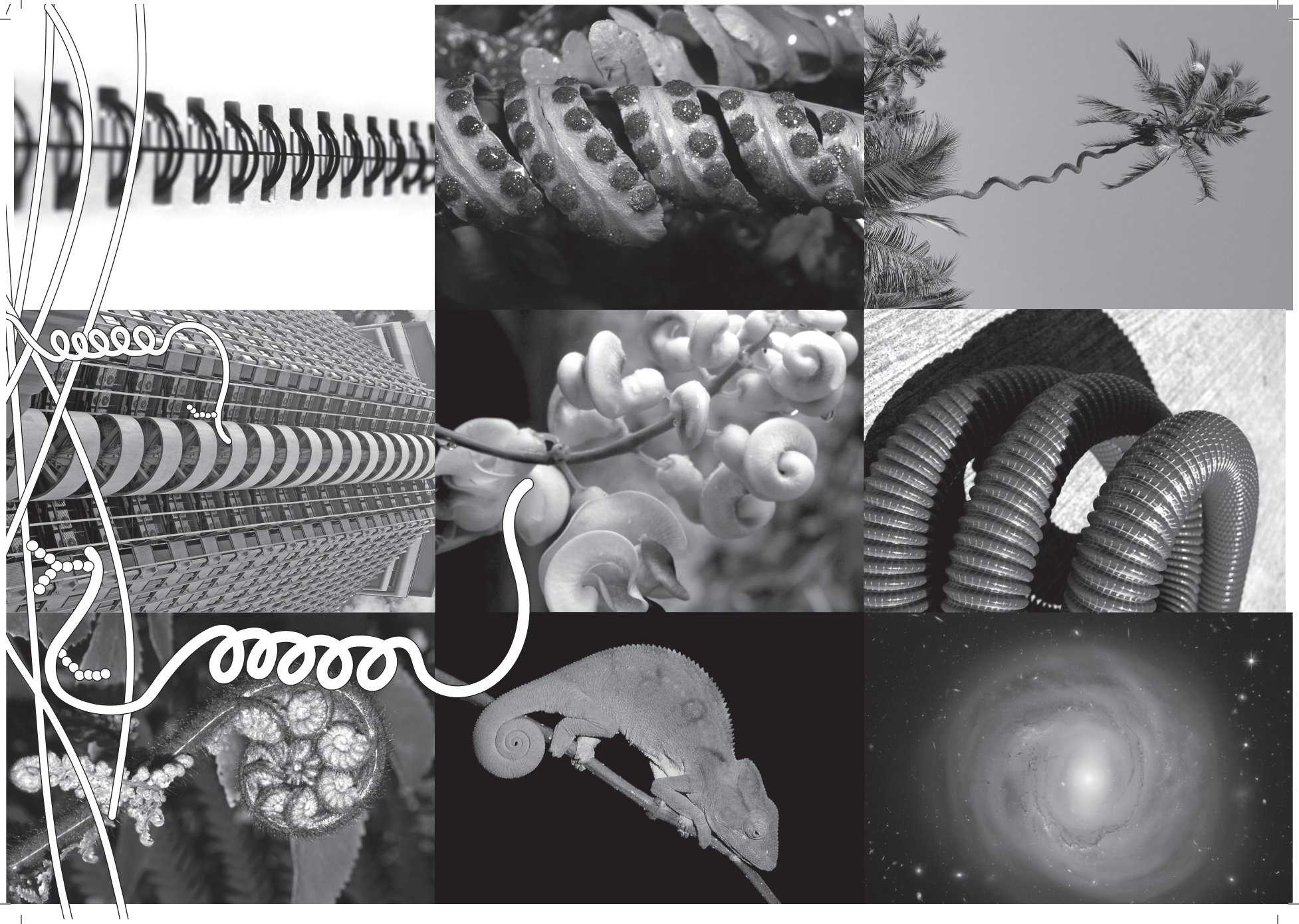

Towards a better understanding of membrane proteins

Synthesis and biophysical characterization of oligomeric model peptides

Op weg naar een beter begrip van membraaneiwitten

Synthese en biofysische karakterisering van oligomere modelpeptiden

Met een samenvatting in het Nederlands

Proefschrift

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Promotor
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Towards a better understanding of membrane proteins **Synthesis and biophysical characterization of oligomeric model peptides**

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General introduction and scope of the thesis

General introduction

‘How do we cure diseases?’ This is a question that has intrigued the human race probably already since the beginning of mankind. The prehistorical medicine man was restrained to a ‘trial and error’ method, expanding his knowledge of nature every generation. Nowadays, an approach is used that starts with addressing the question ‘how does our body work?’ However, understanding the human body on a molecular level is a matter that has proven to be so complex, that one can only try to understand small parts. And even then a single researcher can merely add little pieces to the puzzle. The puzzle that is under investigation in this thesis is the understanding of membrane proteins. The research described in this thesis contributes to the knowledge on the general principles of behaviour of this class of proteins.

Membrane proteins and membranes

Membrane proteins are the gatekeepers of our cell membranes. These membranes are the ‘walls’ that surround and protect all the cells in our bodies. The lipid constituents of the membrane have hydrophilic heads and hydrophobic tails, inducing the formation of a bilayer in the aqueous environment of our body. Because of this organisation there is a significant energy barrier for water soluble molecules to pass the hydrophobic interior of the membrane. The important task of facilitating and regulating transport of all kinds of molecules across this hydrophobic barrier is performed by proteins that reside in the lipid bilayers. These proteins are important in for example signalling, energy transduction and providing a cell with molecules needed to perform its specific function. In addition, membrane proteins play roles in events like cell-cell recognition and enzymatic processes. One can imagine that if for some reason a membrane protein cannot or does not perform its function properly, this may have major consequences for our health. Besides this, because of their transport and signalling function, it is not surprising that membrane proteins are important targets for the majority of newly developed drugs.

Membrane proteins are quite abundant in our bodies. With the sequencing of our entire genome it was found that one out of four proteins that is encoded for by DNA is a membrane protein.[1] Despite the abundance and importance of membrane proteins, relatively little is known about this class of proteins in comparison to water-soluble proteins. This is caused by the difficulties in handling membrane proteins. The hydrophobic nature of these proteins results in problems with expression and crystallization. Though an increasing amounts of crystal structures are being determined, there are still less than 200 known.[2, 3] Moreover, the crystal structures that were obtained are static, while dynamics and the interaction with the membrane are important to understand the functioning of membrane proteins.[4]

To understand how membrane proteins function, more knowledge has to be obtained about the principles that are important for the membrane protein to perform its function. Factors that determine the behaviour of membrane proteins include its structure, dynamics, protein-protein and protein-lipid interactions.[5] Many membrane proteins undergo structural changes to be able to carry out their function. A frequently occurring change is tilting of one or more of the transmembrane helices in a protein.[6] For example in the mechanosensitive channel MscL the orientation of the helices changes drastically upon gating.[7, 8] Alternatively a minor reorientation of only one helix can also be important for function, like in rhodopsin.[9, 10] Another structural change that can be envisaged is a change in conformation of the helix-backbone. Although transmembrane helices have strong conformational preferences[11, 12], adaptations of the backbone are known to occur in some cases[13-15]. Another aspect that can be important for the functioning of membrane proteins is the assembly of transmembrane helices. Many membrane proteins consist of multiple monomeric units that have to meet in the membrane to form a functional entity.[16-18] An illustrative example is the potassium channel KcsA. The assembly of the subunits into a homotetrameric complex is extensively studied.[19-24] Assembly of membrane proteins can also involve several (protein) units in and outside the membrane that together form a functional complex.[25-27]

The structural changes and the forces that reign assembly can be subtle or major, but either way, in most cases they are highly influenced by the surrounding membrane.[28-32] That structural rearrangement can be affected by lipids is nicely demonstrated by the gating of the MscL protein that was mentioned in the previous paragraph. The iris-like tilting of all helices is triggered by membrane tension.[8, 30, 33] Regarding assembly, one can imagine that if the interactions with neighbouring lipids are unfavourable, intermolecular interaction between helices are preferred, driving assembly of monomeric units to oligomeric complexes.[34] This is illustrated by for instance the G-Protein coupled receptor Rhodopsin, in which association is promoted by unfavourable lipid interactions.[35] Differences in lipid composition of a membrane can significantly alter the activity of a membrane protein through the described influence on structure and assembly.[36-38] Thus, understanding the interactions between helices and lipids is of fundamental importance to understand membrane protein structure and function.

As illustrated above, lipids influence membrane proteins, but in turn membrane proteins can also influence lipids.[39] For instance, it was shown in several studies that membrane proteins and peptides alter the phase transition temperature of a lipid bilayer.[40-43] Another example is that membrane proteins can stabilize the lamellar organisation of lipids that would otherwise form a hexagonal phase.[44] The effect of a membrane protein on the membrane may have significant implications. For example, if a protein has a different hydrophobic thickness than the surrounding membrane it would have an unfavourable influence on the neighbouring lipids. This may affect the insertion of the protein. It was shown in previous research that the localization of a protein to a certain membrane is partly governed by their relative hydrophobic thicknesses.[45, 46] Similarly, membrane proteins may induce lipid micro domains to be able to abide in a favourable environment.[47-49] Thus, in the study into the interaction between helices and lipids it is also important to have a closer look at the behaviour of the lipids.

Model systems

As explained above, membrane proteins are difficult to study, but the key to understanding their functioning is knowledge of the general principles of the behaviour of proteins in a membrane. To study protein-lipid interactions, often simple model systems are used. Such systems consist of synthetic α-helical transmembrane peptides

in vesicles of varying lipid content. There are several advantages of using such systems. First, properties of peptides (e.g. flanking residues) and lipids (e.g. acyl chain length) can both be varied. This allows for systematic studies into the factors that are important for protein-protein and protein-lipid interactions. Also, labels can be incorporated essentially everywhere to be able to characterize and study the peptides and lipids. Finally, the simplification of the system from complex membrane protein to transmembrane helix (Figure 1) and from biological membranes to vesicles of synthetic lipids makes biophysical studies of the helix-helix and helix-lipid interactions well achievable.

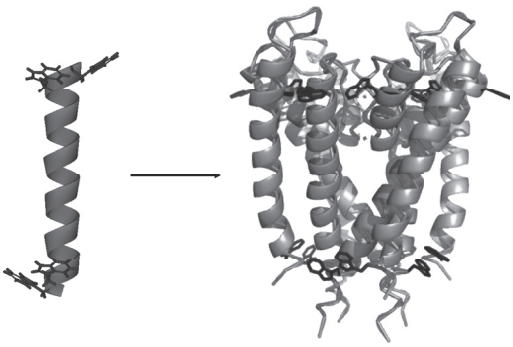

Figure 1. WALP model peptide (left) as mimic for membrane proteins, such as the potassium channel KcsA (right)

An example of a well-defined and -characterized family of model peptides are the so-called 'WALP-peptides', where WALP denotes tryptophan- (W), alanine- (A) and leucine- (L) containing peptide (P). These peptides consist of an α-helical stretch of alternating leucine- and alanine-residues with varying length, flanked on both sides by tryptophan residues (Figure 2), to mimic the membrane spanning part of α-helical membrane proteins. The alternating alanine-leucine sequence and flanking tryptophan residues form a hydrophobic α-helix with an irregular surface. The tryptophan residues anchor the hydrophobic stretch in the membrane through their strong preference to locate at the membrane-water interface, interacting with the lipid headgroups.[50-52] Tryptophan and other aromatic residues are frequently found in this same interfacial position in natural membrane proteins. The WALP peptides have been characterized extensively with various biophysical techniques and were found to incorporate nicely into lipid bilayers.[53-55]

As mentioned before, an advantage of working with synthetic peptides is that the sequence can be systematically varied and labels can be incorporated. Frequently used variations are increasing or decreasing the length of the leucine-alanine stretch (varying n in Figure 2) and re-

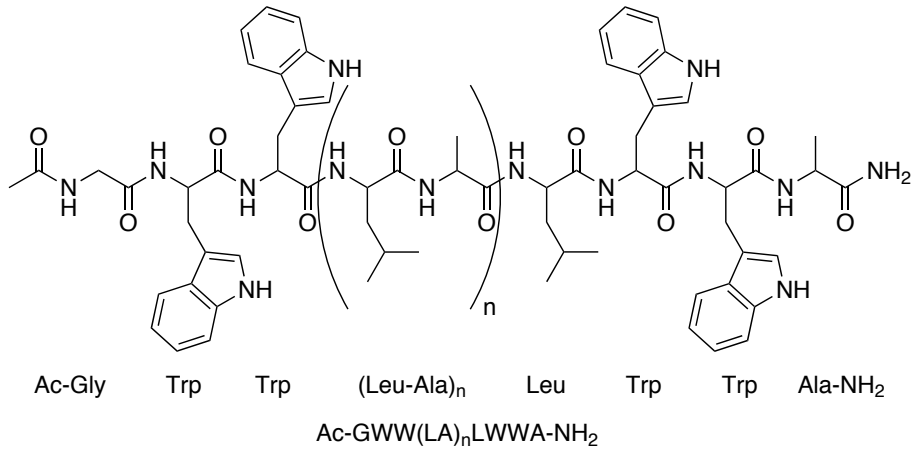

Figure 2. Structure of the transmembrane 'WALP' model peptide

placing the flanking residues with other residues frequently found at the interface. Especially lysine is a commonly used residue, in this case the model peptides are named KALP peptides. To indicate the length of the model peptide, usually a number is stated in the name. For example, if n=8 in Figure 2, the peptide consists of 23 amino acids and is thus called WALP23. Incorporation of labels is mainly achieved via two methods. Often, a cysteine is incorporated in the peptide to which labels (e.g. via a maleïmide linker) can be attached. In addition, isotopically labelled amino acids (e.g. Ala-d_3) are frequently incorporated as substitutes of regular amino acids, for example to use for solid-state NMR measurements.

Depending on the goal and requirements of the measurements different kinds of lipid vesicles can be used. The vesicles that are easiest to prepare are multilamellar vesicles (MLVs, Figure 3A), in which several layers of lipid bilayers surround each other like the layers in an onion. Multilamellar vesicles can be transformed into unilamellar vesicles (Figure 3B) of different sizes, where the size depends on the technique that is employed. Vesicle diameters may vary from 30 nm (small unilamellar vesicles) to 10 µm (giant unilamellar vesicles). In this thesis, mostly MLVs are used, but also large unilamellar vesicles (LUVs) are applied. These are obtained by extrusion of MLVs through membrane filters, yielding vesicles of almost 200 nm in diameter.

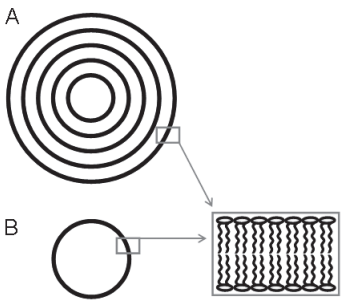

Figure 3. Multilamellar (A) and unilamellar (B) vesicles

Using the described model systems, an elegant and enlightening way to investigate helix-helix and helix-lipid interactions is the use of 'mismatch'. This is a situation in which the hydrophobic thickness of the bilayer does not match the hydrophobic length of the peptides. The hydrophobic thickness of the bilayer is usually defined as the distance between the acyl chain C-2 atoms of two opposing lipids in the two lamellae of the bilayer. The definition of the hydrophobic thickness of the peptides is less clear. It is for example uncertain if the tryptophan residues should be included and if so, what properties of the side chains define the hydrophobic length. In addition, for the tryptophan, as well as the leucine residues, it is the question how the hydrophobic length would depend on the side-chain torsion angles. Therefore, systematic studies are essential. In these studies, for simplicity, the hydrophobic thickness of the WALP peptide is defined as the length of the leucine-alanine backbone (1.5 Å per amino acid), though the effective hydrophobic length will be longer if the tryptophan residues are included. A mismatch situation can be achieved by either varying the hydrophobic thickness of the peptide, or that of the lipids. In Table 1, a few illustrating examples of the hydrophobic thickness of several WALP peptides and bilayers of different lipids are presented.

Table 1. Values of hydrophobic thickness of some model peptides and lipids

Peptide or lipid	Hydrophobic thickness
WALP23	25.5[a]
WALP27	31.5[a]
Di-14:1 PC	20[b]
Di-18:1 PC	27[b]
Di-22:1 PC	34[b]
Di-14:0 PC	23[b]
Di-16:0 PC	25[b]

a. defined as length of Leu-Ala stretch
b. defined as distance between acyl chain C2 atoms of opposing lipids in the two lamella of the bilayer and derived from[56]

Most studies of WALP peptides have been performed in vesicles of matching lipids or under conditions of positive mismatch. In positive mismatch the hydrophobic thickness of the peptides is too long to fit entirely into the hydrophobic part of the bilayer (Figure 4A). The system will then seek the energetically most favourable way to prevent this unfavourable situation. Several events to alleviate the energy cost of having hydrophobic residues exposed to the aqueous environment can be envisaged. For example, the peptide can adapt by tilting (Figure 4B), altering the backbone conformation (Figure 4E) or oligomerization (Figure 4D). The lipids can adapt by stretching the acyl chains (Figure 4C). All these events can be important factors for membrane protein behaviour, as was discussed in a previous paragraph. This makes it interesting to study these responses. By systematically varying the properties of the model system the basic principles of the behaviour and lipid-interactions of natural membrane proteins may be determined. Ultimately, rules and predictions for the functioning of these proteins might be established.

Studies with model systems

With the system of WALP peptides in lipid vesicles so far much basis knowledge has been obtained.[6, 34, 57, 58] In these studies, WALP peptides of varying length were incorporated in vesicles of varying lipid content. As a reference, also the lysine flanked KALP peptides were used. Here, a few results of the studies of WALP23 in vesicles of varying lipid content will be discussed, that are relevant for this thesis.

Tilting of the helices was studied by solid state NMR[59, 60] , fluorescence spectroscopy[61] and molecular dynamics simulations[62]. With fluorescence spectroscopy[61], and solid state NMR using the MACADAM method[60], tilt angles of respectively 24° and 20.5° degrees were found

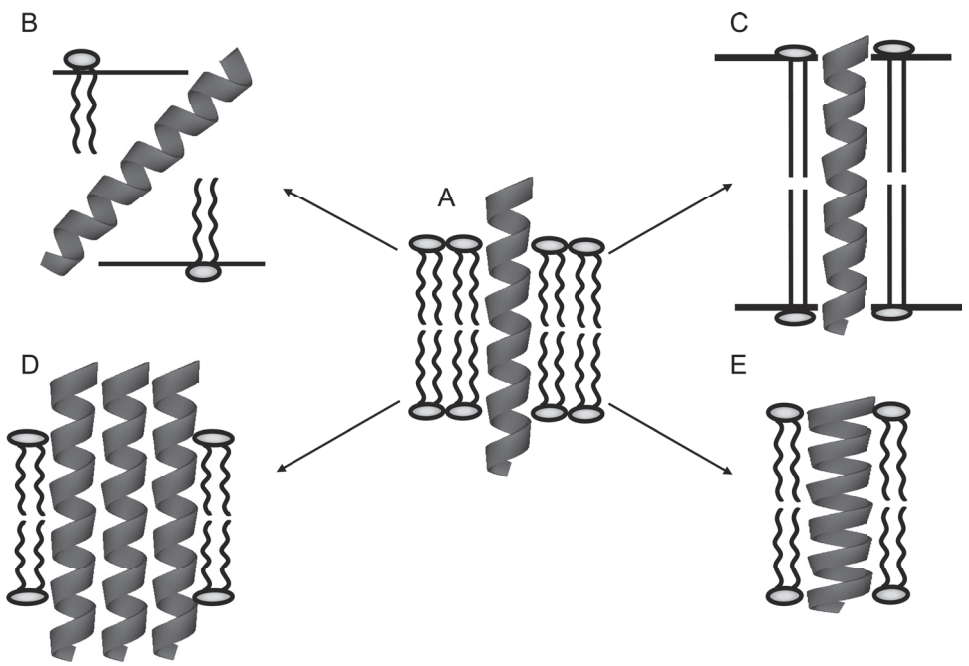

Figure 4. Possible adaptations to hydrophobic mismatch (A) are tilting (B), stretching of the lipids (C), oligomerization (D) and adjusting the backbone (E)

for a WALP23 in DOPC and DMPC respectively. This contrasts the previously reported tilt of 4.5° of a WALP23 in DOPC, determined by solid state NMR following the GALA-method.[63] In these previous studies, the value of the tilt angle was probably too small because the dynamics of the peptide was not sufficiently taken into account in the calculations. On the other hand, using molecular dynamics simulations, an angle of 33° angle was found that could not be matched to the experimental data and may have been overestimated.[62] The fluorescence measurements are on a time scale in which motional averaging does not play a role. The solid state MACADAM measurements allow detailed description of motional averaging, because different interactions are studied, in which the averaging depends on the orientation of the bond with respect to the magnetic field. Therefore, the values obtained with these methods most probably represent 'the correct tilt angles'. It may seem strange that even in a matching situation the helices tilt. This intrinsic tilt is probably caused by the preferred orientation and interaction of the tryptophan residues with the interfacial region of the bilayer. Interestingly, the tilt angle showed small, but systematic changes to increasing tilt angles in response to a positive mismatch situation, as determined from the GALA and the fluorescence studies. This suggests that there is a high energetic cost for tilting and that additional mismatch responses may occur by other mechanisms.

