Chapter VII

Summary and Perspectives

SUMMARY

For decades a large amount of research has dealt with membrane interactions of peptides and proteins as well as peptide-peptide interactions to understand the mechanisms of essential biological processes such as protein-driven vesicle budding and fission, cell penetration and lysis by peptides, and of course protein-driven membrane fusion. The advance of these fields, in combination with recent progress in cell biology, has inspired chemists to mimic these biological processes with simple model systems. However, it becomes apparent that these model systems are more complex than initially thought and the lessons that were learned from natural systems can also be applied here.

The work reported in this thesis applied and extended classical methods for the study of peptide-peptide and peptide-membrane interactions to study the properties of the fusogenic coiled-coil forming lipopeptides in different membrane model systems or in solution. These lipopeptides comprise coiled-coil forming peptides, called E or K, tethered to a membrane anchor via a polyethylene glycol (PEG_{12}) spacer. The DOPE anchored LPE and LPK, the cholesterol anchored CPE and CPK, the untethered peptides E and K and variants of them with altered amino acid sequences were used. The membrane model systems used were lipid monolayers and vesicles of the composition DOPE : DOPE : cholesterol 2 : 1 : 1. Hypotheses were constructed and tested based on the current biochemical and biophysical models of natural membrane fusion.

Initially, a general introduction of concepts and models important for the thesis was given in *Chapter I*. Peptide-peptide interactions in the form of the thermal unfolding of coiled-coil complexes were the focus of *Chapter II*. A common technique employing unfolding curves measured by circular dichroism (CD) spectroscopy was extended by means of a generalized model that covers the 2-state unfolding of peptide oligomers of any stoichiometry. An easy-to-use program, called '*FitDis!*' was developed to fit experimental, concentration dependent, thermal unfolding curves with these models. It was shown that the comparison of the fit results of different models allows conclusions to be drawn on the oligomeric state of the peptide complex. Simulated datasets showed the feasibility of this approach for dimers and trimers and revealed that for higher oligomeric states more data is necessary for improved reliability. Experimental melting curves from coiled coil peptides of known oligomeric state confirmed the applicability of this approach. This method and the developed program will improve the information gained from oligomeric peptide melting transitions and ease the application of this technique to supramolecular systems such as the membrane bound fusogenic lipopeptides.

These fusogenic lipopeptides were studied in detail in the subsequent chapters. In *Chapter III* the interactions of the free peptides E, K and the lipopeptides LPE and

LPK with lipid monolayers were studied. The untethered peptides E, K, and mixtures of both incorporated spontaneously into these monolayers and showed weak, and medium affinity, respectively, to these lipids. However, the monolayer tethered LPE and LPK showed higher affinities. The peptides E, K, and the lipopeptide LPE could be reversibly squeezed out of the monolayers by application of lateral pressure, i.e. a compression of the monolayers. Upon release of the pressure i.e. an expansion of the monolayers LPE immediately reincorporated into the monolayers, while the untethered peptides E and K reincorporated slowly. LPK mostly resisted this squeeze out, indicating higher membrane affinity. Data gained from surface sensitive infrared reflection absorption spectroscopy confirmed the squeeze out of peptide and furthermore supported the model of the peptides being α-helical and incorporated with their helical axis parallel to the monolayer interface. The observed interactions were also found in monolayers containing both LPE and LPK which indicated that these peptides do not interact as a complex with the membrane. It was anticipated that monomeric amphipathic helices of E and K are the monolayer interacting species, and that the stronger interactions of LPK compared to LPE will play a role in lipid bilayer systems such as vesicles.

This prediction was tested in *Chapter IV*, by means of the peptides E_{GW}, K_{GW}, and the lipopeptides LPE_{GW}, LPK_{GW} which were designed, based on the original sequences of E and K. The variation in the peptide sequence was shown to only slightly influence coiled-coil formation and the obtained tryptophan fluorescence emission could be used to probe the polarity of the microenvironment. The microenvironment of K_{GW} and LPK_{GW} became more hydrophobic upon vesicle addition accompanied by an increased helicity as measured by CD spectroscopy, proving the interaction of these peptides with the vesicles. Contrary to this, the complementary E_{GW} and LPE_{GW} showed no change of its microenvironment polarity, although LPE_{GW} showed increased helicity on vesicles. The coiled coil complex formation in solution was found to reduce but not to completely inhibit the K_{GW} – membrane interaction. Surprisingly, the membrane tethered LPK_{GW} could not be readily pulled out of the membrane by coiled coil complex formation, thus it showed an increased membrane affinity compared to K_{GW}. Furthermore, fluorescence quenching experiments with water soluble and membrane bound quenchers supported these conclusions and allowed for the estimation of the penetration depth of K_{GW}. It was found that the peptide incorporates rather shallowly into the membrane, which is in common with an insertion of an α-helix centered close to the glycerol and phosphate groups of the lipids.

