the substrate (both free and bound), L the total concentration of ligand added, [S] the concentration of free substrate, [L] the concentration of unbound ligand and [SL] the concentration of complex one finds:

$$\frac{[S][L]}{[SL]} = k; \quad [S] + [SL] = S; \quad [L] + [SL] = L$$
$$\Rightarrow [SL] = \tfrac{1}{2}\left((S + L + k) - \sqrt{(S + L + k)^2 - 4SL} \right) \tag{12}$$

The reaction enthalpy of one injection is calculated by considering the difference in the concentration [SL] before and after the injection. Thus for a ligand concentration of L before injection and a ligand concentration of L+ΔL after the injection the measured enthalpy is proportional to $[SL]_{(L+\Delta L)}$ - $[SL]_{(L)}$. The envelope of the injection profile corresponds to the derivative:

$$\frac{\partial[SL]}{\partial L} = \tfrac{1}{2} - \frac{S+L+k-2S}{2\sqrt{(S+L+k)^2 - 4SL}} \tag{13}$$

The thermodynamic parameters are obtained from the integration of the injection enthalpies (ΔH) and include the binding constant k = exp(-ΔG/RT) (Figure 7), the number of binding sites from the concentration of ligand and the substrate at the transition mid point, and finally the entropy ΔS from ΔG=ΔH-TΔS. Additional insights in the thermodynamics of the system are obtained from measuring the association curves at different temperatures (Seelig, 2004).

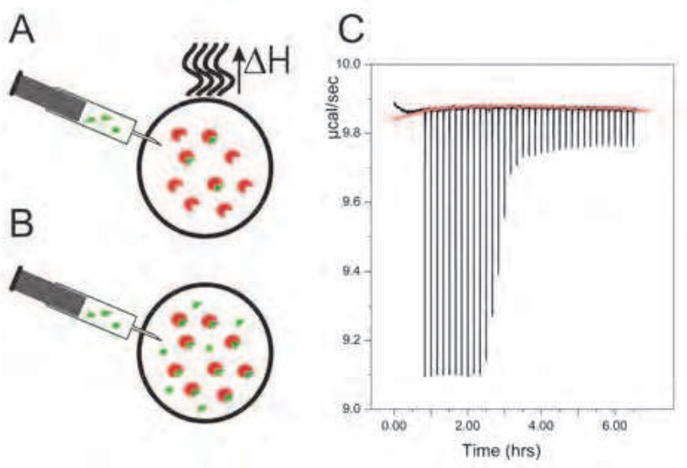

Fig. 6. Illustrates the principles of ITC measurements. A: During the initial phases of a titration the substrate molecules (red 3/4 circles) are in excess and all ligand molecules (green 1/4 circles) associated with a substrate molecule provided that the binding constant is high enough with respect to the substrate concentration. B. At the end of the experiment all substrate molecules are saturated with ligand and no further binding reaction occurs. C. shows the measured heat from individual injections which occur at regular intervals.

4.5 Characterization of topological states by solid-state NMR and ATR-FTIR spectroscopies

The methods presented in sections 4.1-4.4 provide some structural information on the polypeptide-lipid interactions such as the overall secondary structure composition of the peptides and proteins (CD spectroscopy) or about the environment of the tryptophan residues (fluorescence spectroscopy). The spectral changes during membrane association are used to determine e.g. the membrane association constants. Additional and more detailed structural information about the membrane-associated states is obtained from solid-state NMR and attenuated total reflection Fourrier transform infra-red (ATR FTIR) spectroscopies.