Backbone adaptations were studied by attenuated total reflection Fourier transform infrared spectroscopy[53] and solid state NMR[64]. It was shown that the backbone of a WALP peptide is a stable α-helix and no adaptations of the backbone itself to mismatch could be measured.

Oligomerization was studied by atomic force microscopy (AFM)[65], electromagnetic spin resonance (ESR)[66], fluorescence spectroscopy and molecular modelling[67]. In the fluorescence study WALP peptides in fluid bilayers were studied. Initially, WALP peptides were labelled with a pyrene via a cysteine at the C- or N-terminus. Excimer formation, which is indicative of oligomer formation, only occurred when a mixture of both peptides was incorporated in lipid bilayers, suggesting a preference for an antiparallel orientation. In addition, formation of oligomers only seemed to take place when high peptide concentrations were used. Hence, the WALP peptides did not have an intrinsic tendency to aggregate in lipid bilayers. With molecular modelling it was confirmed that an antiparallel orientation was most favourable and that this was caused by the dipole-dipole interactions of the helix-backbones. Tryptophan-pyrene Förster resonance energy transfer (FRET) measurements suggested that the observed association of WALP peptides is influenced by bilayer thickness, showing increased oligomerization with mismatch.[67] The tendency of the WALP peptides to occur as monomers in the fluid bilayer was confirmed by ESR.[66] Above T_m there was no sign of aggregation. The ESR studies also showed that the extent of association of the WALP peptide was influenced by lipid packing and temperature. At 120 K, spin-labelled WALP peptides in a gel phase bilayer associated more strongly in DPPC than in DOPC. At higher temperatures, but below the T_m of the lipids there was less aggregation, possibly corresponding to linear aggregates. Also with AFM, linear aggregates of WALP peptides were observed when incorporated in gel phase DPPC bilayers.[65] From all these studies it can be concluded that the WALP peptides do not form oligomers easily, but the aggregation that does occur is dependent on the physical state of the lipids.

The influence of WALP peptides on the lipid bilayer was studied by differential scanning calorimetry (DSC)[42], electron spin resonance (ESR) and solid state ^{2}H-NMR[68]. With DSC, it was shown that WALP peptides slightly decrease the phase transition enthalpy and temperature of matching lipids. The model peptides seem to favour the fluid phase, probably because the helices have more freedom to adapt in a fluid phase

bilayer in comparison to a gel phase bilayer or because of better matching. Furthermore it was found with ^{2}H-NMR and ESR that WALP peptides slightly, but systematically stretch the acyl chains of mismatching lipids in the fluid phase. The mean bilayer thickness of DMPC was estimated to increase with 1 Å when a WALP23 was incorporated.

Thus, the studies described above show that knowledge can be obtained on helix-helix and helix-lipid interactions by merely studying a WALP peptide in vesicles of varying lipid content. As explained before, this knowledge can help us understand the general principles of membrane protein behaviour.[6, 34, 58] For example, one of the many things that was discovered from the study on the oligomerization of WALP peptides is that dipole-dipole interactions between helices may provide a driving force or stabilizing effect for antiparallel association of helices in polytopic membrane proteins. Altogether, it can be concluded that several adaptations to relieve the unfavourable mismatch situation occur at the same time. However, all responses only occur to a small extent. This suggests that all adaptations have a considerable energy cost. Nevertheless, it is important to note that all occurring responses can be relevant for membrane protein function and that even small adaptations may have huge consequences for functioning. By systematically modifying the model system, knowledge can be obtained about the basic principles and rules of behaviour of membrane proteins. Maybe it will even be possible to predict behaviour, based on structural features. The variation that is the subject of study in this thesis is the size of the model peptide, or the number and arrangement of the transmembrane helices.

Effect of size

Although with the WALP model peptide much can be and has been learned on the basic principles of peptide-lipid interactions, it spans the membrane only once. Since most natural membrane proteins are multispan, it may be questioned if the WALP peptide is an adequate model (Figure 1). Oligomeric assemblies of the WALP peptides would represent a first step towards polytopic membrane proteins. With these covalent assemblies the effect of oligomerization on helix-lipid interactions can be studied in a systematic way. The questions that can be addressed are: 'How does oligomerization influence the effect of lipids on the helices?' and 'How does oligomerization influence the effect of the helices on lipids?'

It is likely that this study will partly answer a stringent question that is raised by the studies with the WALP peptides: 'Why do our model peptides show only small adaptations to mismatch, while natural membrane proteins can undergo huge structural changes?' There could be multiple factors that play a role. It has for example been shown that the flanking tryptophan-residues influence the adaptations of the WALP peptides.[58, 63, 69, 70] Yet, also the size of the membrane protein or the intermolecular interactions may facilitate or restrain structural changes. For example, one can imagine that tilting of one helix is unfavourable for lipid packing, but that tilting of a whole complex of helices can be achieved in a less perturbing way. Therefore, the influence of cross sectional diameter is currently under investigation.

There are several indications that size is one of the factors that influence protein-lipid interactions. A first hint comes from a comparison of the effects on bilayer thickness of WALP peptides and gramicidin In terms of amino acid composition, there is not that large a difference between WALP peptides and gramicidin. In fact, the WALP peptides were originally designed as α-helical equivalents of the β-helical gramicidin dimer. The most evident difference between the two types of peptides is the larger cross sectional diameter of gramicidin and the larger rigidity of the β-helix as compared to the α-helical WALP peptide. With the latter, no alteration of bilayer thickness could be detected, while

gramicidin did show a significant effect, as demonstrated by X-Ray diffraction, solid state NMR and ESR.[68, 71, 72] Second, an important indication of the importance of size comes from studies on phase transition temperatures.[73, 74] Although WALP models show a systematic effect on thermotropic behaviour of lipids, the shifts in phase transition temperatures are maximally 1.5 ºC.[42] In contrast, the effect of large proteins on the phase transition temperature, as a function of mismatch, is enormous. Under mismatching conditions, shifts from 3ºC till as high as 40ºC are observed.[40, 74-76] A third illustration of the potential influence of cross sectional diameter may be the difference in localization properties. Molecular sorting of WALP peptides with matching lipids has not been observed.[77, 78] On the contrary, membrane proteins localize in certain micro domains of the bilayer with matching lipids.[48, 79-81] It is possible that this is caused by the larger effect that multispan membrane proteins would have on the membrane, in comparison to the effect of the single span WALP helix. A fourth example of an assumed effect of size on the behaviour of proteins in membranes is that lateral diffusion of membrane proteins in a lipid bilayer depends on the hydrodynamic radius of proteins.[82, 83] This was shown with a set of fluorescently labelled receptor, channel and transporter proteins and a WALP peptide in giant unilamellar vesicles. Finally, it was found that phospholipid flop may relate to the size of the proteins, with smaller proteins being more effective in promoting lipid transbilayer movement. It was suggested this reflects a functional importance of the flexibility of the proteins.[84]

In the examples mentioned in the previous paragraph WALP peptides and several membrane proteins are compared. The number of transmembrane helices may be an important variable in the studies. However, comparison between the WALP peptide and/or the various membrane proteins is complicated, since there are more differences than cross sectional diameter. The influence of size can be studied more systematically by using model peptides in which the number of transmembrane helices is varied. In covalent oligomers of the WALP peptides all helices would have the same sequence and structure and consequently, the cross sectional diameter is the main variable.

Possibilities for oligomerization of WALP peptides

To systematically study the effect of oligomerization on helix-lipid interactions covalent assemblies of the WALP peptides will have to be synthesized. There are many possibilities to couple two or more peptides covalently. In all methods some functional group has to be incorporated in the peptide. An important prerequisite that has to be met is that this group and the reaction(s) necessary for oligomerization are compatible with the other amino acids in the peptide. The coupling reaction has to be selective and should not damage other residues. This limits the possibilities, but there is still a sufficient amount of functional groups that meet the requirements. Another parameter that might be quite important is the helix-helix distance. On one hand, the helices should have enough motional freedom. On the other hand care has to be taken that they still interact with each other, not leaving space for lipids between the helices. The covalent assemblies were designed by looking at nature and at studies on natural membrane proteins. The designs that were employed in this thesis are schematically depicted in Figure 5 and will be discussed in the coming paragraphs.

A straightforward method to make covalent dimers of the WALP model peptides is via incorporated cysteine-residues. For the research in this thesis the cysteine was positioned in the middle of the helix (Figure 5A). Covalently coupling two cysteine residues requires only mild oxidizing conditions, providing a good compatibility with all other amino acids used in the family of WALP peptides and their analogues. In many studies on interhelical interactions in membrane pro-

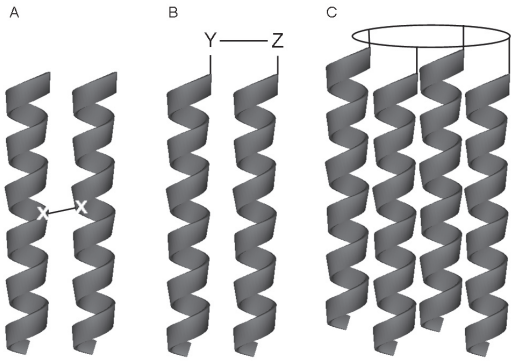

Figure 5. Three possibilities to construct a covalent assembly of WALP peptides

teins, cysteine mutants are used to investigate the interacting interface or proximity of different parts of the protein.[85-89] This indicates that a disulfide bond is sufficiently long to bridge the distance between two transmembrane helices. Thereby, nature itself uses cysteine residues to tie two helices together, thus making a cysteine a logical choice in a membrane protein-mimic. However, since only one functional group is used in this method, asymmetry in the dimer cannot be achieved. For example, if two WALP peptides with a cysteine in the middle of helix are coupled, parallel as well as antiparallel topology may in principle be possible. With a WALP peptide with a cysteine at the C- or N- terminus a specifically parallel dimer could be obtained. However an antiparallel dimer cannot be constructed, since the combination of a WALP peptide with a cysteine at the N-terminus and one with a cysteine at the C-terminus would result in a mixture of an antiparallel and two types of parallel dimers.

When one wants to force a specifically antiparallel topology or achieve another kind of asymmetry in the dimers, two different functional groups will have to be used (Figure 5B). An attractive possibility would be to incorporate an alkyne and an azide in the peptides, with which the so called click reaction can be performed.[90, 91] This copper catalyzed reaction is highly selective and compatible with nearly all naturally occurring amino acids. A triazole ring is formed with the two molecules to which the alkyne and azide are attached, selectively on the 1st and 4th position (Figure 6). The ring could produce a constraint in the dimers. To prevent such a constraint some extra amino acids can be incorporated as spacer in the design of the dimers. In this way a kind of flexible loop between the helices, as is found in many multispan membrane proteins, is created. One drawback of the click reaction is that the use of copper is generally incompatible with the presence of free cysteine residues in the peptide, risking precipitation. Thus the use of cysteine should be avoided or an adequate synthesis route has to be followed. The click reaction is popular in chemistry, but also increasingly used in various biological systems.[92-94] Incorporation of azides and alkynes in biological molecules is frequently achieved by the incorporation of unnatural amino acids containing these functional groups.[95] Thus, incorporating azide and alkyne functionalities in a WALP peptide via such amino acid analogues would provide an excellent possibility to make all kinds of (asymmetric) dimers.

When more than two helices have to be coupled together, either multiple (orthogonal) groups have to be used or the helices have to be coupled to some kind of scaffold. Using multiple orthogonal groups means elaborate and complicated synthesis work. Also, folding of the WALP oligomer into the desired conformation upon incorporation in the bilayer might not be straightforward. Therefore, using a scaffold

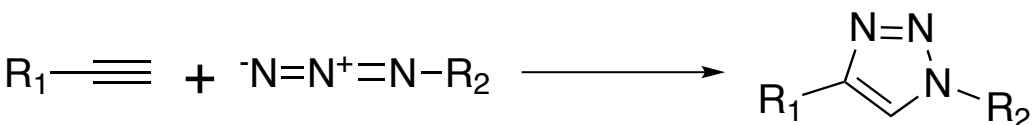

Figure 6. The click reaction: azide and alkyne selectively reacting to a triazole

would be much more convenient (Figure 5C). When scaffold and functional groups are chosen strategically the synthesis of several kinds of oligomers is well feasible. As functional groups, again an azide and alkyne are appealing to use. The WALP monomers could then be attached to the scaffold via the highly selective click-reaction. Sufficient spacing between the scaffold and the helices would be important to provide enough flexibility of the helices. Additionally, it would be a advantage if the scaffold promotes a well-defined orientation and packing of the helices. This can be achieved by using a scaffold with a well-defined three-dimensional structure, where the functional groups are all oriented in the same direction. A suitable scaffold to make tetrameric assemblies would be the cyclic peptide introduced by Mutter et al.[96, 97] The template assembled synthetic protein (TASP) approach is frequently used to make covalent assemblies of peptides, some of which are even functional ion channels.[98-104]

Methods to characterize and compare monomers and dimers

As soon as oligomers are obtained and reconstituted into lipid bilayers, the effect of size can be investigated by several techniques. Yet, first some basic properties of the reconstituted systems themselves need to be characterized. For example, it has to be verified if the stability of the α-helices in the dimers is comparable to that of the monomeric helices and if they incorporate to a similar extend. In addition, the bilayer should not show abnormalities. When the system has been shown adequate, the behaviour and influence on lipids of the monomeric and oligomeric model peptides can be compared. Below, the main techniques that are applied in this thesis to characterize and compare monomers and dimers will be discussed.

A straightforward choice of the technique to use for the first characterization of the model peptides is circular dichroism (CD).[105, 106] The principle of this technique is that the absorption of right and left circularly polarized light by the amide bonds of the peptide backbone in the far-UV region is dependent on the secondary structure. The shape of a CD-spectrum is thus characteristic for the secondary structure of the protein or peptide measured. Additional information that can be derived from this technique is the extent of incorporation. WALP peptides that are not incorporated will aggregate and do not contribute to the CD signal.[53] Circular dichroism is also a convenient technique to assess the stability of proteins. For soluble proteins this is approached by measuring the changes in secondary structure when treating the protein with denaturants.[107] Recently, this technique is also increasingly being applied to membrane proteins.[108] Thus, it should also be possible to derive the stability of the model peptides under denaturing conditions by CD. A limitation of CD is that all kinds of structures, like aromatic rings and double bonds in lipids may show absorbance in the near-UV region, thereby making the measurements more difficult, and that the presence of lipid vesicles may induce scattering artefacts. However, there is still ample possibility to obtain valuable information. Measurements of proteins and peptides can be performed under appropriate conditions in solution, in detergent or even in lipid bilayers.[106]

With attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) a wealth of information on the influence of oligomerization on peptides and lipids can be obtained. By merely measuring the vibrations of the bonds in the molecules, all kinds of knowledge on the properties and behaviour of peptides and lipids can be acquired. In ATR-FTIR simply multilamellar vesicles can be used and only micrograms of material are needed. The vesicle suspension is spread over an internal reflection element (IRE) and dried under an N_2-flow, forming multiple layers of oriented lipids. In the ATR setup the IR beam is directed into the IRE, forming an evanescent wave when reflecting of the internal surface that is in contact with the sample. The vibrational bands of peptides and lipids have

proven to be quite informative. The secondary structure of a peptide or protein structure can be derived from the position and shape of the amide I and II bands in its ATR-FTIR spectrum.[109] Like with CD, aggregated peptides do not contribute to the signal, so from the relative intensities of the lipid-normalized bands it can be derived if the peptides are incorporated adequately.[53] The stability of the protein can be explored by measuring the changes in intensity of the amide II band when exposing the protein to D_2O.[110] The intensity of this band decreases when hydrogen is exchanged for deuterium. The rate of exchange depends on the stability of the secondary (and tertiary) structure of the protein and on its mode of incorporation. Amide-hydrogen atoms in stable hydrogen bonds are shielded from exchange. Dichroic ATR-FTIR spectra of the peptides in vesicles give information on the orientation of the peptide with respect to the membrane normal.[110, 111] With this method, spectra are recorded with the incident light polarized perpendicular or parallel relative to the incidence plane. The orientation of the dipole of the peptide with respect to the ATR plate can be calculated from the differences in intensity of the amide I signal in the parallel and perpendicular spectrum, when normalized to the lipid carbonyl signal. Thus, tilt angles can be derived, but no information is obtained on rotational angles. From the lipid vibrational bands, changes in the conformation of the acyl chains can be derived. Temperature-induced gauche bonds in the normally all-trans acyl chains shift the CH_2 stretching modes to higher wavenumbers. With this principle phase transitions of lipids can be measured by measuring the position of the band as a function of temperature.

Another technique to characterize the behaviour and influence on lipids of monomeric and dimeric model peptides is solid state NMR. The basic principle through which information is derived in this type of NMR is the substantial influence of directionally dependent interactions in the solid state. This may be applied to determine the orientation and dynamics of model peptides. The first attempt to determine the orientation of a WALP peptide was by using the GALA-method.[59, 63, 112] With this method the tilt and rotation with respect to the bilayer is calculated from ^{2}H-NMR spectra of a set of WALP peptides with deuterated alanine residues on multiple positions along the backbone. It was discovered later that the calculated tilt was underestimated due to insufficient correction for the dynamics of the peptide. In all WALP-analogues synthesized in this thesis one alanine residue with a CD_3 side chain was incorporated and the shape of the recorded ^{2}H-NMR spectra was used exclusively as a fingerprint, determined by the orientation and extent and mode of motional averaging of the peptides. The multilamellar vesicles in which the peptides are incorporated can be characterized by ^{31}P-NMR.[113, 114] Lamellar, hexagonal or other phases yield spectra with a shape characteristic for the organisation of the lipids. The lamellar organization is characterized by a low field shoulder and a high field peak. Abnormalities in the vesicles will cause deformations in the spectra. The width or residual chemical shift anisotropy of the spectrum is characteristic for a specific lipid. For PC in the lamellar phase, usually a width of around 45 ppm is found. Information on the order of the acyl chains can be derived by measuring ^{2}H-NMR spectra of lipids with deuterated acyl chains.[68] When using *sn-2* chain perdeuteratured lipids, order parameter profiles can be obtained and bilayer thicknesses calculated.[68, 115, 116] In spectra of lipids with both chains perdeuterated the assignment of the peaks becomes complicated, but ordering of acyl chains can still be detected by possible changes in the full width at the half-height (FWHH) of the spectra.

Scope of this thesis

The aim of the research presented in this thesis is to investigate the influence of the cross sectional diameter and the corresponding helix-helix interactions of membrane proteins on their behaviour and lipid interactions. In literature, there are several indications that the diameter of the membrane spanning part of membrane proteins is important, but none are conclusive. In this thesis, transmembrane model peptides are employed to perform the, to our knowledge, first systematic study on the influence of cross sectional diameter on membrane protein properties and lipid interactions. This is approached by the synthesis and study of covalent assemblies of the so-called WALP peptides.

First, three WALP analogues were designed with a cysteine at the 11th, 12th or 13th position of the peptide, to obtain dimers with three different interfaces. In **chapter 2**, the synthesis and purification of covalent dimers of these analogues, their incorporation into lipid vesicles and the structural characterization of the monomers and dimers is described. All peptides incorporated properly into lipid bilayers and proved to form highly stable α-helices, as illustrated by CD measurements under several conditions. Then, in **chapter 3**, a study on the influence of dimerization on the structural stability, orientation, dynamics and lipid effects of the peptides is presented. The behaviour of the monomers and dimers and their influence on lipid acyl chains was investigated by ATR-FTIR, solid state NMR and simulations. Surprisingly, the tilt angle of the employed dimers with respect to the membrane normal seemed comparable to that of the monomers. However, dimerization did affect the dynamics of the peptides and their influence on lipids significantly.

Second, WALP analogues with azide or alkyne functionalities at the termini were designed to construct dimers with a forced parallel or antiparallel topology via click chemistry. The synthesis, purification, structural characterization and biophysical study results of these dimers are presented in **chapter 4**. It was demonstrated by CD and ATR-FTIR that the monomers as well as dimers formed well-defined and stable α-helices. Dichroic ATR-FTIR spectra showed that the dimers had an increased tilt with respect to the monomers and that there were possibly differences in tilt between the parallel and antiparallel dimers. Remarkably, with solid state NMR and ATR-FTIR, only minor ordering of lipid acyl chains could be detected and no differentiation for monomers versus dimers. The results differed markedly from the results for the dimers that were covalently coupled in the middle of the helix (chapter 3), suggesting an important role for helix-helix interactions.

The ambitious third objective was to obtain covalent tetramers of the WALP peptides and to analyze their behaviour and lipid interactions. For synthesis, the frequently applied TASP approach[99] was combined with the highly selective click reaction[90, 91]. Azide containing WALP analogues were designed to couple to a scaffold that was functionalized with four alkynes. In **chapter 5**, the explorative synthesis, characterization and purification of these assemblies is described. It was indicated by SDS-PAGE, MALDI-TOF and GPC that tetramers were formed. However, the covalent assemblies could not be isolated and therefore the protein-lipid interactions of these tetramers could not by studied.

From the studies of the two types of dimers, new insights were obtained on membrane protein behaviour and lipid interactions. The results and their implications are discussed in **chapter 6**.