A close-up image of the secondary structures of the membrane tethered CPE, CPK and untethered, membrane bound K could be obtained in *Chapter V* using temperature dependent infrared (IR) and circular dichroism spectroscopy. Vesicle

tethered CPE showed CD unfolding curves and temperature dependency of its IR amide I' bands that indicate unfolding of homomeric coiled coil complexes. In contrast, vesicle bound K and membrane tethered CPK showed no cooperative unfolding and stable IR amide I' bands. This showed that the membrane bound helix was rather stable and might fray on the termini. Furthermore the amide I' band shape of the membrane bound K, homo coiled K, and the coiled coil complex EK was investigated by means of the isotopically labeled variant ^{13}CK. It was found that in all these spectra, the amide I' band two band pattern which was observed is caused by amphipathic helices, and thus the membrane bound structure shows high similarities to the coiled-coil bound structure of K.

Taken together all the data and conclusions from *Chapters III – V* drew the image of an asynchronous behavior of the membrane tethered lipopeptides. Tethered K incorporated as a helix, with a parallel orientation to the membranes, while tethered E formed significant amounts of α-helical homo coils on the membrane surface. Both states were also expected in the post fusion membranes. Hence an asymmetric mechanism was proposed in which the K interaction is the membrane curvature-inducing or membrane distorting process that promotes fusion.

To test this hypothesis, K variants with shortened lysine side chains were designed in *Chapter VI*, with the aim being to suppress the membrane interaction. It was anticipated that the long lysine side chains snorkel, i.e. bend towards the polar interface to have a more favourable conformation in the membrane. The sequence variations strongly reduced the membrane affinity of the untethered K variants, hence the snorkeling contributed strongly to the membrane binding. However, the variations also strongly influenced the coiled-coil binding propensity, which strongly reduced the number of reasonable sequences for lipopeptide synthesis. Finally, two cholesterol anchored lysine shortened lipopeptides CPO_3K_3 and $CPK_2O_2K_2$ were synthesized. However, vesicles decorated with these molecules still showed the hallmarks of full fusion with CPE decorated liposomes, and an α-helical interaction of CPO_3K_3 and $CPK_2O_2K_2$ with the membrane was found by CD. Thus, the outcome of the fusion experiments was not sufficient to disprove the hypothesis, that K-membrane interaction is necessary to promote the vesicle fusion.

Taken together, the work reported here led to a new perspective on lipopeptide mediated vesicle fusion and one important general insight: an alleged easy model system such as the fusogenic lipopeptides can reveal complex interdependent interactions and equilibria in a close-up view. Also, finding the right magnifying glass can be a challenging and time-consuming task.

PERSPECTIVES

Within this thesis several important aspects that need to be focused on were revealed and these indicate the direction further research should pursue. It might turn out to be a challenging task to find definite proof for the hypothesis that peptide K induces the necessary membrane disruption to enable lipid reorganization during fusion. However, experimentally testable predictions based on this hypothesis can be made, which might deliver indirect proof or disproof. For instance, different systems based on coiled-coil forming lipopeptides are currently designed to be tested in fusion experiments. If a system is found that efficiently docks liposomes but does not fuse them, full fusion should be triggered by the addition of untethered peptide K. The wanted system does not necessarily need to be based on coiled-coil forming peptides; other molecular recognition systems might suffice for that purpose. Another approach to deliver indirect proof is to attempt to suppress the peptide membrane interaction by variation of the lipid composition. Incorporation of charged lipids, variation of the acyl chains or variation of cholesterol concentration will influence the membrane binding of K and maybe even E. The challenge of this approach will be the preservation of the intrinsic ability of the lipids to fuse, which is why this approach might not deliver a definite proof.

From a mechanistic point of view the effect of the K incorporation on the membrane structure is very important. The central question is whether the incorporation causes curvature in the membrane and if this is positive or negative curvature. A detailed NMR study is being undertaken at the moment and calorimetric studies and X-ray or neutron scattering experiments are further possible ways to approach this. For instance DSC studies can reveal the influence of peptide K on the phase transition of PE lipids from the fluid L_{α} to the inverse hexagonal H_{II} phase, which contains negative curvature. A reduction of this transition temperature would indicate negative curvature creation, and increased promotion of positive curvature. These insights might feed back into the peptide design with the aim of specifically influencing the curvature creation by the helical membrane insertion. In such studies also molecular dynamics simulations will be helpful to test the rationality of the developed models.

Plenty of possibilities appear concerning the new design of fusogenic lipopeptides. One might use databases or rational design to find sequences that are able to form similar amphipathic helices as K, i.e. that can form coiled-coils and bind to membranes; one could try to find heterotrimeric sequences, which allow for triggering of fusion by addition of a free binding partner; or one could employ curvature-sensing amphipathic helices to specifically recognize and fuse small vesicles.

From the possible routes mentioned here it becomes apparent that much potential is seen in a combined approach of biophysical studies, coupled with targeted peptide design, because this will open exciting opportunities for the understanding and thus the rational improvement of artificial membrane fusion systems.