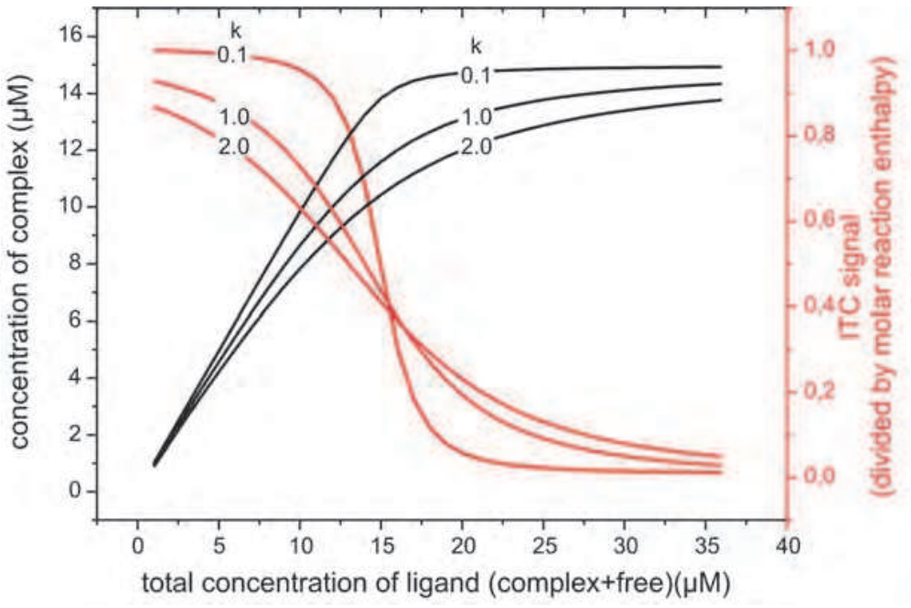

Fig. 7. Simulations of ITC experiments. The concentration of the complex (black line) and the relative heat of reaction as a function of ligand concentration are shown for three different ITC experiments in the presence of 15µM of substrate at binding constants k of 0.1, 1 and 2 µM.

Whereas in solution fast reorientational motions average the alignment dependence of all NMR interactions and only the isotropic values are obtained this is not the case in solid or semi-solid samples, such as lipid bilayer assemblies, where the resonance positions are strongly dependent on the orientation of the molecules relative to the magnetic field of the spectrometer (B_o). Therefore, in solid-state NMR spectroscopy chemical shifts, quadrupolar and dipolar splittings are all anisotropic and are routinely used to derive angular information relative to B_o. For example in membranes that are uniaxially aligned with their normal parallel to B_o the ^{15}N chemical shift of a peptide bond exhibits an anisotropy between 50 and 230 ppm (Bechinger & Sizun, 2003). Due to the particular properties of the ^{15}N chemical shift interaction with the magnetic field (which is described by a second rank tensor) ^{15}N labelled sites within helical peptides exhibit chemical shifts around 200 ppm when transmembrane and < 100 ppm when oriented along the surface (Fig. 8). Therefore, the approximate tilt angle of a membrane-associated peptide can be determined directly from this measurement (Bechinger & Sizun, 2003). Accurate information on the tilt and the rotational pitch angles is obtained when other measurements such as the ^{2}H quadrupolar splitting of methyl-deuterated alanines is also taken into consideration (Bechinger et al., 2011). More recently it has also been shown that for peptides that undergo fast rotational diffusion of the membrane normal the partially averaged spectral line shapes of non-oriented samples provides similar information about the helix alignment (Prongidi-Fix et al., 2007).

ATR-FTIR spectroscopy of uniaxially oriented samples provides structural information on membrane-associated polypeptides. Whereas the resonance frequency of the amide I band is a reliable indicator of the secondary structure composition (Goormaghtigh et al., 1994), the dichroic ratio R^{ATR}, that is obtained by comparing the ATR-FTIR intensities when the sample is irradiated with light of polarization parallel and perpendicular to the surface, is an indicator of the membrane topology (Bechinger et al., 1999). In contrast to solid-state NMR spectroscopy where the in-plane and transmembrane alignments result in two distinct

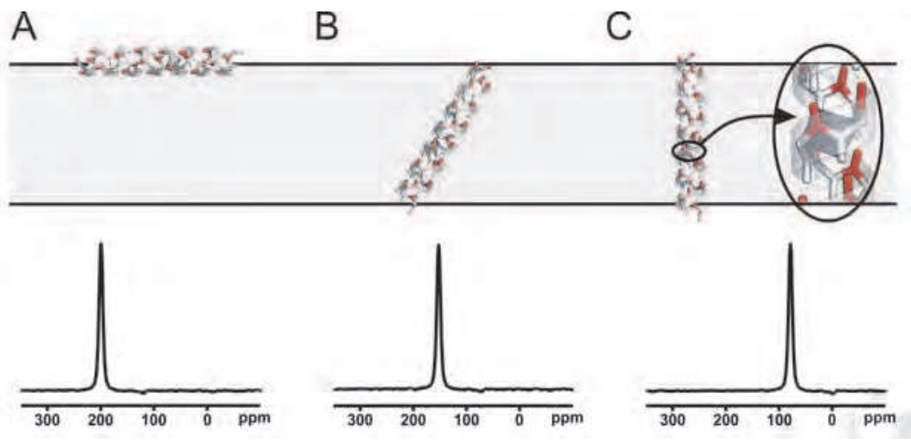

Fig. 8. Illustrates how the ^{15}N chemical shift measured from a ^{15}N labelled peptide bond within a helical domain correlates with the helix tilt angle for an in-planar (A), a tillted (B) or a transmembrane orientation (C).

resonances of very different chemical shift (Fig. 8), and therefore exchange processes can also be studied, the FTIR signal of the two states results in the same resonance position albeit with different dichroic ratio (Fig. 9). Therefore the fraction of transmembrane alignment p_{TM} is evaluated from

$$R^{ATR} = \frac{2p_{TM}(R_a^{ATR} - R_b^{ATR}) + R_b^{ATR}(R_a^{ATR} + 2)}{p_{TM}(R_b^{ATR} - R_a^{ATR}) + R_a^{ATR} + 2} \quad (14)$$

where R_a^{ATR} and R_b^{ATR} are the dichroic maxima and minima, respectively (Bechinger et al., 1999). Oriented CD spectroscopy, which is not discussed in detail here, provides a third method to analyse the topology of membrane-associated polypeptides (Wu et al., 1990).