References

1. Wallin, E. and von Heijne, G., Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms, *Protein Sci*, **1998**, 7(4), p. 1029-38
2. White, S. H., Biophysical dissection of membrane proteins, *Nature*, **2009**, 459(7245), p. 344-6
3. Lacapere, J. J., Pebay-Peyroula, E., Neumann, J. M., and Etchebest, C., Determining membrane protein structures: still a challenge!, *Trends Biochem Sci*, **2007**, 32(6), p. 259-70
4. Schuldiner, S., When biochemistry meets structural biology: the cautionary tale of EmrE, *Trends Biochem Sci*, **2007**, 32(6), p. 252-8
5. Langosch, D. and Arkin, I. T., Interaction and conformational dynamics of membrane-spanning protein helices, *Protein Sci*, **2009**, 18(7), p. 1343-58
6. Holt, A. and Killian, J. A., Orientation and dynamics of transmembrane peptides: the power of simple models, *Eur Biophys J*, **2009**, 39(4), p. 609-621
7. Biggin, P. C. and Sansom, M. S., Channel gating: twist to open, *Curr Biol*, **2001**, 11(9), p. R364-6
8. Sukharev, S., Betanzos, M., Chiang, C. S., and Guy, H. R., The gating mechanism of the large mechanosensitive channel MscL, *Nature*, **2001**, 409(6821), p. 720-4
9. Wegener, A. A., Chizhov, I., Engelhard, M., and Steinhoff, H. J., Time-resolved detection of transient movement of helix F in spin-labelled pharaonis sensory rhodopsin II, *J Mol Biol*, **2000**, 301(4), p. 881-91
10. Kusnetzow, A. K., Altenbach, C., and Hubbell, W. L., Conformational states and dynamics of rhodopsin in micelles and bilayers, *Biochemistry*, **2006**, 45(17), p. 5538-50
11. Page, R. C., Kim, S., and Cross, T. A., Transmembrane helix uniformity examined by spectral mapping of torsion angles, *Structure*, **2008**, 16(5), p. 787-97
12. Vos, W. L., Schor, M., Nazarov, P. V., Koehorst, R. B., Spruijt, R. B., and Hemminga, M. A., Structure of membrane-embedded M13 major coat protein is insensitive to hydrophobic stress, *Biophys J*, **2007**, 93(10), p. 3541-7
13. Franzin, C. M., Gong, X. M., Teriete, P., and Marassi, F. M., Structures of the FXYD regulatory proteins in lipid micelles and membranes, *J Bioenerg Biomembr*, **2007**, 39(5-6), p. 379-83
14. Siebert, F., Infrared spectroscopy applied to biochemical and biological problems, *Methods Enzymol*, **1995**, 246, p. 501-26
15. Otero-Cruz, J. D., Baez-Pagan, C. A., Caraballo-Gonzalez, I. M., and Lasalde-Dominicci, J. A., Tryptophan-scanning mutagenesis in the alphaM3 transmembrane domain of the muscle-type acetylcholine receptor. A spring model revealed, *J Biol Chem*, **2007**, 282(12), p. 9162-71
16. Jagannathan, B. and Golbeck, J. H., Breaking biological symmetry in membrane proteins: the asymmetrical orientation of PsaC on the pseudo-C2 symmetric Photosystem I core, *Cell Mol Life Sci*, **2009**, 66(7), p. 1257-70
17. Bohnert, M., Pfanner, N., and van der Laan, M., A dynamic machinery for import of mitochondrial precursor proteins, *FEBS Lett*, **2007**, 581(15), p. 2802-10
18. Evans, W. H., De Vuyst, E., and Leybaert, L., The gap junction cellular internet: connexin hemichannels enter the signalling limelight, *Biochem J*, **2006**, 397(1), p. 1-14
19. van Dalen, A., van der Laan, M., Driessen, A. J., Killian, J. A., and de Kruijff, B., Components required for membrane assembly of newly synthesized K+ channel KcsA, *FEBS Lett*, **2002**, 511(1-3), p. 51-8
20. Molina, M. L., Encinar, J. A., Barrera, F. N., Fernandez-Ballester, G., Riquelme, G., and Gonzalez-Ros, J. M., Influence of C-terminal protein domains and protein-lipid interactions on tetramerization and stability of the potassium channel KcsA, *Biochemistry*, **2004**, 43(47), p. 14924-31
21. van den Brink-van der Laan, E., Chupin, V., Killian, J. A., and de Kruijff, B., Stability of KcsA tetramer depends on membrane lateral pressure, *Biochemistry*, **2004**, 43(14), p. 4240-50
22. Williamson, I. M., Alvis, S. J., East, J. M., and Lee, A. G., The potassium channel KcsA and its interaction with the lipid bilayer, *Cell Mol Life Sci*, **2003**, 60(8), p. 1581-90
23. Sansom, M. S., Shrivastava, I. H., Bright, J. N., Tate, J., Capener, C. E., and Biggin, P. C., Potassium channels: structures, models, simulations, *Biochim Biophys Acta*, **2002**, 1565(2), p. 294-307
24. Barrera, F. N., Renart, M. L., Molina, M. L., Poveda, J. A., Encinar, J. A., Fernandez, A. M., Neira, J. L., and Gonzalez-Ros, J. M., Unfolding and refolding in vitro of a tetrameric, alpha-helical membrane protein: the prokaryotic potassium channel KcsA, *Biochemistry*, **2005**, 44(43), p. 14344-52
25. Neagoe, I. and Schwappach, B., Pas de deux in groups of four--the biogenesis of KATP channels, *J Mol Cell Cardiol*, **2005**, 38(6), p. 887-94
26. Groemping, Y. and Rittinger, K., Activation and assembly of the NADPH oxidase: a structural perspective, *Biochem J*, **2005**, 386(Pt 3), p. 401-16
27. Graham, L. A., Flannery, A. R., and Stevens, T. H., Structure and assembly of the yeast V-ATPase, *J Bioenerg Biomembr*, **2003**, 35(4), p. 301-12
28. Tatulian, S. A. and Tamm, L. K., Secondary structure, orientation, oligomerization, and lipid interactions of the transmembrane domain of influenza hemagglutinin, *Biochemistry*, **2000**, 39(3), p. 496-507
29. Lee, A. G., How lipids affect the activities of integral membrane proteins, *Biochim Biophys Acta*, **2004**, 1666(1-2), p. 62-87
30. Poolman, B., Spitzer, J. J., and Wood, J. M., Bacterial osmosensing: roles of membrane structure and electrostatics in lipid-protein and protein-protein interactions, *Biochim Biophys Acta*, **2004**, 1666(1-2), p. 88-104
31. Lee, A. G., Lipid-protein interactions in biological membranes: a structural perspective, *Biochim Biophys Acta*, **2003**, 1612(1), p. 1-40
32. van den Brink-van der Laan, E., Killian, J. A., and de Kruijff, B., Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile, *Biochim Biophys Acta*, **2004**, 1666(1-2), p. 275-88
33. Sukharev, S., Mechanosensitive channels in bacteria as membrane tension reporters, *Faseb J*, **1999**, 13 Suppl, p. S55-61
34. Nyholm, T. K., Ozdirekcan, S., and Killian, J. A., How protein transmembrane segments sense the lipid environment, *Biochemistry*, **2007**, 46(6), p. 1457-65
35. Botelho, A. V., Huber, T., Sakmar, T. P., and Brown, M. F., Curvature and hydrophobic forces drive oligomerization and modulate activity of rhodopsin in membranes, *Biophys J*, **2006**, 91(12), p. 4464-77
36. Froud, R. J., Earl, C. R., East, J. M., and Lee, A. G., Effects of lipid fatty acyl chain structure on the activity of the (Ca2+ + Mg2+)-ATPase, *Biochim Biophys Acta*, **1986**, 860(2), p. 354-60
37. Montecucco, C., Smith, G. A., Dabbeni-sala, F., Johannsson, A., Galante, Y. M., and Bisson, R., Bilayer thickness and enzymatic activity in the mitochondrial cytochrome c oxidase and ATPase complex, *FEBS Lett*, **1982**, 144(1), p. 145-8
38. Cornelius, F., Modulation of Na,K-ATPase and Na-ATPase activity by phospholipids and cholesterol. I. Steady-state kinetics, *Biochemistry*, **2001**, 40(30), p. 8842-51
39. Marsh, D., Protein modulation of lipids, and vice-versa, in membranes, *Biochim Biophys Acta*, **2008**, 1778(7-8), p. 1545-75
40. George, R., Lewis, R. N., and McElhaney, R. N., Studies on the purified Na+,Mg(2+)-ATPase from Acholeplasma laidlawii B membranes: a differential scanning calorimetric study of the protein-phospholipid interactions, *Biochem Cell Biol*, **1990**, 68(1), p. 161-8
41. Papahadjopoulos, D., Moscarello, M., Eylar, E. H., and Isac, T., Effects of proteins on thermotropic phase transitions of phospholipid membranes, *Biochim Biophys Acta*, **1975**, 401(3), p. 317-35
42. Morein, S., Killian, J. A., and Sperotto, M. M., Characterization of the thermotropic behavior and lateral organization of lipid-peptide mixtures by a combined experimental and theoretical approach: effects of hydrophobic mismatch and role of flanking residues, *Biophys J*, **2002**, 82(3), p. 1405-17

43. Blazyk, J. F. and Newman, J. L., Calorimetric studies of lipid phase transitions in native and heat-denatured membranes of beef heart submitochondrial particles, *Biochim Biophys Acta*, **1980**, 600(3), p. 1007-11
44. Taraschi, T. F., de Kruijff, B., and Verkleij, A. J., The effect of an integral membrane protein on lipid polymorphism in the cardiolipin-Ca2+ system, *Eur J Biochem*, **1983**, 129(3), p. 621-5
45. Pelham, H. R. and Munro, S., Sorting of membrane proteins in the secretory pathway, *Cell*, **1993**, 75(4), p. 603-5
46. Munro, S., Localization of proteins to the Golgi apparatus, *Trends Cell Biol*, **1998**, 8(1), p. 11-5
47. Wallace, E. J., Hooper, N. M., and Olmsted, P. D., Effect of hydrophobic mismatch on phase behavior of lipid membranes, *Biophys J*, **2006**, 90(11), p. 4104-18
48. Dumas, F., Sperotto, M. M., Lebrun, M. C., Tocanne, J. F., and Mouritsen, O. G., Molecular sorting of lipids by bacteriorhodopsin in dilauroylphosphatidylcholine/distearoylphosphatidylcholine lipid bilayers, *Biophys J*, **1997**, 73(4), p. 1940-53
49. Mouritsen, O. G. and Bloom, M., Mattress model of lipid-protein interactions in membranes, *Biophys J*, **1984**, 46(2), p. 141-53
50. de Planque, M. R., Kruijtzer, J. A., Liskamp, R. M., Marsh, D., Greathouse, D. V., Koeppe, R. E., 2nd, de Kruijff, B., and Killian, J. A., Different membrane anchoring positions of tryptophan and lysine in synthetic transmembrane alpha-helical peptides, *J Biol Chem*, **1999**, 274(30), p. 20839-46
51. de Planque, M. R., Bonev, B. B., Demmers, J. A., Greathouse, D. V., Koeppe, R. E., 2nd, Separovic, F., Watts, A., and Killian, J. A., Interfacial anchor properties of tryptophan residues in transmembrane peptides can dominate over hydrophobic matching effects in peptide-lipid interactions, *Biochemistry*, **2003**, 42(18), p. 5341-8
52. Demmers, J. A., van Duijn, E., Haverkamp, J., Greathouse, D. V., Koeppe, R. E., 2nd, Heck, A. J., and Killian, J. A., Interfacial positioning and stability of transmembrane peptides in lipid bilayers studied by combining hydrogen/deuterium exchange and mass spectrometry, *J Biol Chem*, **2001**, 276(37), p. 34501-8
53. de Planque, M. R., Goormaghtigh, E., Greathouse, D. V., Koeppe, R. E., 2nd, Kruijtzer, J. A., Liskamp, R. M., de Kruijff, B., and Killian, J. A., Sensitivity of single membrane-spanning alpha-helical peptides to hydrophobic mismatch with a lipid bilayer: effects on backbone structure, orientation, and extent of membrane incorporation, *Biochemistry*, **2001**, 40(16), p. 5000-10
54. Ganchev, D. N., Rijkers, D. T. S., Snel, M. M., Killian, J. A., and de Kruijff, B., Strength of integration of transmembrane alpha-helical peptides in lipid bilayers as determined by atomic force spectroscopy, *Biochemistry*, **2004**, 43(47), p. 14987-93
55. Demmers, J. A., Haverkamp, J., Heck, A. J., Koeppe, R. E., 2nd, and Killian, J. A., Electrospray ionization mass spectrometry as a tool to analyze hydrogen/deuterium exchange kinetics of transmembrane peptides in lipid bilayers, *Proc Natl Acad Sci U S A*, **2000**, 97(7), p. 3189-94
56. Lewis, B. A. and Engelman, D. M., Lipid bilayer thickness varies linearly with acyl chain length in fluid phosphatidylcholine vesicles, *J Mol Biol*, **1983**, 166(2), p. 211-7
57. Killian, J. A., Synthetic peptides as models for intrinsic membrane proteins, *FEBS Lett*, **2003**, 555(1), p. 134-8
58. Killian, J. A. and von Heijne, G., How proteins adapt to a membrane-water interface, *Trends Biochem Sci*, **2000**, 25(9), p. 429-34
59. Strandberg, E., Ozdirekcan, S., Rijkers, D. T. S., van der Wel, P. C., Koeppe, R. E., 2nd, Liskamp, R. M., and Killian, J. A., Tilt angles of transmembrane model peptides in oriented and non-oriented lipid bilayers as determined by 2H solid-state NMR, *Biophys J*, **2004**, 86(6), p. 3709-21
60. Holt, A., Rougier, L., Reat, V., Jolibois, F., Saurel, O., Czaplicki, J., Killian, J. A., and Milon, A., Order parameters of a transmembrane helix in a fluid bilayer: case study of a WALP peptide, *Biophys J*, **2010**, 98(9), p. 1864-72
61. Holt, A., Koehorst, R. B., Rutters-Meijneke, T., Gelb, M. H., Rijkers, D. T. S., Hemminga, M. A., and Killian, J. A., Tilt and rotation angles of a transmembrane model peptide as studied by fluorescence spectroscopy, *Biophys J*, **2009**, 97(8), p. 2258-66
62. Ozdirekcan, S., Etchebest, C., Killian, J. A., and Fuchs, P. F., On the orientation of a designed transmembrane peptide: toward the right tilt angle?, *J Am Chem Soc*, **2007**, 129(49), p. 15174-81
63. Ozdirekcan, S., Rijkers, D. T. S., Liskamp, R. M., and Killian, J. A., Influence of flanking residues on tilt and rotation angles of transmembrane peptides in lipid bilayers. A solid-state 2H NMR study, *Biochemistry*, **2005**, 44(3), p. 1004-12
64. Andronesi, O. C., Pfeifer, J. R., Al-Momani, L., Ozdirekcan, S., Rijkers, D. T. S., Angerstein, B., Luca, S., Koert, U., Killian, J. A., and Baldus, M., Probing membrane protein orientation and structure using fast magic-angle-spinning solid-state NMR, *J Biomol NMR*, **2004**, 30(3), p. 253-65
65. Sparr, E., Ganchev, D. N., Snel, M. M., Ridder, A. N., Kroon-Batenburg, L. M., Chupin, V., Rijkers, D. T. S., Killian, J. A., and de Kruijff, B., Molecular organization in striated domains induced by transmembrane alpha-helical peptides in dipalmitoyl phosphatidylcholine bilayers, *Biochemistry*, **2005**, 44(1), p. 2-10
66. Scarpelli, F., Drescher, M., Rutters-Meijneke, T., Holt, A., Rijkers, D. T. S., Killian, J. A., and Huber, M., Aggregation of transmembrane peptides studied by spin-label EPR, *J Phys Chem B*, **2009**, 113(36), p. 12257-64
67. Sparr, E., Ash, W. L., Nazarov, P. V., Rijkers, D. T. S., Hemminga, M. A., Tieleman, D. P., and Killian, J. A., Self-association of Transmembrane {alpha}-Helices in Model Membranes: IMPORTANCE OF HELIX ORIENTATION AND ROLE OF HYDROPHOBIC MISMATCH, *J Biol Chem*, **2005**, 280(47), p. 39324-39331
68. de Planque, M. R., Greathouse, D. V., Koeppe, R. E., 2nd, Schafer, H., Marsh, D., and Killian, J. A., Influence of lipid/peptide hydrophobic mismatch on the thickness of diacylphosphatidylcholine bilayers. A 2H NMR and ESR study using designed transmembrane alpha-helical peptides and gramicidin A, *Biochemistry*, **1998**, 37(26), p. 9333-45
69. de Planque, M. R. and Killian, J. A., Protein-lipid interactions studied with designed transmembrane peptides: role of hydrophobic matching and interfacial anchoring, *Mol Membr Biol*, **2003**, 20(4), p. 271-84
70. Strandberg, E., Morein, S., Rijkers, D. T. S., Liskamp, R. M. J., van der Wel, P. C., and Killian, J. A., Lipid dependence of membrane anchoring properties and snorkeling behavior of aromatic and charged residues in transmembrane peptides, *Biochemistry*, **2002**, 41(23), p. 7190-8
71. Harroun, T. A., Heller, W. T., Weiss, T. M., Yang, L., and Huang, H. W., Experimental evidence for hydrophobic matching and membrane-mediated interactions in lipid bilayers containing gramicidin, *Biophys J*, **1999**, 76(2), p. 937-45
72. Weiss, T. M., van der Wel, P. C., Killian, J. A., Koeppe, R. E., 2nd, and Huang, H. W., Hydrophobic mismatch between helices and lipid bilayers, *Biophys J*, **2003**, 84(1), p. 379-85
73. Killian, J. A., Hydrophobic mismatch between proteins and lipids in membranes, *Biochim Biophys Acta*, **1998**, 1376(3), p. 401-15
74. Sperotto, M. M., Ipsen, J. H., and Mouritsen, O. G., Theory of protein-induced lateral phase separation in lipid membranes, *Cell Biophys*, **1989**, 14(1), p. 79-95
75. Riegler, J. and Mohwald, H., Elastic Interactions of Photosynthetic Reaction Center Proteins Affecting Phase Transitions and Protein Distributions, *Biophys J*, **1986**, 49(6), p. 1111-1118
76. Piknova, B., Perochon, E., and Tocanne, J. F., Hydrophobic mismatch and long-range protein/lipid interactions in bacteriorhodopsin/phosphatidylcholine vesicles, *Eur J Biochem*, **1993**, 218(2), p. 385-96
77. van Duyl, B. Y., Rijkers, D. T. S., de Kruijff, B., and Killian, J. A., Influence of hydrophobic mismatch and palmitoylation on the association of transmembrane alpha-helical peptides with detergent-resistant membranes, *FEBS Lett*, **2002**, 523(1-3), p. 79-84
78. Ridder, A. N., Spelbrink, R. E., Demmers, J. A., Rijkers, D. T.

S., Liskamp, R. M. J., Brunner, J., Heck, A. J., de Kruijff, B., and Killian, J. A., Photo-crosslinking analysis of preferential interactions between a transmembrane peptide and matching lipids, *Biochemistry*, **2004**, 43(15), p. 4482-9
79. Fernandes, F., Loura, L. M., Prieto, M., Koehorst, R., Spruijt, R. B., and Hemminga, M. A., Dependence of M13 major coat protein oligomerization and lateral segregation on bilayer composition, *Biophys J*, **2003**, 85(4), p. 2430-41
80. Martens, J. R., O'Connell, K., and Tamkun, M., Targeting of ion channels to membrane microdomains: localization of KV channels to lipid rafts, *Trends Pharmacol Sci*, **2004**, 25(1), p. 16-21
81. Briggs, J. A., Wilk, T., and Fuller, S. D., Do lipid rafts mediate virus assembly and pseudotyping?, *J Gen Virol*, **2003**, 84(Pt 4), p. 757-68
82. Ramadurai, S., Holt, A., Krasnikov, V., van den Bogaart, G., Killian, J. A., and Poolman, B., Lateral diffusion of membrane proteins, *J Am Chem Soc*, **2009**, 131(35), p. 12650-6
83. Saffman, P. G. and Delbruck, M., Brownian motion in biological membranes, *Proc Natl Acad Sci U S A*, **1975**, 72(8), p. 3111-3
84. Kol, M. A., van Dalen, A., de Kroon, A. I., and de Kruijff, B., Translocation of phospholipids is facilitated by a subset of membrane-spanning proteins of the bacterial cytoplasmic membrane, *J Biol Chem*, **2003**, 278(27), p. 24586-93
85. Wu, J. and Kaback, H. R., A general method for determining helix packing in membrane proteins in situ: helices I and II are close to helix VII in the lactose permease of Escherichia coli, *Proc Natl Acad Sci U S A*, **1996**, 93(25), p. 14498-502
86. van Montfort, B. A., Schuurman-Wolters, G. K., Wind, J., Broos, J., Robillard, G. T., and Poolman, B., Mapping of the dimer interface of the Escherichia coli mannitol permease by cysteine cross-linking, *J Biol Chem*, **2002**, 277(17), p. 14717-23
87. Soskine, M., Steiner-Mordoch, S., and Schuldiner, S., Crosslinking of membrane-embedded cysteines reveals contact points in the EmrE oligomer, *Proc Natl Acad Sci U S A*, **2002**, 99(19), p. 12043-8
88. Audia, J. P., Roberts, R. A., and Winkler, H. H., Cysteine-scanning mutagenesis and thiol modification of the Rickettsia prowazekii ATP/ADP translocase: characterization of TMs IV-VII and IX-XII and their accessibility to the aqueous translocation pathway, *Biochemistry*, **2006**, 45(8), p. 2648-56
89. Schwem, B. E. and Fillingame, R. H., Cross-linking between helices within subunit a of Escherichia coli ATP synthase defines the transmembrane packing of a four-helix bundle, *J Biol Chem*, **2006**, 281(49), p. 37861-7
90. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B., A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes, *Angew Chem Int Ed Engl*, **2002**, 41(14), p. 2596-9
91. Tornoe, C. W., Christensen, C., and Meldal, M., Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides, *J Org Chem*, **2002**, 67(9), p. 3057-64
92. Hein, C. D., Liu, X. M., and Wang, D., Click chemistry, a powerful tool for pharmaceutical sciences, *Pharm Res*, **2008**, 25(10), p. 2216-30
93. Lutz, J. F. and Zarafshani, Z., Efficient construction of therapeutics, bioconjugates, biomaterials and bioactive surfaces using azide-alkyne "click" chemistry, *Adv Drug Deliv Rev*, **2008**, 60(9), p. 958-70
94. Haberkant, P. and van Meer, G., Protein-lipid interactions: paparazzi hunting for snap-shots, *Biol Chem*, **2009**, 390(8), p. 795-803
95. de Graaf, A. J., Kooijman, M., Hennink, W. E., and Mastrobattista, E., Nonnatural amino acids for site-specific protein conjugation, *Bioconjug Chem*, **2009**, 20(7), p. 1281-95
96. Dumy, P., Eggleston, I. M., Esposito, G., Nicula, S., and Mutter, M., Solution structure of regioselectively addressable functionalized templates: an NMR and restrained molecular dynamics investigation, *Biopolymers*, **1996**, 39(3), p. 297-308
97. Dumy, P., Eggleston, I. M., Cervigni, S., Sila, U., Sun, X., and Mutter, M., A convenient synthesis of cyclic peptides as regioselectively addressable functionalized templates (RAFT), *Tetrahedron Letters*, **1995**, 36(8), p. 1255-1258
98. Tuchscherer, G., Scheibler, L., Dumy, P., and Mutter, M., Protein design: on the threshold of functional properties, *Biopolymers*, **1998**, 47(1), p. 63-73
99. Mutter, M. and Tuchscherer, G., Non-native architectures in protein design and mimicry, *Cell Mol Life Sci*, **1997**, 53(11-12), p. 851-63
100. Nyanguile, O., Mutter, M., and Tuchscherer, G., Synthesis of antiparallel 4a-helix bundle TASP by chemoselective ligation, *Letters in Peptide Science*, **1994**, 1, p. 9-16
101. Grove, A., Mutter, M., Rivier, J. E., and Montal, M., Template-assembled synthetic proteins designed to adopt a globular, four-helix bundle conformation form ionic channels in lipid bilayers, *J Am Chem Soc*, **1993**, 115, p. 5919-5924
102. Becker, C. F., Oblatt-Montal, M., Kochendoerfer, G. G., and Montal, M., Chemical synthesis and single channel properties of tetrameric and pentameric TASPs (template-assembled synthetic proteins) derived from the transmembrane domain of HIV virus protein u (Vpu), *J Biol Chem*, **2004**, 279(17), p. 17483-9
103. Bayley, H., Designed membrane channels and pores, *Curr Opin Biotechnol*, **1999**, 10(1), p. 94-103
104. Matsubara, A., Asami, K., Akagi, A., and Nishino, N., Ion-channels of cyclic template-assembled alamethicins that emulate the pore structure predicted by the barrel-stave model, *Chemical Communications*, **1996**, 17, p. 2069
105. Greenfield, N. J., Applications of circular dichroism in protein and peptide analysis, *Trends Anal Chem*, **1999**, 18(4), p. 236
106. Kelly, S. M., Jess, T. J., and Price, N. C., How to study proteins by circular dichroism, *Biochim Biophys Acta*, **2005**, 1751(2), p. 119-39
107. Kelly, S. M. and Price, N. C., The application of circular dichroism to studies of protein folding and unfolding, *Biochim Biophys Acta*, **1997**, 1338(2), p. 161-85
108. Booth, P. J. and Curnow, P., Folding scene investigation: membrane proteins, *Curr Opin Struct Biol*, **2009**, 19(1), p. 8-13
109. Goormaghtigh, E., Ruysschaert, J. M., and Raussens, V., Evaluation of the information content in infrared spectra for protein secondary structure determination, *Biophys J*, **2006**, 90(8), p. 2946-57
110. Vigano, C., Manciu, L., Buyse, F., Goormaghtigh, E., and Ruysschaert, J. M., Attenuated total reflection IR spectroscopy as a tool to investigate the structure, orientation and tertiary structure changes in peptides and membrane proteins, *Biopolymers*, **2000**, 55(5), p. 373-80
111. Bechinger, B., Ruysschaert, J. M., and Goormaghtigh, E., Membrane helix orientation from linear dichroism of infrared attenuated total reflection spectra, *Biophys J*, **1999**, 76(1 Pt 1), p. 552-63
112. van der Wel, P. C., Strandberg, E., Killian, J. A., and Koeppe, R. E., 2nd, Geometry and intrinsic tilt of a tryptophan-anchored transmembrane alpha-helix determined by (2)H NMR, *Biophys J*, **2002**, 83(3), p. 1479-88
113. Killian, J. A., Salemink, I., de Planque, M. R., Lindblom, G., Koeppe, R. E., 2nd, and Greathouse, D. V., Induction of nonbilayer structures in diacylphosphatidylcholine model membranes by transmembrane alpha-helical peptides: importance of hydrophobic mismatch and proposed role of tryptophans, *Biochemistry*, **1996**, 35(3), p. 1037-45
114. Harzer, U. and Bechinger, B., Alignment of lysine-anchored membrane peptides under conditions of hydrophobic mismatch: a CD, 15N and 31P solid-state NMR spectroscopy investigation, *Biochemistry*, **2000**, 39(43), p. 13106-14
115. Davis, J. H., The description of membrane lipid conformation, order and dynamics by 2H-NMR, *Biochim Biophys Acta*, **1983**, 737(1), p. 117-71
116. Petrache, H. I., Dodd, S. W., and Brown, M. F., Area per lipid and acyl length distributions in fluid phosphatidylcholines determined by (2)H NMR spectroscopy, *Biophys J*, **2000**, 79(6), p. 3172-92

2

Dimeric assemblies of transmembrane model peptides; Design, synthesis, purification and characterization

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Abstract

To study the influence of cross sectional diameter on the behaviour and lipid interactions of membrane proteins, a systematic approach is required. A model system of synthetic α-helices in lipid vesicles would enable this requirement. Here, so-called WALP peptides (Ac-GWW$(LA)_8$LWWA-NH_2) are used as models for transmembrane segments of natural membrane proteins. To be able to study the influence of oligomerization on protein-lipid interactions, as first step, WALP analogues were designed and synthesized to make covalent dimers. It was shown that pure dimers in lipid vesicles can best be obtained by first synthesizing and purifying dimers and second incorporating them into vesicles. The secondary structure and stability of monomers and dimers was established by CD-measurements.

Introduction

Membrane proteins are an abundant and important class of proteins. Roughly one out of four proteins that is encoded for by DNA is a membrane protein.[1] They play major roles in cellular processes like signalling, energy transduction and cell-cell recognition. Because they are the 'gatekeepers' of our cells, it is not surprising that they are targets for the majority of newly developed drugs.[2, 3] Despite their significant role in health and disease there is not so much known about membrane proteins, mainly because of the problems due to their hydrophobic nature. Especially when compared to globular proteins, there are only few crystal structures available.[4, 5] In addition, these crystal structures are static, while dynamics and interactions with the surrounding lipids are usually important for membrane protein function.

To learn more about the role of membrane proteins in health and disease knowledge has to be obtained on the molecular mechanisms by which they function. As stated before, structure and function of these hydrophobic membrane proteins can be highly influenced by the surrounding lipids.[6] To study the fundamentally important protein-lipid interactions often simplified systems have to be used. A first step towards a less complicated system is the investigation of purified proteins reconstituted in synthetic bilayers.[7-11] One step further is the use of model systems, such as the WALP peptides (Ac-GWW$(LA)_n$LWWA-NH_2) in vesicles of varying lipid composition. The advantage of using these model systems is that properties of peptides and lipids can both be systematically varied. The α-helical WALP peptides mimic the features of natural membrane proteins and their behaviour in lipid bilayers has been well characterized.[12-14] However, most proteins have multiple membrane spanning segments. To better mimic this situation in simple model systems, we synthesized, as a first step, cysteine-containing WALP peptides that can be oxidized to form covalent dimers. Here we will describe the design, synthesis, purification and characterization of these dimers.

Three WALP analogues, designated as C11-, C12- and C13-WALP, with cysteine residues at varying positions along the putative α-helix (Figure 1) were used to obtain dimers. Two methods of obtaining dimers from these cysteine containing WALP peptides can be envisaged. Monomers can be incorporated in vesicles and then dimerized. This would be a convenient method, because the incorporation of monomers is well established.[15] Nevertheless, there is a risk that not all WALP peptides will form dimers, so a mix of monomers and dimers in the bilayer will result. To circumvent this, dimers can be first synthesized and purified in solution and subsequently incorporated in vesicles. In this way, pure dimers can be obtained, so there is no risk of the presence of unreacted monomers in the bilayer. However, a drawback is that the (extent of) inclusion of a dimer in a bilayer has not been characterized so far.

In this chapter we report on the synthesis and characterization of the WALP analogues and the dimerization of the monomers in a bilayer and in solution. It will be shown that dimerization in solution and subsequent incorporation of the dimers in a bilayer is the best method to obtain a sample for comparison of monomers and dimers. The secondary structure and stability of all peptides was established using circular dichroism. The peptides proved to be highly stable α-helices in DDM, SDS and DMPC.

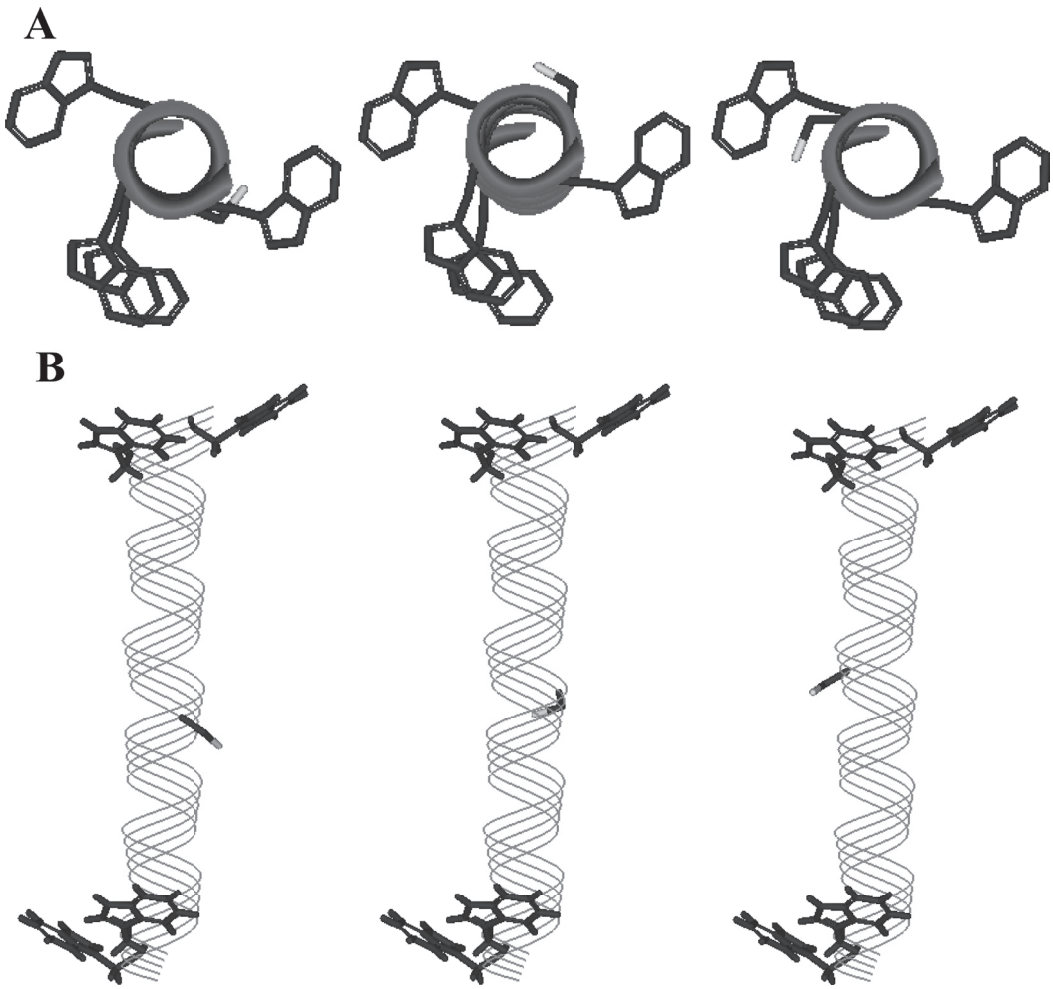


Figure 1. Top view (A) and side view (B) of the C11-WALP (left), C12-WALP (middle) and C13-WALP (right) with the tryptophan residues at the termini and the cysteine residues in the middle of the helices represented as sticks.

Results

Design of the monomers

Three WALP analogues were designed with a cysteine at the 11th, 12th or 13th position in the amino acid sequence (Table 1). These positions (Figure 1) were chosen for several reasons. From previous research there are indications that the orientation of the helices with respect to each other might be preferentially antiparallel.[16] By placing the position of the covalent bond in the middle of the helix the preferential orientation (parallel or antiparallel) of all three WALP analogues in the bilayer is not expected to be important for the dimerization. Furthermore the cysteine residues are all at a different face of the helix, but the depth of the residues in the membrane does not differ significantly. In this way, a possible preferential helix-helix interface can be determined. In addition, if there is a preferential interface, at least one of the combinations of model-peptides should be able to form dimers.

Table 1. WALP analogues designed to synthesize covalent dimers

Peptide	Sequence
C11-WALP	Ac-GWWLALALAL**C**LALALALALWWA-NH_2
C12-WALP	Ac-GWWLALALALA**C**ALALALALWWA-NH_2
C13-WALP	Ac-GWWLALALALAL**C**LALALALWWA-NH_2

Synthesis of the monomers

The cysteine containing WALP analogues were synthesized by manual solid phase peptide synthesis on a Tentagel S-RAM resin, using an Fmoc-strategy,[17] checking couplings and deprotections with the Kaiser test[18]. ESI-MS analysis showed that the analogues were successfully synthesized. Only the molecular weights of the products were found (Table 2). Besides the $[M+2H]^{2+}$ peaks, also some sodium and potassium adducts were detected. The characterization of the peptide with HPLC seemed less conclusive at first. Each WALP analogue showed a broad signal of four peaks, as illustrated in Figure 2 for the C11-WALP. There were no packing defects in the column, because other peptides did show sharp peaks on the same column and the peak pattern of the C11-WALP was comparable to that obtained with another column with similar properties (data not shown). It is also unlikely that the other peaks were from shorter peptides (deletions), formed during synthesis, because these would be detected by ESI-MS, but only the mass of the product was found. The presence of covalent dimers formed during synthesis or cleavage could cause one of the four peaks. To explore this possibility, the C11-, C12- and C13-WALP peptides were run on a tris-tricine gel. Only one band at monomer height was visible, thus no dimers were present (data not shown). In addition, the peptides were treated with DTT prior to the HPLC run, to reduce possible disulfide bonds. If the formation of dimers was the cause of (one of) the four peaks the HPLC spectrum should be altered after DTT-treatment, but the spectrum was identical to the spectrum of the non-treated WALP analogues (data not shown). Finally, a possible cause for the presence of the multiple peaks is that the peptides are present in multiple conformational and/or aggregational states. As a control, a preparative HPLC-run of the C11-WALP was performed. The four peaks were collected in four fractions and characterized by ESI-MS and HPLC. All peaks showed solely the molecular weights of the C11-WALP with ESI-MS. In HPLC, if all peaks represented another peptide-species it would be expected that in an analytical run of the first fraction, peak one would be enriched, in an analytical run of the second fraction, peak 2 would be enriched, etc. However, the HPLC of all four fractions showed an enrichment of peak 3. This indicated that the position of the peak(s) only depends on environment (the peptide was now injected directly from the preparative fraction, thus from another solvent). This supports the presence of

multiple aggregational states of the C11-WALP as the origin of the multiple peaks.

Table 2. ESI-MS analysis of WALP analogues

Peptide	$[M+2H]^{2+}$ (calculated)	$[M+2H]^{2+}$ (found)
C11-WALP	1278.08	1278.33
C12-WALP	1257.04	1257.33
C13-WALP	1278.08	1278.39

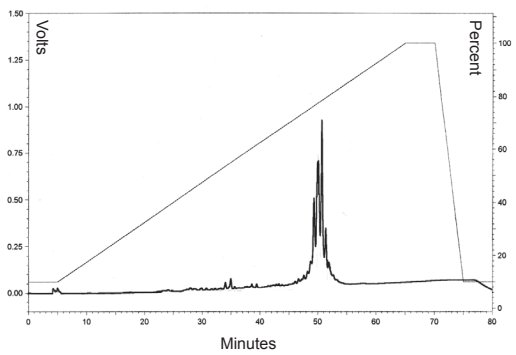


Figure 2. HPLC spectrum of C11-WALP injected from a solution in trifluoroethanol

Synthesis of dimers in a bilayer

First, obtaining a sample of dimers in a bilayer was attempted by introducing monomers in a bilayer and oxidizing these to dimers. For this purpose, peptide containing multilamellar vesicles were prepared and incubated at 40°C overnight. The oxidizing agent in this case was plainly oxygen from the air. As can be seen in Figure 3A, almost no dimers were formed when oxidizing the C11-WALP in a bilayer of DOPC, not even at a P/L ratio as high as 1:5. The same result was obtained with the C12- and C13-WALP (data not shown). In Figure 3B it can be seen that potassium hexacyanoferrate(II) and copper(II) chloride enhanced dimerization. Also DMSO and iodine were shown to have a catalytic function (data not shown). However, with none of the catalysts, pure dimers could be obtained. In addition, oxidizing catalysts have to be used with care, since the tryptophan might be sensitive to some agents. For example, it was shown for the sample with hexacyanoferrate(II) that the absorption spectrum of tryptophan was altered after incubation.

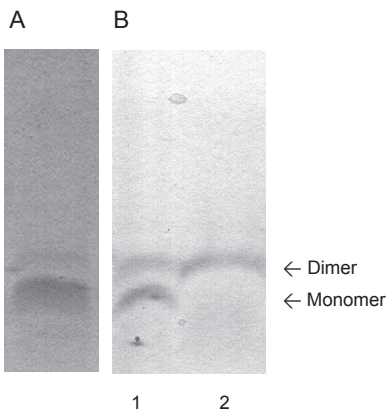


Figure 3. Tricine-SDS-PAGE of C11-WALP/DOPC, 1:5 (A) and C13-WALP/DOPC, 1:25 (B) with hexacyanoferrate(II) (lane 1) and copper(II)chloride (lane 2) as catalysts

To establish the amount of dimers formed more quantitatively, to determine a possible preferential interface, HPLC was used (see next paragraph for validation). The results of several dimerization reactions in multilamellar vesicles with and without catalyst are shown in Table 3 for the three cysteine-containing WALP analogues. As was already illustrated by tris-tricine gel electrophoresis, also with HPLC it was found that almost no dimers were formed when only using oxygen from the air as catalyst. With DMSO the dimerization was slightly enhanced. With $CuCl_2$ as a catalyst a slight differentiation in dimerization of the different analogues seemed to occur. However, this was not reproducible and more likely related to small variations due to the experimental procedure, like the extent to which the cysteine residues were reached by the catalyst (see concluding discussion). Thus, with this method, no preferential interface could be detected.