5. Conclusion

A number of biophysical approaches have been developed for the thermodynamic analysis of the association of and the interactions between biological macromolecules and/or supramolecular systems. These techniques allow one to quantitatively describe the association constants and number of binding sites but also other thermodynamic quantities such as reaction enthalpy and entropy. This data is important to understand the regulatory mechanisms that govern life and which are key for health and disease.

6. Acknowledgment

Much of the work presented in this paper has only been possible through collaborations with many other teams and the many previous and present co-workers of our own laboratory. Of the former we would like to mention in particular the fruitful interactions with Antoine Kichler, Erick Goormaghtigh, Jean-Marie Ruysschaert, Gilles Guichard, Bernard Desbat, Marie-Hélène Metz-Boutigue and Gilles Prévost. We gratefully acknowledge the financial support over the years by Vaincre la Mucoviscidose, the Agence Nationale de la Recherche, the European Commission, the Alsace region, the CNRS, the French ministry of research and the University of Strasbourg.

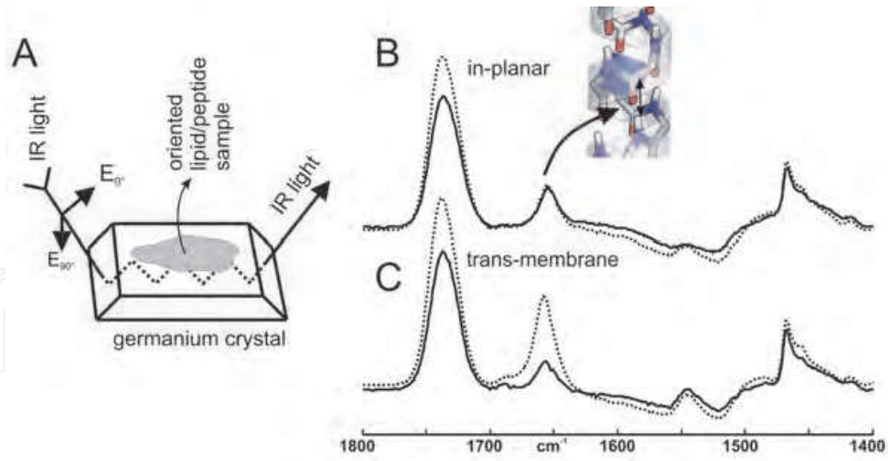

Fig. 9. A The experimental setup for attenuated total reflection infrared spectroscopy (ATR-FTIR) using linear polarized light. Infrared light is sent trough a germanium crystal in such a manner that several total reflections occur at the inner surface. The absorption spectrum is due to the evanescent field along the surface of the germanium crystal and thereby also penetrated the oriented lipid/peptide membrane. The intensities shown correspond to the carboxyl stretching motion of the peptide backbone. Dashed lines are recorded with 90° polarized light and solid lines are recorded with 0° polarized light. In the in-planar conformation the carboxyl stretching vibration has similar intensities for both polarizations (B), whereas the 90°polarized spectrum exhibits a significant higher intensity for transmembrane alignments (C).

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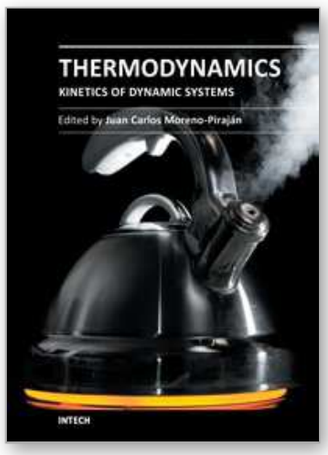

Thermodynamics - Kinetics of Dynamic Systems
Edited by Dr. Juan Carlos Moreno PirajÃ¡n

ISBN 978-953-307-627-0
Hard cover, 402 pages
Publisher InTech
Published online 22, September, 2011
Published in print edition September, 2011

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How to reference
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Aisenbrey Christopher and Bechinger Burkhard (2011). Equilibria Governing the Membrane Insertion of Polypeptides and Their Interactions with Other Biomacromolecules, Thermodynamics - Kinetics of Dynamic Systems, Dr. Juan Carlos Moreno PirajÃ¡n (Ed.), ISBN: 978-953-307-627-0, InTech, Available from: http://www.intechopen.com/books/thermodynamics-kinetics-of-dynamic-systems/equilibria-governing-the-membrane-insertion-of-polypeptides-and-their-interactions-with-other-biomac

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