Table 3. Percentage of dimers formed with the different WALP analogues in DOPC vesicles in an aqueous buffer, with a P/L ratio of 1:25, with and without extra catalyst, according to HPLC

Peptide	No catalyst	1% DMSO	0.05 mM $CuCl_2$
C11-WALP	2	9	17
C12-WALP	5	8	20
C13-WALP	5	10	11

Synthesis of dimers in solution

To obtain pure dimeric assemblies in a bilayer, dimers were first prepared and purified and then incorporated. Dimers could be obtained by simply dissolving the peptides in TFE, adding a little TEA and stirring at 40°C for one week. Dimerization was characterized by HPLC, using a C4 column with buffers and a gradient designed especially for these hydrophobic peptides. In Figure 4A the HPLC spectrum of a C11-WALP that has been partly oxidized to form dimers is shown. As can be seen, a group of peaks appears at a higher retention time than that of the monomers. To confirm that the upcoming peaks originated from dimers, DTT was added to the sample. This reducing agent should and indeed did decrease the intensity of the peaks at higher retention time (Figure 4B). Mass spectrometry on these large hydrophobic molecules without any charge, was not possible. However, the formation of dimers as observed with HPLC and the purification by preparative HPLC was confirmed by gel electrophoresis (Figure 5). The yields of pure dimer, starting from monomer, were on average around 20%. The HPLC spectra of the dimers did not change over time, indicating that the assemblies were stable (data not shown).

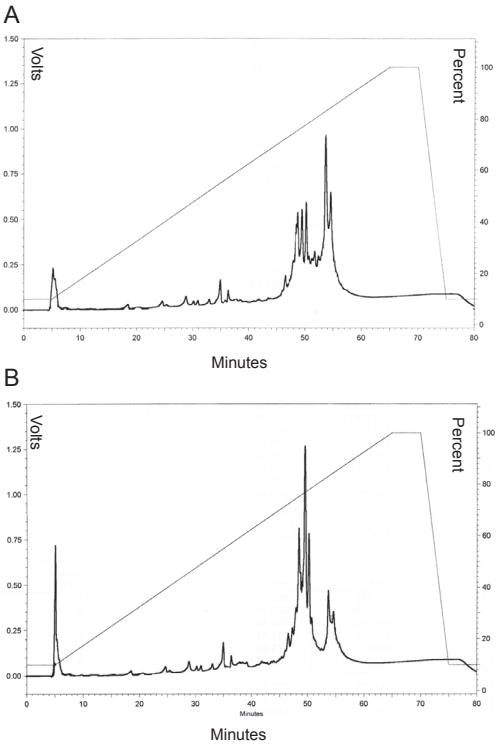


Figure 4. HPLC spectra of C11-WALP after an oxidation reaction (A) and after reduction with DTT of the products of the oxidation reaction (B)

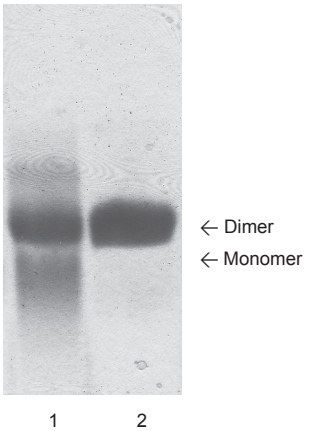


Figure 5. Tricine-SDS-PAGE of C11-WALP after an oxidation reaction (lane 1) and after HPLC purification (lane 2)

Structural characterization of monomers and dimers

A convenient method for the structural characterization of the monomers and dimers is checking their secondary structure by circular dichroism (CD). As a first step, the peptides were measured in SDS. In Figure 6A the CD-spectra of the C11-WALP monomer and dimer in SDS are shown. As can be seen from the minima at 222 and 208 nm and the maximum at 192 nm, both peptides form well-defined α-helices. Equal results were obtained for the C12- and C13-WALP dimers and their monomeric counterparts. The stability of the α-helices was tested by treating the peptides in SDS with various concentrations of the denaturants urea or guanidine hydrochloride. A typical example of the stability of the secondary structure of the peptides is shown in Figure 6B. The spectrum of the C11-WALP dimer in SDS was unaltered after treatment with 5 M urea and even after incubation of the urea-sample at 90°C for one hour, illustrating that the peptide stayed α-helical. Similarly, all monomers and dimers proved to be extremely stable, staying α-helical under all conditions used. The secondary structure and stability of all monomers and dimers was also determined in DDM. SDS is a strong detergent, usually breaking intermolecular interactions, but stabilizing α-helical secondary structures. DDM is a 'weaker' detergent, which usually leaves intermolecular interactions intact. Therefore, the stabilizing effect of DDM is probably less than that of the SDS.[19] Remarkably, the CD-spectra of all peptides in DDM, with and without denaturant were similar to that in SDS (data not shown).

To determine the secondary structure of all peptides in a lipid environment, the monomers and dimers were incorporated in large unilamellar vesicles (LUVs) of saturated lipids. The peptide concentration after extrusion was determined by absorption spectroscopy (see experimental section), to correct the CD-spectra. Typically, between 20 and 30% of the peptides was lost. All peptides were α-helical in DMPC, as illustrated for the C11-WALP monomer and dimer in Figure 7. The region from 190-200 nm was disregarded, because the concentration of the samples with dimer was too high, giving artefacts in the detection in this region. The influence of denaturants on the peptides was also tested in DMPC. Again, all monomers and dimers proved to be extremely stable, staying α-helical after (heat) treatment with several concentrations of urea or guanidine hydrochloride (data not shown).

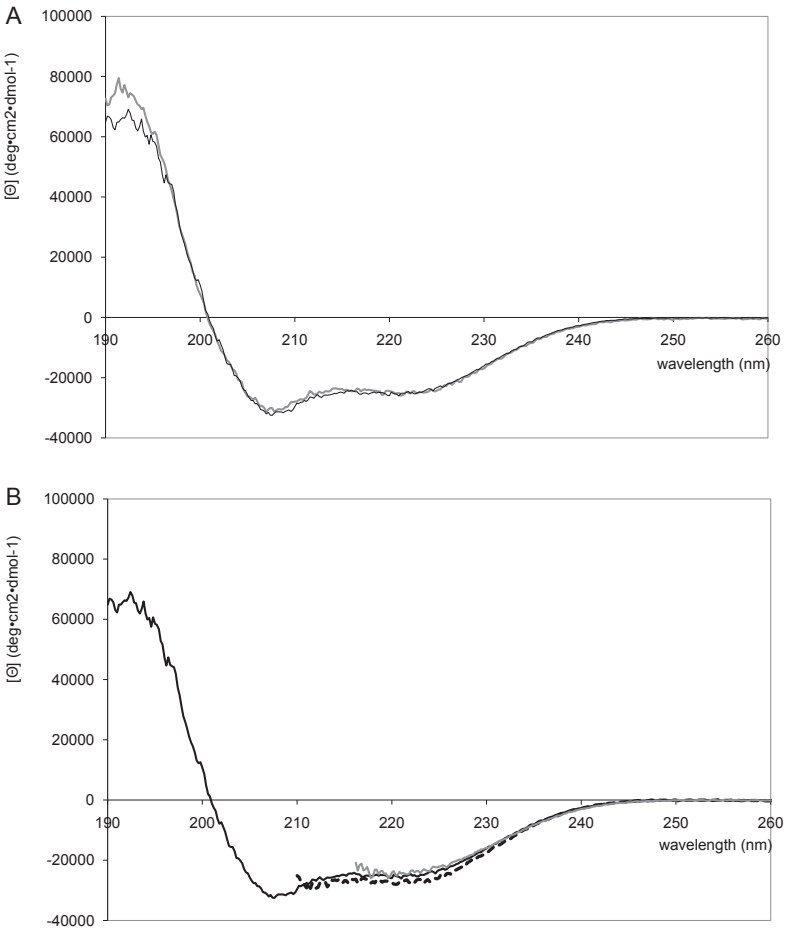


Figure 6. CD spectra of C11-WALP monomer (grey) and dimer (black) in SDS (A) and spectra of the C11-WALP dimer in SDS (B) without denaturant (solid black line), with 5M urea (dotted black line) and incubated with 5M urea at 90ºC (solid grey line)

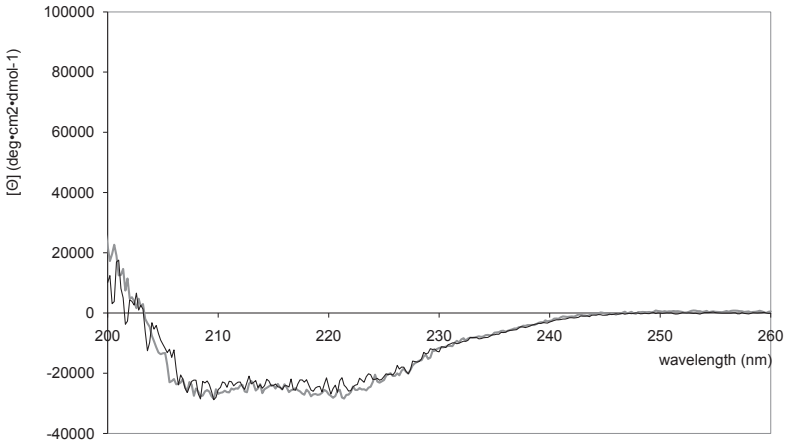


Figure 7. CD spectra of C11-WALP monomer (grey) and dimer (black) in DMPC

Concluding discussion

Three cysteine-containing WALP analogues (Table 1) were designed to make covalent dimers. According to ESI-MS the C11-, C12- and C13-WALP were successfully synthesized. However, HPLC showed multiple peaks for each monomer. It was shown by additional experiments that this was not caused by the presence of shorter peptides or dimers. The occurrence of the multiple peaks is probably caused by the presence of the peptides in different conformational or aggregational states. Similar behaviour was observed previously[20, 21] with the synthesis and purification of transmembrane segments of glycophorin-A, prion protein (110-137) and fibroblast growth factor receptor (368-397).

Dimerization of the monomeric WALP analogues in a bilayer was never complete. There were always monomers left. Therefore, this method is not suitable to obtain samples for comparison of monomers and dimers. The incompleteness of the dimerization might be due to several factors. First, the WALP analogues have to come in close contact to be able to form a disulfide bridge between the two cysteine residues. It was shown previously that the concentration of WALP peptides in the bilayer has to be quite high for dimerization to occur.[16] Proximity of two WALP peptides in fluid bilayers was only observed for P/L ratio's of 1:25 and higher. Second, the sides of the helices that contain the cysteine residues have to face each other to be able to form covalent dimers. It has been shown for WALP peptides with a cysteine at the C- or N-terminus that, under the same conditions as used here, significant amounts of dimers were formed (unpublished data). This indicates that the necessity for the cysteine-containing sides to face each other might indeed be an important cause of the difficult dimerization. In relation to this, also helices that are oriented parallel when they come into proximity, might not have an interaction that is favourable enough for the cysteine residues to reach each other. Third, the extent to which the cysteine residues can be reached by the several catalysts might complicate dimerization. Most of the catalysts applied here are water soluble and some even charged. However, the cysteine is located in the middle of the bilayer, in the hydrophobic environment of the lipid acyl chains. During freeze-thaw cycles the vesicles become leaky and a catalyst will be able to perform its function, but during the overnight incubation of the vesicles, most molecules will not be able to reach the thiol group. The accessibility of the cysteine residues might also be the reason that when catalysts are used, there seem to be more dimers formed according to gel electrophoresis than to HPLC. The (boiling in) SDS will break the vesicles, forming mixed micelles. As a result, the catalysts will probably be able again to reach the cysteine-residues. Thus, it is likely that during the process of boiling the vesicles in SDS, additional dimers will be formed, but only when catalysts are present.

Pure dimers could best be obtained by first oxidizing the monomeric WALP analogues and then isolating the dimers by HPLC. Since WALP peptides do not dissolve in aqueous media, the oxidation was performed in an organic solvent. The basic conditions, required for the formation of the disulfide bonds, were achieved by adding an organic base. Purification of the WALP dimers was not straightforward. A previously developed method of purification by HPLC[22], using an aqueous phosphorous acid/triethylamine buffer at pH 2.25 and iso-propanol in buffer B, yielded an adequate separation of the dimers from the monomers. However, purification with this buffer would require removal of residual salts after preparative HPLC. Therefore, another system of TFA-buffers was developed (see experimental section), which also yielded satisfactory separation results. HPLC results and purification were confirmed by gel electrophoresis. In Figure 5 an additional band can be seen above the band of

the dimer. This band probably results from an aggregated complex of WALP peptides. These bands were present in some gels, but they were not found systematically.

Using CD, it was shown that all monomers and dimers were α-helical under varying conditions. Spectra were recorded with the peptides incorporated in the detergents DDM and SDS, but also in bilayers of DMPC. The shape of the peptide/DMPC-spectra was slightly different than that of the peptide/detergent-spectra. In DDM and SDS the minimum at 208 is more pronounced and the crossing point with the x-axis is shifted slightly to the left. Apparently, DDM and SDS micelles are stronger α-helix inducers for these peptides than DMPC bilayers.

Another important result obtained by CD was that the α-helical structure of both monomers and dimers of the WALP analogues was highly stable. In literature, the stability and (un)folding kinetics of membrane proteins is increasingly being investigated.[23] In such studies denaturants are frequently used as destabilizing agents. Like with the determination of the folding kinetics of the ß-barrel protein OmpA by refolding of the urea denatured-state into small unilamellar vesicles.[24] Also, differences in stability of the protein in varying detergents or lipid bilayers are used. For instance for the reversible unfolding of the integral membrane protein diacylglycerol kinase by varying the ratio of n-decyl ß-D-maltoside and SDS.[25] Combinations of both are used as well. Like the differences in destabilization of the sodium-coupled branched-chain amino acid carrier (LIV-II carrier) by guanidine hydrochloride, when it is incorporated in either detergent or in lipid bilayers.[26] Based on these articles the stability of the monomers and dimers was tested in DMPC, SDS and DDM. The choice of denaturants is limited when CD is used, because of the scattering resulting from the structures of the molecules. In this study mostly urea and guanidine hydrochloride were used in several concentrations and also heating the sample. Though there was a significant amount of scattering in the region 190-210, from the rest of the spectrum it can be concluded that the secondary structure of all peptides was unaffected under all conditions used.

Obviously, the synthesized WALP analogues, consisting of only one helix or two covalently coupled helices, are less complex than natural membrane proteins. Still, it is not self-evident that every designed transmembrane helix automatically forms α-helices that are stable to denaturation. More importantly, one can imagine that dimerization can induce unfavourable interactions in the WALP analogues or cause restrictions. The dimers could therefore be more prone to destabilization. In addition, the different interfaces of the three dimers can influence their stability. Especially the respective orientations of the tryptophan residues (Figure 1) may be important. Simply because the tryptophan residues can sterically hinder each other, but also because of the possibilities of tryptophan residues to partition at the lipid interface. Tryptophan anchoring plays a major role in peptides.[27-29] In the stability studies no differences were found between the dimers. This confirms the result that no evidence for a preferential interface was found in the dimerization of the three WALP analogues in the bilayer.

Finally, it can be derived from the CD-experiments that all monomer and dimers properly incorporated into large unilamellar vesicles of DMPC. First of all, in the extrusion-step, performed to obtain unilamellar vesicles, (aggregated) peptide can be lost in the membrane filter. No systematic differences in loss of peptide were found for dimers in comparison to monomers, which indicates that they have similar incorporation properties. In addition, after correcting for peptide-concentration, path length and number of amino acids, all CD-spectra had the same intensity. Because aggregated peptides do not contribute to the CD-signal,[15] this implies that all monomers and dimers are incorporated to the same extent.

Experimental section

General

All chemicals were obtained from commercial sources and used without further purification. Analytical HPLC was performed on a Shimadzu Class-VP HPLC, equipped with a Shimadzu SPD-10AVP UV-VIS detector. Preparative HPLC was performed on an automated Gilson preparative HPLC system. Masses were measured on a Micromass LC-T spectrometer. CD spectra were recorded on a Jasco J-810 spectropolarimeter with Jasco CDF-426S temperature control unit. The gel electrophoresis setup was purchased from BioRad Laboratories B.V. Stock solutions of 2-12 mM lipids were prepared by dissolving an appropriate amount of lipid in chloroform. The concentration of lipid stocks was determined according to the method of Rouser.[30] Peptides were dissolved in TFE. The concentration of peptide stocks and CD-samples was determined by absorption spectroscopy on a Perkin Elmer Lambda 18 spectrometer, using the tryptophan absorption at 280 nm with an extinction coefficient of 22400.[15] Typical concentrations of peptide stock solutions were 0.5-2 mM

Synthesis

C11-, C12- and C13-WALP

The peptides were synthesized manually on a Tentagel S-RAM resin, using a standard Fmoc/tBu strategy.[17] For coupling HOBt, HBTU and DiPEA were used, for deprotection piperidine/NMP (20/80 v/v). Kaisertests[18] were performed to verify each coupling and deprotection. To allow for characterization by solid state ^{2}H-NMR,[31] (Chapter 3) an Fmoc-Ala(d3)-OH was incorporated instead of a regular Fmoc-Ala-OH on the 17th position (7th coupling). The coupling of the first 10 amino acids was performed on a 0.75 mmol scale. After this, the synthesis of the C11-, C12- and C13-WALP peptides was finished on a 0.25 mmol scale, using 1/3 of the resin for each WALP analogue. The peptides were deprotected and cleaved of the resin by shaking in TFA/TIS/H_2O (95/2.5/2.5 v/v/v) for 3 hrs, precipitated in MTBE/n-hexane (1:1 v/v, 4°C) and lyophilized from tert-butanol/H_2O (1:1 v/v) to obtain fluffy white powders. Crude yields of C11-, C12- and C13-WALP were 60, 64 and 60 % respectively. According to analytical HPLC they were all more than 95% pure. C11-WALP: R_t 50.7 min, ESI-MS: calculated; 2552.44 ($C_{129}H_{192}D_3N_{28}O_{24}S$, exact mass), found; 1277.84 $[M+2H]^{2+}$, 1288.83 $[M+H+Na]^{2+}$, 1299.33 $[M+2Na]^{2+}$. C12-WALP: R_t 49.1 min, ESI-MS: calculated; 2510.39 ($C_{126}H_{186}D_3N_{28}O_{24}S$, exact mass), found; 1256.85.84 $[M+2H]^{2+}$, 1285.38 $[M+Na+K]^{2+}$, 1292.89 $[M+K]^{2+}$. C13-WALP: R_t 50.7 min, 2552.44 ($C_{129}H_{192}D_3N_{28}O_{24}S$, exact mass), found; 1277.90 $[M+2H]^{2+}$, 1288.35 $[M+H+Na]^{2+}$ 1297.86 $[M+2Na]^{2+}$.

General procedure(s) to oxidize C11-, C12- and C13-WALP in a bilayer

In all samples the peptide content was kept constant at 0.5 mg·ml^{-1} and P/L ratio's between 1:5 and 1:25 were used. Appropriate amounts of peptide in TFE and lipid in chloroform were mixed vigorously, after which the solvents were evaporated under a N_2-flow. The film was further dried, usually overnight, hydrated with a 100 mM NaCl, 50 mM TRIS buffer (pH ~8.5). To enhance dimerization, either 1-10% DMSO, 20 µl of a 5 mM $CuCl_2$ solution, 20 µl of a 5 mM hexacyanoferrate(II) solution or 20 µl of a 5 mM iodine solution was added. The vesicle suspension was then freeze-thawed 10-50 times, with occasional vortexing, until a milky-white suspension was obtained. After overnight incubation at 40ºC the vesicles were spun down and the pellet lyophilized. If DMSO was used, the pellet was first washed with H_2O two times. The pellet was either dissolved in loading buffer to characterize with gel electrophoresis or in TFE to characterize with HPLC.

General procedure(s) to oxidize C11-, C12- and C13-WALP in solution

When a small scale (1-2 mg) dimerization was performed the WALP analogue was dissolved in TFE and TEA was added to obtain an overall concentration of 0.5 mg·ml^{-1} and a TFE/TEA ratio of 3:1 v/v. This solution was left at 40°C overnight. On a larger scale (30-50 mg) the peptide was dissolved in TFE (2-5 ml) and the pH brought to 8-9 with TEA. The reaction was stirred for 5-7 days at 40°C. After this the TFE and TEA was removed in vacuo and the peptide dissolved in tert-butanol/H_2O (1:1 v/v) and lyophilized.

Characterization and purification

HPLC

For analytical HPLC two methods were used. The first characterizations were performed using TEAP buffers and an adsorbosphere XL butyl column as described previously.[22] The HPLC spectra shown in Figure 2 and 4 were obtained using these conditions. For easier purification, a switch was made to another method. The peptide was then eluted of a prosphere C4 column (300Å, 5u, 250x4.6mm) with a gradient of 10 to 100% buffer B in 40 minutes, where buffer A was H_2O/acetonitrile (80/20 v/v) + 0.1% TFA and buffer B was acetonitrile/iso-propanol/H_2O (50/45/5 v/v/v) + 0.1% TFA. A flow rate of 0.75 ml·min^{-1} was used and UV-detection at 220 and 280 nm. Preparative HPLC was performed similarly, but using a preparative prosphere C4 column (300Å, 10u, 250x22mm) and a gradient of 10 to 100% buffer B in 100 minutes followed by maintaining at 100% buffer B for 15 minutes.

Gel electrophoresis

The samples were dissolved in a loading buffer of 0.1 M TRIS-Cl, pH 6.8, 24% glycerol (v/v), 8% SDS (w/v) and bromophenol blue at a concentration of 2 mg·ml^{-1}. Tricine-SDS-PAGE to separate molecules with molecular weights of 1-100 kDa was used.[32] As separating gel the composition described in the article as 16.5%T, 6%C was applied. An exception on the protocol was that for the stacking gel a 3M TRIS solution of pH 6.8 was used. The gel was run on a voltage of 30V for 30 minutes, followed by a voltage of 100V for 3 hours. Gels were fixated in destaining solution (H_2O/methanol/acetic acid, 315/150/35 v/v/v) for 10-30 minutes, stained with Coomassie brilliant blue G-250 for minimally one hour and destained overnight.

Circular dichroism

For the unilamellar vesicle samples the appropriate amount of peptide (from a 0.5-2 mM stock in TFE) were mixed with the appropriate amount of DMPC (from a 10 mM stock in chloroform) to obtain a P/L ratio of 1:100. Solvents were evaporated under a N_2-flow and the resulting films further dried under vacuum overnight. The film was hydrated with a 10 mM phosphate buffer of pH 7 (500 µl) to obtain a peptide concentration of 50 µM, freeze-thawed ten times and extruded ten times over 200 nm Whatman membrane filters to obtain large unilamellar vesicles (LUVs). CD-spectra were measured with the sample in a 1 mm cuvet, in the range 260-190 nm, with a scan speed of 20 nm·min^{-1}, averaging 10 scans, in continuous mode, with a data pitch of 0.2 nm, 1 nm bandwidth and 1 second response time, keeping the temperature at 30ºC. The molar ellipticity per residue was calculated using the following formula:

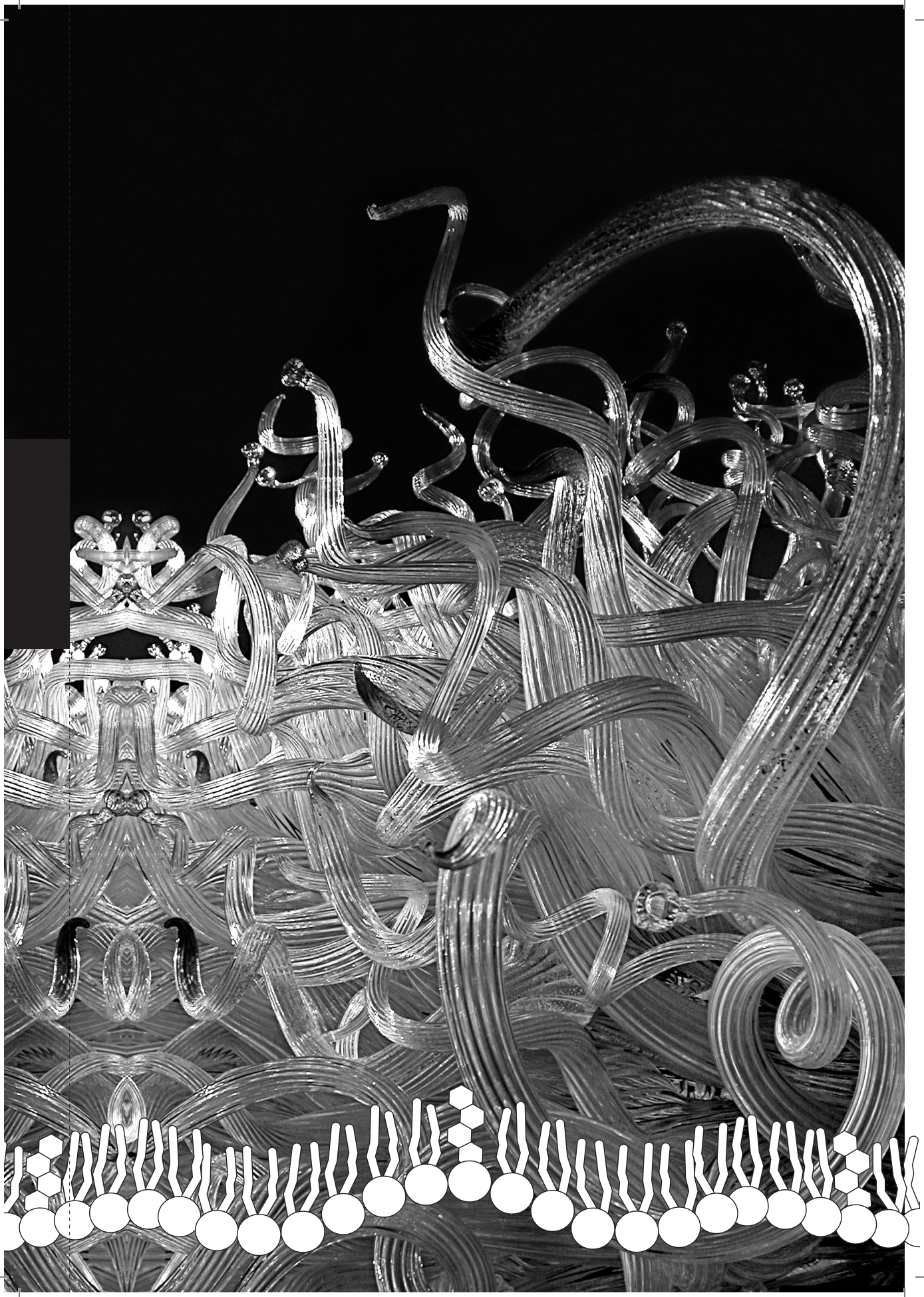
$$[\theta] = \frac{\theta}{l * AAn * c}$$

where θ is the ellipticity (in degrees), l is the path length of the cuvet (in cm), AAn is the number of amino acids in the peptide and c is the concentration of the peptide (in dmol·cm^{-3}), which was determined after the CD-measurement using absorption spectroscopy, to correct for possible loss of peptide during extrusion.

For the concentration determination (see also ‘General’) an aliquot of the sample was added to 1 ml TFE, which disrupts the vesicles.The SDS and DDM samples were prepared as described previously.[33] In a typical preparation 20 µl of a 0.4 M detergent solution in H_2O was added to the appropriate amount of peptide in 20 µl TFE. The peptide concentration was held constant at 50 µM and a P/detergent ratio of 1:400 was used. The sample was vortexed, diluted with 450 µl H_2O and vortexed again, yielding a clear solution which was frozen and lyophilized. The fluffy white powder was dissolved in either 400 µl 10 mM phosphate buffer, pH 7 or in a mixture of 10 mM phosphate buffer, pH 7 and 10 M urea in buffer (1:1 v/v) to obtain a clear sample. CD-spectra were measured with the same settings as used for the di-14:0 PC samples and normalized using the same formula, except that the samples with denaturant were measured in the range of 260-210 nm and when appropriate at 90ºC.

References

1. Wallin, E. and von Heijne, G., Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms, *Protein Sci*, **1998**, 7(4), p. 1029-38
2. Bkaily, G., Avedanian, L., and Jacques, D., Nuclear membrane receptors and channels as targets for drug development in cardiovascular diseases, *Can J Physiol Pharmacol*, **2009**, 87(2), p. 108-19
3. Schindler, J. and Nothwang, H. G., Enrichment of brain plasma membranes by affinity two-phase partitioning, *Methods Mol Biol*, **2009**, 528, p. 119-26
4. Lacapere, J. J., Pebay-Peyroula, E., Neumann, J. M., and Etchebest, C., Determining membrane protein structures: still a challenge!, *Trends Biochem Sci*, **2007**, 32(6), p. 259-70
5. White, S. H., Biophysical dissection of membrane proteins, *Nature*, **2009**, 459(7245), p. 344-6
6. Lee, A. G., How lipids affect the activities of integral membrane proteins, *Biochim Biophys Acta*, **2004**, 1666(1-2), p. 62-87
7. Demmers, J. A., van Dalen, A., de Kruijff, B., Heck, A. J., and Killian, J. A., Interaction of the K+ channel KcsA with membrane phospholipids as studied by ESI mass spectrometry, *FEBS Lett*, **2003**, 541(1-3), p. 28-32
8. Cuevas, F. J., Jameson, D. M., and Sotomayor, C. P., Modulation of Reconstituted Pig Kidney Na(+)/K(+)-ATPase Activity by Cholesterol in Endogenous Lipid Vesicles: Role of Lipid Domains, *Biochemistry*, **2006**, 45(46), p. 13855-13868
9. Ma, A. W., Redka, D. S., Pisterzi, L. F., Angers, S., and Wells, J. W., Recovery of oligomers and cooperativity when monomers of the M2 muscarinic cholinergic receptor are reconstituted into phospholipid vesicles, *Biochemistry*, **2007**, 46(26), p. 7907-27
10. Jittikoon, J., East, J. M., and Lee, A. G., A fluorescence method to define transmembrane alpha-helices in membrane proteins: studies with bacterial diacylglycerol kinase, *Biochemistry*, **2007**, 46(38), p. 10950-9
11. Schuldiner, S., Lebendiker, M., and Yerushalmi, H., EmrE, the smallest ion-coupled transporter, provides a unique paradigm for structure-function studies, *J Exp Biol*, **1997**, 200(Pt 2), p. 335-41
12. Killian, J. A., Synthetic peptides as models for intrinsic membrane proteins, *FEBS Lett*, **2003**, 555(1), p. 134-8
13. Nyholm, T. K., Ozdirekcan, S., and Killian, J. A., How protein transmembrane segments sense the lipid environment, *Biochemistry*, **2007**, 46(6), p. 1457-65
14. Holt, A. and Killian, J. A., Orientation and dynamics of transmembrane peptides: the power of simple models, *Eur Biophys J*, **2009**, 39(4), p. 609-621
15. de Planque, M. R., Goormaghtigh, E., Greathouse, D. V., Koeppe, R. E., 2nd, Kruijtzer, J. A., Liskamp, R. M., de Kruijff, B., and Killian, J. A., Sensitivity of single membrane-spanning alpha-helical peptides to hydrophobic mismatch with a lipid bilayer: effects on backbone structure, orientation, and extent of membrane incorporation, *Biochemistry*, **2001**, 40(16), p. 5000-10
16. Sparr, E., Ash, W. L., Nazarov, P. V., Rijkers, D. T. S., Hemminga, M. A., Tieleman, D. P., and Killian, J. A., Self-association of Transmembrane {alpha}-Helices in Model Membranes: IMPORTANCE OF HELIX ORIENTATION AND ROLE OF HYDROPHOBIC MISMATCH, *J Biol Chem*, **2005**, 280(47), p. 39324-39331
17. Jones, J., *Amino Acid and Peptide Synthesis*. 2nd ed. 2002, New York: Oxford University Press.
18. Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I., Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides, *Anal Biochem*, **1970**, 34(2), p. 595-8
19. Kleinschmidt, J. H., Wiener, M. C., and Tamm, L. K., Outer membrane protein A of E. coli folds into detergent micelles, but not in the presence of monomeric detergent, *Protein Sci*, **1999**, 8(10), p. 2065-71
20. Glover, K. J., Martini, P. M., Vold, R. R., and Komives, E. A., Preparation of insoluble transmembrane peptides: glycophorin-A, prion (110-137), and FGFR (368-397), *Anal Biochem*, **1999**, 272(2), p. 270-4
21. Fisher, L. E. and Engelman, D. M., High-yield synthesis and purification of an alpha-helical transmembrane domain, *Anal Biochem*, **2001**, 293(1), p. 102-8
22. Rijkers, D. T. S., Kruijtzer, J. A., Killian, J. A., and Liskamp, R. M. J., A convenient solid phase synthesis of S-palmitoyl transmembrane peptides, *Tetrahedron Letters*, **2005**, 46, p. 3341-3345
23. Booth, P. J. and Curnow, P., Folding scene investigation: membrane proteins, *Curr Opin Struct Biol*, **2009**, 19(1), p. 8-13
24. Kleinschmidt, J. H. and Tamm, L. K., Secondary and tertiary structure formation of the beta-barrel membrane protein OmpA is synchronized and depends on membrane thickness, *J Mol Biol*, **2002**, 324(2), p. 319-30
25. Lau, F. W. and Bowie, J. U., A method for assessing the stability of a membrane protein, *Biochemistry*, **1997**, 36(19), p. 5884-92
26. Uratani, Y., Kobayashi, M., Yokoyama, Y., Maeda, T., Mitaku, S., and Hoshino, T., Phospholipids stabilize the secondary structure of the sodium-coupled branched-chain amino acid carrier of Pseudomonas aeruginosa, *Biochim Biophys Acta*, **1999**, 1435(1-2), p. 71-83
27. Kachel, K., Asuncion-Punzalan, E., and London, E., Anchoring of tryptophan and tyrosine analogs at the hydrocarbon-polar boundary in model membrane vesicles: parallax analysis of fluorescence quenching induced by nitroxide-labeled phospholipids, *Biochemistry*, **1995**, 34(47), p. 15475-9
28. Killian, J. A. and von Heijne, G., How proteins adapt to a membrane-water interface, *Trends Biochem Sci*, **2000**, 25(9), p. 429-34
29. de Planque, M. R., Bonev, B. B., Demmers, J. A., Greathouse, D. V., Koeppe, R. E., 2nd, Separovic, F., Watts, A., and Killian, J. A., Interfacial anchor properties of tryptophan residues in transmembrane peptides can dominate over hydrophobic matching effects in peptide-lipid interactions, *Biochemistry*, **2003**, 42(18), p. 5341-8
30. Rouser, G., Fkeischer, S., and Yamamoto, A., Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, *Lipids*, **1970**, 5(5), p. 494-6
31. Strandberg, E., Ozdirekcan, S., Rijkers, D. T. S., van der Wel, P. C., Koeppe, R. E., 2nd, Liskamp, R. M., and Killian, J. A., Tilt angles of transmembrane model peptides in oriented and non-oriented lipid bilayers as determined by 2H solid-state NMR, *Biophys J*, **2004**, 86(6), p. 3709-21
32. Schagger, H. and von Jagow, G., Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal Biochem*, **1987**, 166(2), p. 368-79
33. Killian, J. A., Trouard, T. P., Greathouse, D. V., Chupin, V., and Lindblom, G., A general method for the preparation of mixed micelles of hydrophobic peptides and sodium dodecyl sulphate, *FEBS Lett*, **1994**, 348(2), p. 161-5



3

Dimeric assemblies of transmembrane model peptides; The effect of cross sectional diameter on protein-lipid interactions

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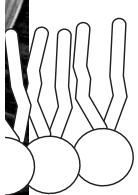

Abstract

In this chapter, the influence of dimerization of transmembrane helices on protein-lipid interactions is investigated. Properties of dimeric assemblies of transmembrane model peptides, covalently coupled in the middle of the helix, are compared with properties of their monomeric counterparts. The orientation and dynamics of the peptides and their influence on lipids are studied by ATR-FTIR, solid state NMR and MD simulations. It is shown that dimerization decreases the dynamics of the peptides, while their acyl chain ordering effect per neighbouring lipid is more pronounced. Surprisingly, the orientation of the employed dimers with respect to the membrane normal seems similar to that of the monomers. The results provide new insights into the basic principles of membrane protein behaviour.

Introduction

The interactions between proteins and lipids in membranes are of fundamental importance for membrane structure and function. For example, factors like lipid packing or lateral pressure are significant determinants of membrane protein structure, function and dynamics.[1-3] Vice versa, membrane proteins influence lipids by perturbing the membrane[4-6] or having interactions with lipids neighbouring the transmembrane segments[7-11]. Together with the large variety of proteins and lipids, this makes the complexity of biological membranes enormous, which complicates the study of their behaviour and mutual influence. To better understand protein-lipid interactions we have chosen the simple and systematic approach to investigate artificial transmembrane peptides in vesicles of synthetic lipids. The advantages of using such model systems are that properties of peptides and lipids can be systematically varied and labels to allow biophysical characterization can be incorporated essentially everywhere. In our model systems, WALP peptides (Ac-GWW$(LA)_n$LWWA-NH_2) are employed as mimics for the membrane spanning segments of membrane proteins. The behaviour of the WALP model peptide and its influence on lipid membranes has been extensively studied and important insights on protein-lipid interactions have been obtained.[12-14]

Since membrane proteins usually have multiple membrane spanning segments, one may question whether the single membrane spanning WALP helix is sufficient as a model for the behaviour of membrane proteins. To learn more about polytopic membrane proteins, while still having the advantage of using simple model systems, in this study we aim to compare the properties and lipid interactions of dimeric assemblies of WALP peptides to that of their monomeric equivalents. One consequence of dimerization is an increase in the effective cross sectional diameter of the protein. In literature, there are several indications that this cross sectional diameter affects membrane protein behaviour and lipid interactions. First, the effect membrane proteins have on the phase transition of lipids as function of mismatch[15-18] is larger than the effect of single span model peptides[19]. Second, the lipid stretching effect of the β-helix forming gramicidin dimer, that has the same hydrophobic length but a larger diameter in comparison to our model peptide, is significantly more than that of a WALP peptide.[14] Third, the dynamics, such as lateral diffusion, of membrane proteins depends on the radius of the membrane protein.[20-22] Fourth, localization of membrane spanning segments to matching domains, has been shown to occur for membrane proteins[15, 23, 24], but not for WALP peptides[25, 26]. Finally, the extent of translocation of lipids by a subset of membrane proteins seems to correlate to their diameter.[27] It is possible that these differences in behaviour of the model peptides and various natural membrane proteins are caused by a difference in size or cross-sectional diameter. Yet, it is evident that when comparing WALP peptides and natural proteins with different numbers of transmembrane helices, there are more variables than only cross sectional diameter. Hence, in this chapter we will start with a more systematic approach to determine the influence of diameter on the behaviour and lipid interactions of transmembrane helices. To this purpose, the properties and lipid interactions of WALP dimers and monomers are compared.

In chapter 2 we reported on the synthesis of dimeric assemblies of three WALP analogues, the C11-, C12- and C13-WALP (Table 1). These analogues were designed to all have the cysteine residues positioned at a different face of the helix. Consequently, the dimers have varying helix-helix and helix-lipid interfaces. In addition, with the position of the cysteine in the middle of the helices, antiparallel and parallel orientations of the helices are in principle both possible. Consequently, the helices in the dimers can adopt the most favourable orientation with respect to

each other.[28] As shown in chapter 2, the dimers, synthesized in solution, properly incorporate into lipid bilayers and form stable α-helices.

Table 1. WALP analogues synthesized to make covalent dimers

Peptide	Sequence
C11-WALP	Ac-GWWLALALAL**C**LALALALALWWA-NH_2
C12-WALP	Ac-GWWLALALALA**C**ALALALALWWA-NH_2
C13-WALP	Ac-GWWLALALALAL**C**LALALALWWA-NH_2

Using these dimers and their monomeric counterparts, we here studied the consequences of a hydrophobic mismatch situation. In this situation the hydrophobic length of the peptide does not match the hydrophobic thickness of the lipids. Under conditions of positive mismatch hydrophobic residues of the helix would be exposed to the aqueous environment. To avoid this unfavourable situation, the helices and/or the lipids have to adapt. Possible responses to positive mismatch are tilting of the helices[29], alteration of backbone conformation[30], oligomerization[28] and stretching of the lipid acyl chains[14]. Thus, mismatch can be used as a tool to gain insight into the importance of dimerization and hence of cross sectional diameter and helix-helix interactions in the behaviour of the peptides.

The structural properties of the monomers and dimers were examined by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). The influence of dimerization on the orientation and dynamics of the transmembrane helices was studied by ATR-FTIR, solid state ^{2}H-NMR and molecular dynamics simulations. In addition, the influence of the monomers and dimers on the ordering of acyl chains was compared by solid state ^{2}H-NMR and ATR-FTIR measurements of the lipids. The results show differences between monomers and dimers, which may have important implications for our understanding of membrane protein behaviour and lipid interactions.

Results

Characterization of structure and stability of monomers and dimers

Attenuated total reflection- Fourier transform infrared experiments

All monomers and dimers were incorporated in di-14:0 PC and ATR-FTIR spectra were recorded. It was shown in chapter 2 that the C11-, C12- and C13-WALP monomers did not form covalent assemblies during vesicle preparation under non-catalytic conditions. Thus, they can be used as monomeric references in the comparison of monomers and dimers. In Figure 1 the amide I and II region of the representative spectra of the C12-WALP monomer (solid black line) and dimer (dashed black line) in di-14:0 PC are shown. As references (grey lines), also the spectra of some water soluble proteins with a random coil, β-sheet and α-helical structure are given.[31] The amide I band located in the region 1700-1600 cm^{-1} is assigned to the C=O stretching vibration (ν(C=O)) of the amide bond and is the most sensitive indicator of secondary structure. The amide I bands of proteins with random coil or α-helical structure have a maximum that is shifted to the left with respect to the maximum of β-sheet proteins. This is evident when comparing the β-sheet forming protein avidin (solid grey) with the other proteins. With respect to random coil, α-helices can have the same wavenumber position, but the bands are narrower. This is illustrated by the random coil protein metallothionein II (dashed grey), which has approximately the same amide I band position as the α-helical protein myoglobin (dotted grey), but a much larger full width at the half-height (FWHH). A further left-shift and narrowing would indicate even stronger α-helix formation. Thus, the C12-WALP monomer and dimer are highly defined α-helices with respect to the soluble proteins as shown by the position and FWHH of the amide I bands in Figure 1. The results for the C11- and C13-WALP monomers and dimers were similar (data not shown). The amide I bands of all monomeric and dimeric WALP peptides had maxima at 1657-1659.6 cm^{-1} and FWHH values of around 17 cm^{-1}. The differences in maxima of the amide I bands of all six model peptides were minor and non-systematic. The amide II vibration located in the region 1590-1500 cm^{-1} is assigned to the N-H bending vibration (δ(N-H)) of the amide bond. The strong α-helical character of the peptides was confirmed by the position of this band, which had a maximum at 1545 cm^{-1} for all mono- and dimers. The area of the amide I band with respect to the lipid carbonyl peak (1700-1780 cm^{-1}) is representative for the peptide amide/lipid ester ratio in the sample. When normalized to the ν(C=O) the amide I and II band intensities were comparable for all model peptides (data not shown). Since the helix-lipid ratio was the same in all samples, this implies that there were no systematic differences in incorporation of the monomers and dimers.

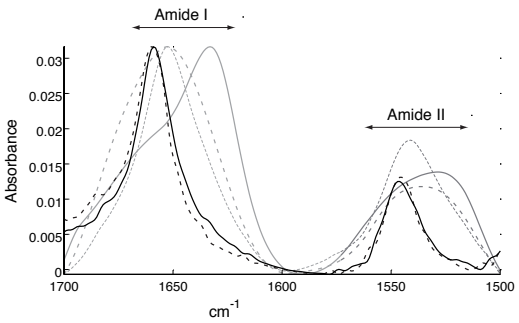

Figure 1. FTIR spectra of monomer (black) and dimer (dashed black) of C12-WALP in di-14:0 PC, together with spectra (retrieved from 31) of the soluble proteins avidin (solid grey) with β-sheet structure, metallothionein II (dashed grey) with random coil structure and myoglobin (dotted grey) with α-helical structure, for comparison

Next, the stability of the secondary structure of all dimers as compared to that of a monomeric peptide in di-14:0 PC was studied by H/D exchange. With this method the area under the amide II band is measured in time while the sample is exposed to a D_2O saturated N_2-flow. If exchange takes place, the area under the amide II band (1545 cm^{-1}) decreases while the amide I band (1659 cm^{-1}) shifts to the right. The exchange of the amide hydrogen is a measure of the stability of the helical structure embedded in

the membrane.[32] In Figure 2 the amide I and II regions of some exchange spectra of the C11-WALP dimer are shown. In the first 30 minutes (three grey lines) hardly any exchange occurs, indicating that the helices are stable. After normalization to the lipid ν(C=O), to correct for film swelling, it was found that the hydrogen content levelled at 90%. The exchange kinetics of the C11-WALP dimer was comparable to that of the C11-WALP monomer and also the other dimers showed similar exchange (data not shown). No systematic differences were found between the dimers and monomer, suggesting equal stability of the secondary structure. To further test the stability of the α-helices in the C11-WALP dimer, harsher conditions were applied. The sample was lyophilized, resuspended in D_2O and heated overnight at 80ºC and another night at 90 ºC. After each night a spectrum was recorded. As is shown in Figure 2 (two black lines), exchange did occur under these excessive conditions. From the last spectrum it was calculated that the exchange was 75% (hence, still even 25% amide hydrogen content left).

Comparison of orientation and dynamics of monomers and dimers

Attenuated total reflection Fourier transform infrared experiments

In addition to secondary structure and stability, also the orientation of the peptides in the bilayer was established by ATR-FTIR. The principle of orientation determination with this technique is that infrared light is absorbed most efficiently by bonds that have their dipole oriented parallel to the electric field vector of the incident light. Thus, the ratio of absorption of parallel versus perpendicular polarized light (the dichroic ratio) by a specific bond is directly related to its orientation with respect to the germanium crystal. Figure 3 shows two regions of the ATR-FTIR spectra recorded with the incident light polarized parallel (||) or perpendicular (⊥) to the incidence plane. In addition, the dichroic spectrum of the parallel minus the perpendicular spectrum (||-⊥) is shown. These specific spectra are of the C11-WALP dimer in di-14:0 PC, but are repre-

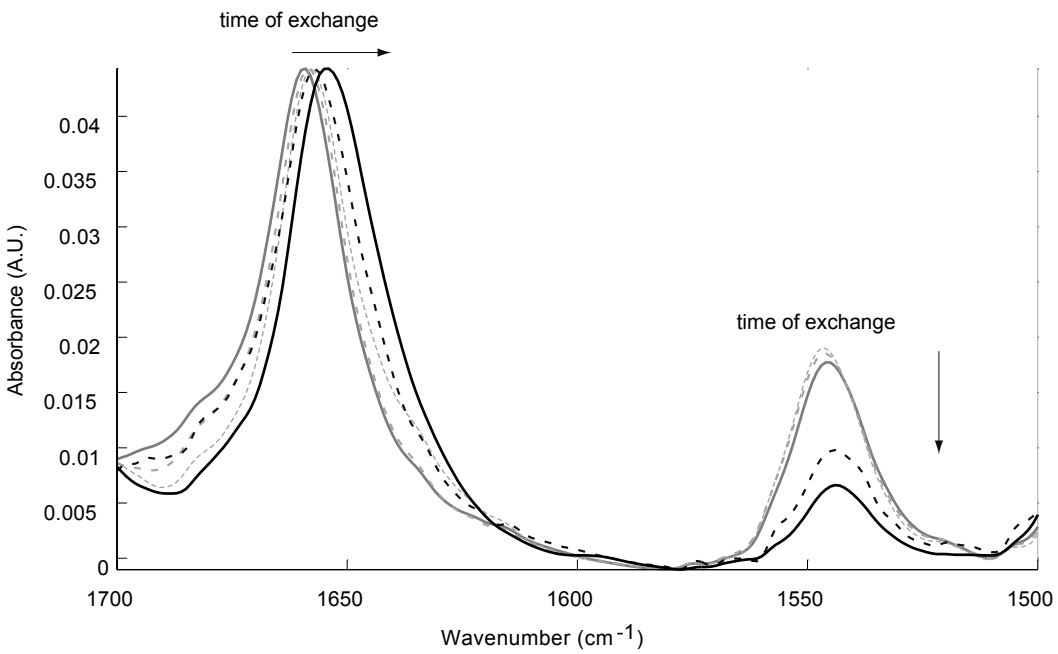

Figure 2. Exchange of C11-WALP dimer in di-14:0 PC at t=0 (solid grey) t=3 min (broken grey) t=30 min (dotted grey), after 1 night at 80°C in D2O (dashed black) and 2 nights at 90°C in D2O (solid black)

sentative for all monomers and dimers (data not shown). Before subtraction the spectra were normalized to the lipid v(C=O) vibration (1740 cm^{-1}). It has been shown in previous research that this band can be used to correct for the difference in the relative powers of the evanescent waves.[33] From the difference spectrum information was derived on the orientation of the lipids and peptides in the system. The di-14:0 PC is expected to form highly ordered multilayers of lipids, with acyl chains oriented normal to the germanium plate. The bands in the 3000-2800 cm^{-1} region, displayed in Figure 3A, originate from the $v(CH_2)$ and $v(CH_3)$ vibrations of the sn-1 and sn-2 chains of the lipids. The positive values for the CH_3 bands (2960, 2870 cm^{-1}) and the negative values for the CH_2 bands (2915, 2850 cm^{-1}) in the difference spectrum indicate that the lipids are well-oriented. The proper orientation of the lipids was confirmed by the fact that the CH_2

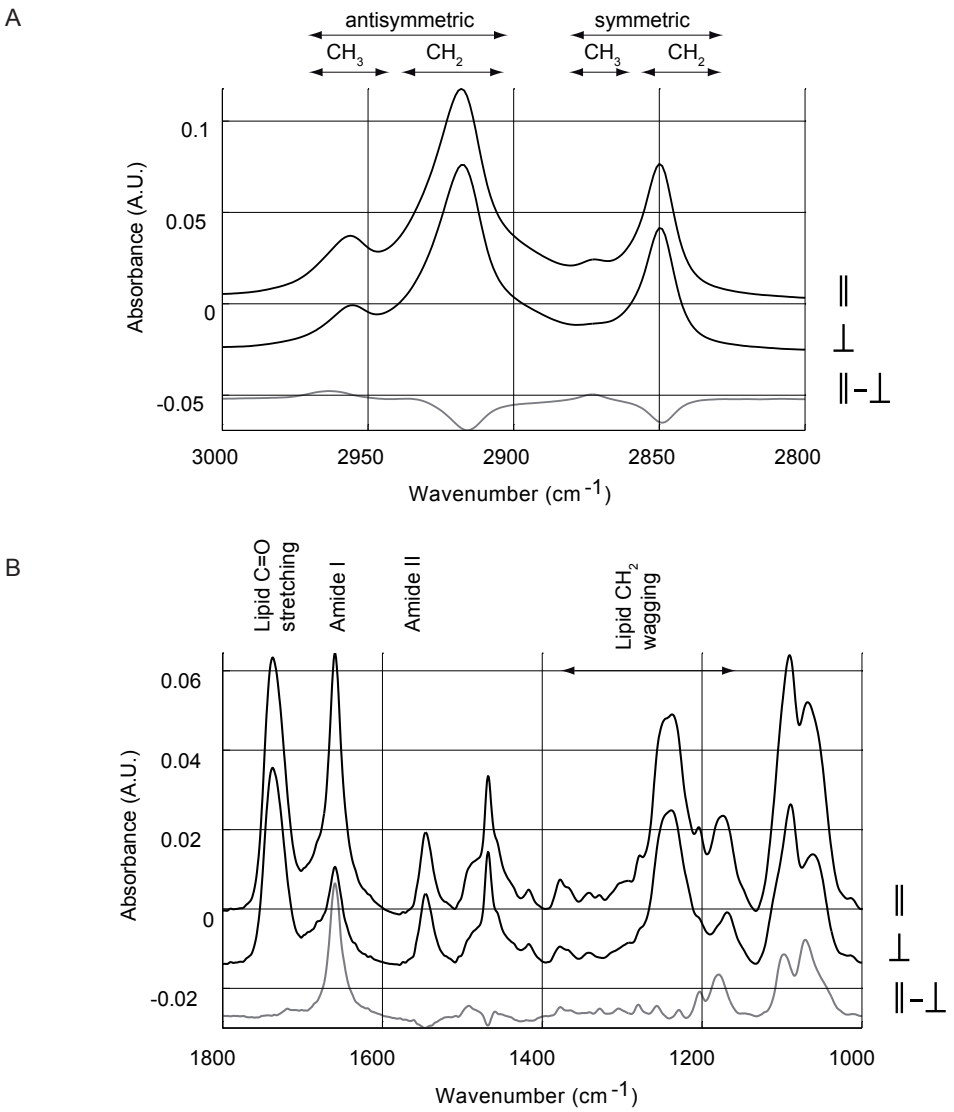

Figure 3. The regions 3000-2800 cm-1 (A) and 1800-1000 cm-1 (B) of the parallel (black ||) and perpendicular (black ⊥) polarized ATR-FTIR spectra of the C11-WALP dimer in di-14:0 PC, together with the dichroic difference spectrum of parallel minus perpendicular (grey || - ⊥). All spectra, including the difference spectrum, have the same scaling

wagging vibrations resulted in a series of bands from 1180-1330 cm^{-1} (Figure 3B) with positive deviation. This band progression (values 1179, 1203, 1229, 1257, 1280, 1304, 1328 cm^{-1}) originates from the resonance between the lipid ester group and the all-trans sn-1 hydrocarbon chain. The stronger absorbance of the parallel polarized light implies that the acyl chains are normal to the germanium surface. The orientation of the peptides can best be derived from the amide I band (1659 cm^{-1}). The positive deviation in the dichroic spectrum in Figure 1 denotes that the v(C=O) vibration is normal to the crystal surface. This signifies that the peptides were transmembrane, because the dipole of the carbonyl stretching vibration is rather parallel to the helix. The fact that the amide II band (1545 cm^{-1}) is negative in the dichroic spectrum indicates that the orientation of the peptide perpendicular to the membrane plane is well defined. Indeed, because several non-δ(N-H) contributions make the band sensitive to variations in the system, such deviations are only observed if the distribution of peptide orientations is homogeneous and/or if the tilt is relatively small.

The tilt of all model peptides with respect to the membrane normal was calculated from the dichroic ratios of the amide I bands. From the dichroic ratio R^{ATR}, defined as $A_{\parallel}/A_{\perp}$, an order parameter can be derived which is related to the tilt angle. R^{ATR} was calculated by dividing the amide I band areas of the parallel and perpendicular spectrum (Table 2). The areas were obtained by using an integration tool in the software-package 'Matlab'. To calculate the order parameter from the dichroic ratio, several additional parameters had to be obtained. The refractive indices of the internal reflection element, sample and outer medium were obtained from literature (see experimental section).[33] In addition, the film thickness had to be derived. This parameter can be obtained from the dichroic ratio of an isotropic distribution of dipoles or a dipole oriented at the magic angle; R^{iso}.[33] Following previous research, the R^{iso} values were calculated from the lipid v(C=O) band areas of the parallel and perpendicular spectra (Table 2). To convert the order parameter, that now could be derived from R^{ATR}, to the so-called maximum tilt angle θ, two assumptions were made. First, the peptide v(C=O) dipole is not completely parallel to the helix long axis. We applied the widely used angle of 27º, obtained for bacteriorhodopsin.[34] Second, perfect helix and membrane order were assumed. The order of the membrane can be derived from

Table 2 Dichroic ratios and orientations of all monomers and dimers with respect to ATR-surface

Peptide	$R^{ATR, a}$	$R^{iso, b}$	θ (º) [c]	θ (º) [d]
C11-WALP monomer	2.92, 2.56[e]	1.29, 1.29[e]	18, 23[e]	21
C12-WALP monomer	2.75, 2.57[e]	1.29, 1.29[e]	20, 23[e]	22
C13-WALP monomer	2.76	1.32	22	22
C11-WALP dimer	2.77	1.29	20	20
C12-WALP dimer	2.98, 2.53[e]	1.27, 1.29[e]	15, 24[e]	20
C13-WALP dimer	2.81	1.30	20	20

a. dichroic ratio of amide I bands
b. dichroic ratio of lipid v(C=O) bands
c. maximum tilt angles
d. average
e. duplicate measurement

the dichroic ratios of the $\gamma_w(CH_2)$. High ratios indicate high degrees of order. For the C11-WALP dimer values of ~9 were calculated for the CH_2 wagging vibrations, implying that the acyl chains were well oriented. In Table 2 the maximum tilt angles θ are depicted, obtained from all calculated, assumed and known parameters using the ATROPTIC 1.0 software tool 'stdwave'. All monomers and dimers exhibited similar tilt angles of around 20º. For three of the WALP analogues the orientation was re-measured with another aliquot of the same sample, to yield an approximate experimental error of 2-5º.

Solid state ^2H nuclear magnetic resonance fingerprinting

In the C11-, C12- and C13-WALP analogues an Ala-d_3 was incorporated at the 17th position. In an oriented system, the quadrupolar splitting in the spectrum depends on the orientation and dynamics of the C-D_3 bond and thus of the peptide in the magnetic field. Because of fast rotation of the peptide around the bilayer normal, the same information can be derived from unoriented systems, as was shown in previous research.[29] In Figure 4 the spectrum of the C12-WALP monomer in di-18:1 PC, at a P/L ratio of 1:100, is depicted. The splitting for this spectrum was 6.0 kHz. The C11- and C13-WALP monomers showed similar splittings (data not shown). These $\Delta\nu_q$ values are comparable to those found in previous measurements on a WALP23 with the label on the 17th position in unoriented di-18:1 PC-vesicles.[29]

The ^2H-NMR spectra of all dimers in multilamellar vesicles of di-18:1 PC were also measured. The C12-WALP dimer showed an increase in splitting of 1.7 kHz with respect to that of the monomer (Figure 4). In addition, the outer peaks may be slightly broadened in comparison that of the monomer. This demonstrates that the C12-WALP monomers and dimers behave differently in the bilayer. There may be a change in tilt or dynamics of the peptides. The fact that the C12-WALP dimer exhibited one single splitting indicates that both helices in the dimer behave similarly. Unfortunately the dimers of the C11- and C13-WALP in bilayers of both di-14:1 and di-18:1 PC showed spectra with an isotropic-like shape. The spectrum of the C13-WALP dimer in di-18:1 PC, recorded at 40ºC, is shown in Figure 4. This sample was also measured at 60ºC (data

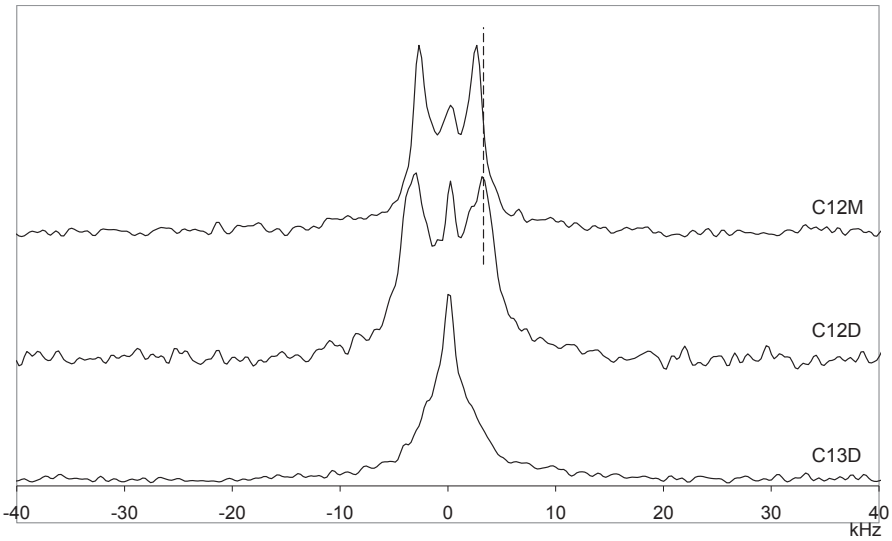

Figure 4. 2H-NMR spectra of C12-WALP monomer (C12M), C12-WALP dimer (C12D) and C13-WALP dimer (C13D) in di-18:1 PC with helix/lipid ratios of 1:100

not shown), which yielded a comparably shaped spectrum, indicating that the origin of the shape was not a restriction of motional averaging of the dimer. In addition, the shape was probably not caused by deformations of the bilayer. Solid state ^{31}P-NMR spectra of all samples were characteristic for the expected organization of bilayers into the lamellar liquid crystalline phase (L_α) (data not shown). Apparently, the dimers of the C11- and C13-WALP analogue behave differently than the C12-WALP dimer. The possible explanation(s) for the isotropic-like shape of the spectra can be manifold (see discussion) and it was decided not to perform any further experiments with the C11- and C13-WALP dimers.

To further explore the difference between the C12-WALP dimer and the monomer in di-18:1 PC, ^{2}H-NMR spectra of both peptides were recorded in a series of (mis)matching lipids. The calculated splittings of the C12-WALP mono- and dimers in di-14:1, 16:1, 18:1, 20:1 and 22:1 PC are shown in Figure 5. The splitting of the monomers is more or less constant in all lipid bilayers, which indicates that there is no change in tilt and/or dynamics for the peptides. The $\Delta\nu_q$ of the dimers is consistently higher than that of the monomer in all lipids. This suggests that, in comparison to the monomer, the dimer may have less dynamical averaging and/or an increased tilt and/or a different rotational angle. For the dimers a minimum is found for matching conditions and the splitting increases with both positive and negative mismatch (Figure 5). The position of the minimum suggests that the main cause for the differences in splittings is a change in dynamics of the dimer (see concluding discussion).

Coarse-grained simulations

To complement the ATR-FTIR and ^{2}H-NMR data, coarse-grained (CG) simulations using the MARTINI force field were performed. The main advantage of using CG is that a longer sampling time can be achieved at lower computational cost.[35] The orientation and dynamics of a WALP23 were compared with that of a parallel and an antiparallel dimer of the C12-WALP analogue in di-18:1 PC. Snapshots of the dimers are shown in Figure 6, where it is also visible that the disulfide bonds have different conformations. Two simulations of 10 μs (40 μs physical time) were performed for the monomers and both dimers.

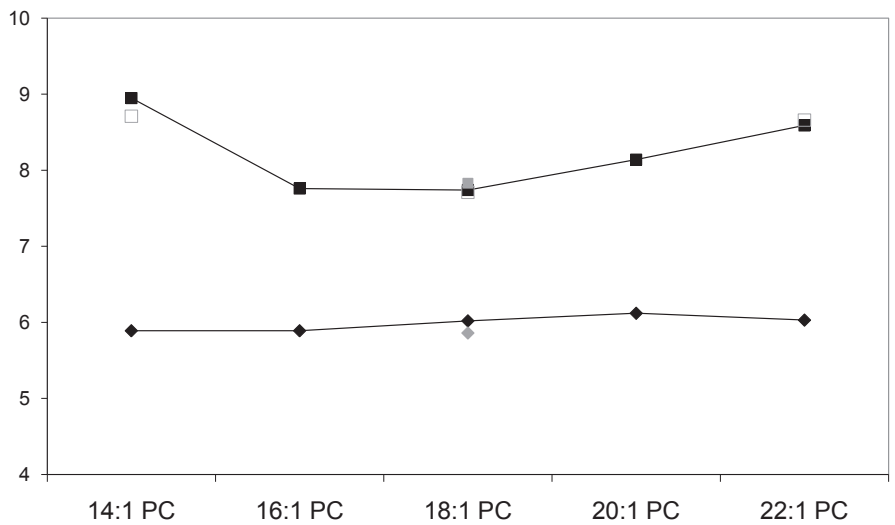

Figure 5. 2H-NMR splittings of C12-WALP monomers (diamonds) and dimers (squares) in vesicles of varying lipid content, with helix/lipid ratio of 1:100 (solid black), duplicate experiments in 18:1 PC (solid grey) and with helix/lipid ratio of 1:50 (open grey)

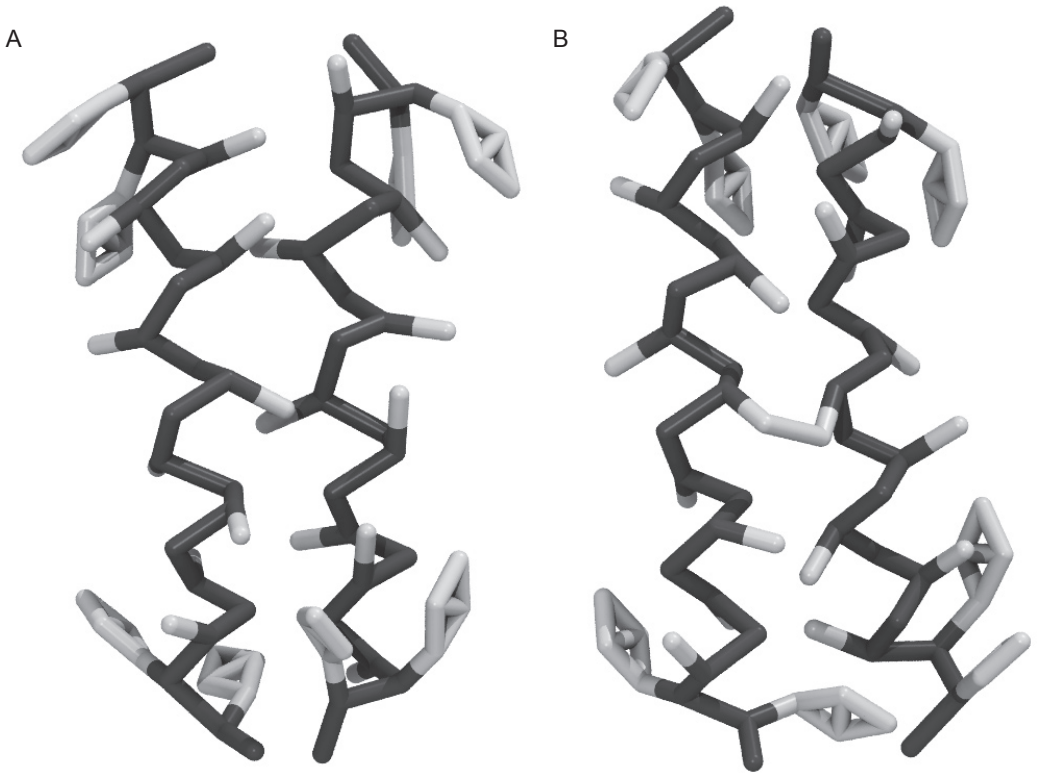

Figure 6. Snapshots of antiparallel (A) and parallel (B) dimer of C12-WALP

In Figure 7A, the tilt distributions in two simulations (SIM1 and SIM2) for the two helices (helix 1 and helix 2) in the antiparallel and parallel dimers (upper and lower panel) are shown together with the results for the monomer (WALP23 monomer). As can be seen, the distributions are reproducible between the two simulations. This implies that the tilt has converged properly. For the monomeric WALP23 an average tilt angle of 10º is found. Strikingly, the tilt angles of all helices in the dimers are higher than the tilt of the monomeric helix. The change is subtle, with differences of 2-6º, but significant. Another remarkable result is the difference between the parallel and antiparallel dimer. In the parallel dimer the helices have different tilt angles, where in the antiparallel dimer both helices have the same tilt. This is probably related to the respective conformation of the disulfide bridge and to the helix-helix packing. Also the distributions of the azimuthal rotation, depicted in Figure 7B, show differences for the three simulated peptides. First, the distribution of rotations is narrower for the helices in both dimers than for the helix in the monomer. This makes sense since the rotation of the helices in the dimers is restrained by the disulfide bond and helix-helix packing. More noticeable are the rotational differences between the parallel and antiparallel dimers. The helices in the parallel dimer (helix 1 and 2, lower panel) have different rotation angles, while the helices in the antiparallel dimer (helix 1 and 2, upper panel) both have the same rotation. In the second simulation, the rotational angles of the helices in the parallel dimer are reproduced. However, another azimuthal rotation is found for both helices in the antiparallel dimer. This suggests that there are two possible states for the antiparallel dimer. In between the two conformations there must be a high free energy barrier, since the peptides are

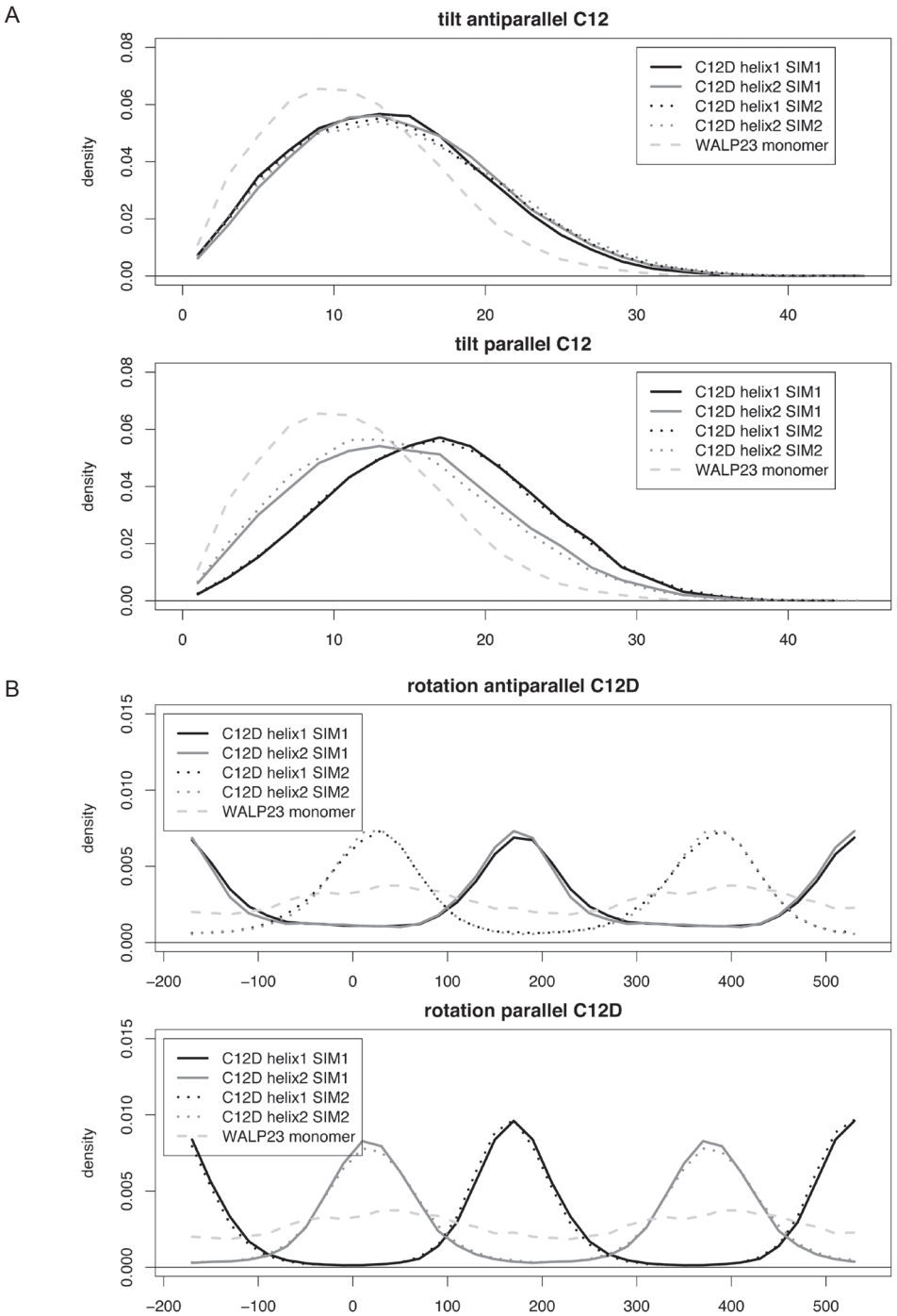

Figure 7. Distributions of the tilt (A) and rotational (B) angles in CG simulation 1 (solid line) and 2 (dotted line) of helix 1 (black) and helix 2 (grey) of the antiparallel (top panel) and parallel (bottom panel) dimers of the C12-WALP analogue (C12D) and a WALP23 (black broken line) in di-18:1 PC. Each distribution has been duplicated for convenience

stuck in one state and no transition occurs within the 10 μs of the simulation.

The mean square displacements (MSDs) of the monomer and the C12-WALP dimer in the CG simulations are presented in Figure 8A. The dimers showed similar diffusion for both orientations in both simulations (data not shown). Therefore only the line of the mean square displacement of the antiparallel dimer is shown. The graph demonstrates that WALP23 diffuses faster than the C12-WALP dimer. Because of the finite size of the simulation box, an artificial movement of the bilayer relative to water occurs in MD simulations.[36] This artificial motion was systematically taken out and subsequently the self-diffusion constant was calculated from the MSD curve using Einstein's law.[36] To evaluate a standard deviation on the diffusion constant, the MSD was evaluated on each half simulation giving four values per peptide. The self-diffusion constants were calculated to be $(10.7\pm1.8)\cdot10^{-8}$ cm^2/s for

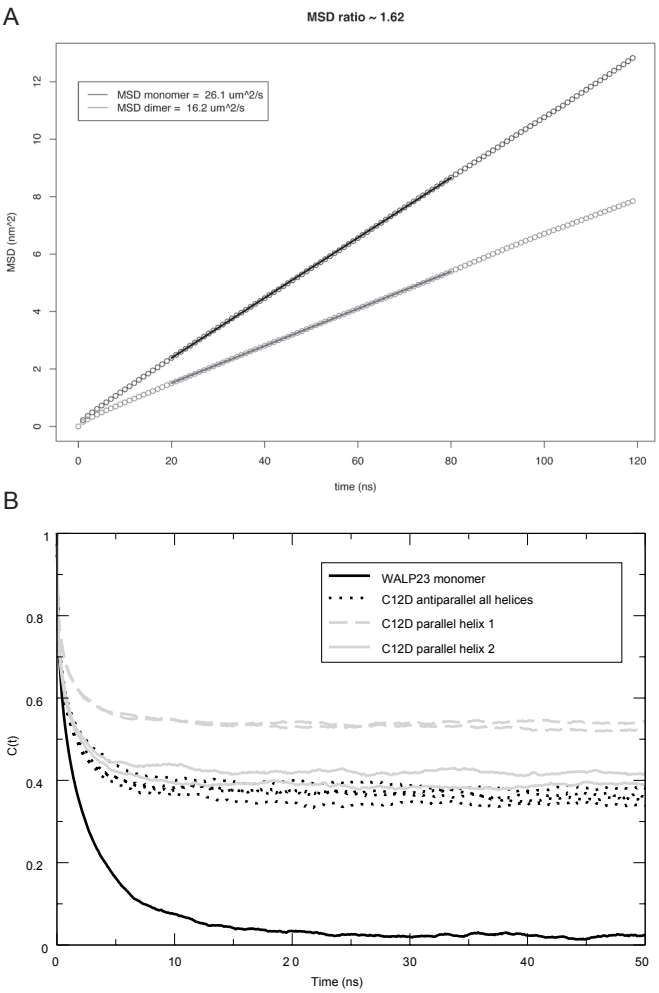

Figure 8. Mean square displacement of WALP23 monomer (black) and C12-WALP antiparallel dimer (grey) that is representative for both dimers in both simulations (A) and rotational autocorrelation function of helix in WALP23 monomer (solid black line), helices in C12-WALP antiparallel dimer (dotted lines), and helix 1 (dashed grey lines) and helix 2 (solid grey lines) in C12-WALP parallel dimer, calculated using a first order Legendre polynomial.

WALP23 and (7.6±0.8)·10^{-8} cm^2/s for the C12-WALP dimer, from the MSD curves with a fitting procedure described previously.[36] The value for WALP23 is on the same order of magnitude as a recent value determined by fluorescence in similar lipids (*i.e.* ~ 5 x10^{-8} cm^2/s).[21] The over-estimation of the calculated value as compared to the experimental one is expected, since there is less friction in CG simulations. According to the simulations, the diffusion is approximately 1.4 times slower for the dimers in comparison to the monomer at a 1:200 P/L ratio. To gain insight into the dynamics of the helix rotation in the C12-WALP dimers and WALP23 monomer the rotational autocorrelation function (C(t)) was computed (Figure 8B). The decay of the function is faster for the monomeric helix than for any of the helices in the dimers, which reflects slower relaxation dynamics for the latter. The C(t) value at infinite time gives the order parameter and is thus related to the spatial restriction of the motion of the rotation vector. The motion of each helix within the dimer is systematically more restricted in comparison to the monomer. The slower dynamics and the larger restriction of motion in the dimer compared to the monomer is intuitively explained by the presence of the S-S bridge and helix-helix packing. Interestingly, the motion of helix 1 in the parallel dimer (dashed gray lines) is more restricted than that of helix 2 (solid gray lines). This is consistent with the distributions of rotation presented in Figure 7B (bottom panel), in which the distributions of helix 1 (black lines) is narrower than that of helix 2 (grey lines). The range of motions of both helices in the antiparallel dimer is identical to that of helix 2 in the parallel dimer. The differences in restriction of motion between the helices in the dimers might be due to the conformation of the disulfide bridge which is *trans* in the antiparallel dimer and *cis* in the parallel dimer (Figure 6).

Comparison of the effect on lipids of monomers and dimers

Solid state ^2H nuclear magnetic resonance experiments

The effect of mono- and dimers on the acyl chain ordering of di-14:0 PC was investigated by solid-state ^2H-NMR. To this purpose, spectra of multilamellar vesicles of di-14:0 PC-d_{54}, which has both acyl chains perdeuterated, were measured at 40ºC with and without peptides. An increased spectrum width denotes that the order of the acyl chains is increased by the peptides. In Figure 9 two sets of spectra are shown to compare the effects of C12- and C13-WALP analogues with their dimeric assemblies. For convenience and to allow direct comparison, the helix/lipid ratio is used, instead of the peptide/lipid ratio. It can be seen that all peptides had an ordering effect on the acyl chains. The mono- and dimer of the C12-WALP had similar effects on the order of the lipids at each particular helix/lipid ratio (Figure 9A). For the C13-WALP mono- and dimer it was shown that at both helix/lipid ratios, the dimer had a slightly more pronounced effect than the monomer (Figure 9B).

To adequately compare the effect of monomers and dimers, the differences in the amount of lipids surrounding the monomers and dimers has to be taken into account. The amount of lipids neighbouring the peptides can be approximated using the cross sectional diameter of both components. From AFM data on linear aggregates of WALP peptides it can be derived that the diameter of the model peptides is 1 nm.[37] The lipid area of di-14:0 PC is 60 $Å^2$ (fully hydrated, 30ºC) yielding a diameter of 0.9 nm.[38] With this information it was estimated that the monomers have 12 neighbouring lipids and the dimers 16. Following this assumption, the amount of lipids, per 120 lipids in total, which can be alongside a helix, was calculated for the applied P/L ratios. In Table 3 these amounts are given, together wit the full width at the half-height (FWHH) values

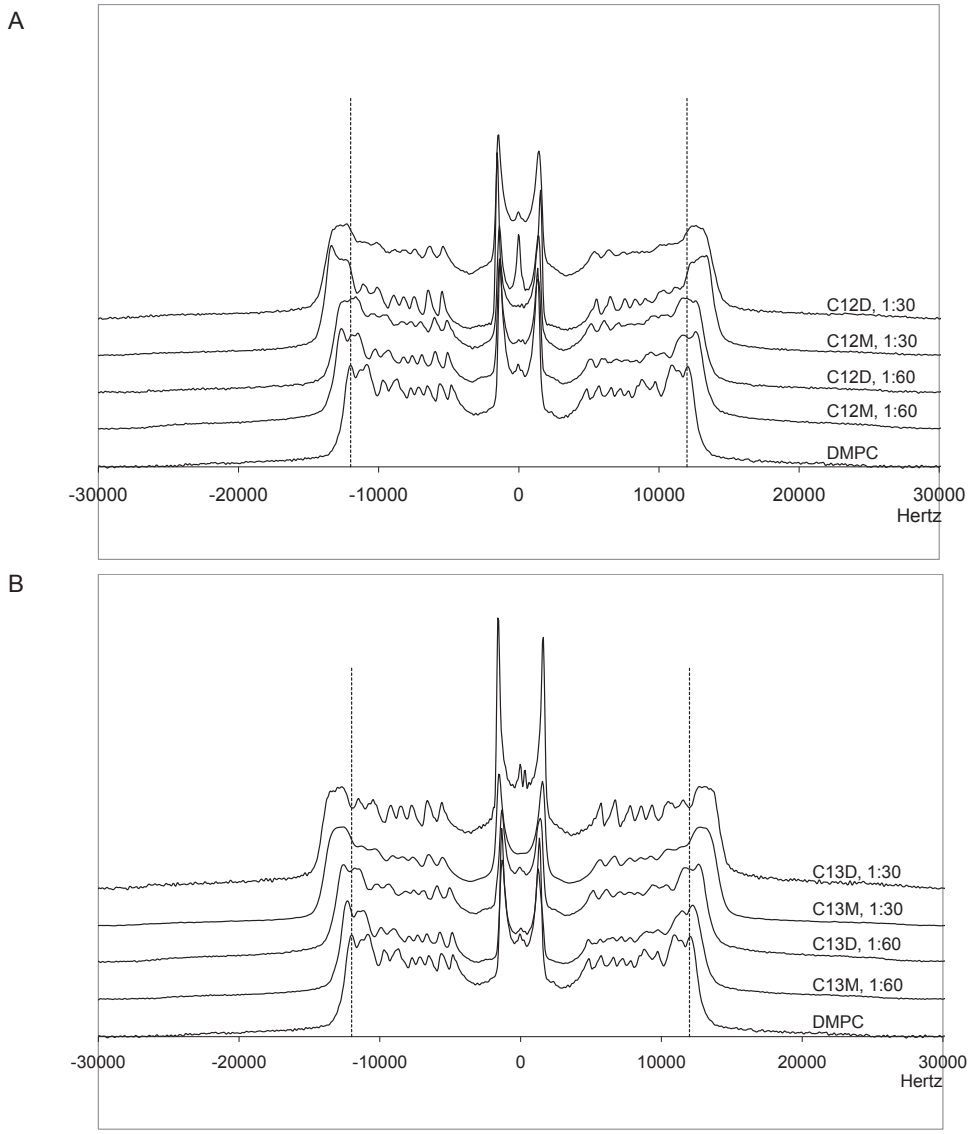

Figure 9. 2H-NMR spectra of di-14:0 PC-d54, at 40ºC, without peptide (DMPC) or with the C12-WALP monomer (C12M), C12-WALP dimer (C12D), C13-WALP monomer (C13M) or C13-WALP dimer (C13D) at helix/lipid ratios denoted at the graphs. Spectra to compare the C12-WALP mono- and dimers at two helix/lipid ratios (A) or the C13-WALP mono- and dimers at two helix/lipid ratios (B) are shown.

of the spectra in Figure 9. Typical errors are estimated to be maximally 0.2 kHz, as also determined in previous research on di-14:0 PC-d_{27} [39]. Hence, the increases in FWHH values are significant. At each helix/lipid ratio, the dimers show a similar or larger FWHH than the monomers, even though fewer lipids are adjacent to the dimers in comparison to monomers. Thus, the effect on the acyl chains per lipid is more pronounced for the dimers than for the monomers. When comparing the C12- and C13-WALP it can be seen that the differences between monomers and dimers are more pronounced for the C13-WALP.

Table 3. Comparison of lipids surrounding monomers and dimers with different peptide/lipid ratios and ordering effect of the WALP analogues on the di-14:0 PC-d54 lipids

Peptide	Helix/ lipid	Surrounding lipids[a]	C12-WALP		C13-WALP	
			FWHH[b]	Broadening[c]	FWHH[b]	Broadening[c]
Monomer	1:30	48	27.9	2.6	28.1	2.9
Dimer	1:30	32	28.0	2.7	28.5	3.2
Monomer	1:60	24	26.6	1.4	26.0	0.7
Dimer	1:60	16	26.7	1.4	26.6	1.4
No peptide	-	0	25.3	-	25.3	-

a. Approximation of amount of lipids neighbouring a helix when an amount of 120 lipids in total is regarded
b. Full width at the half-height (kHz) of the spectra in Figure 9, with an estimated maximum error of 0.2 kHz[39]
c. Difference in full width at the half-height (kHz) with respect to 'no peptide'

Attenuated total reflection- Fourier transform infrared experiments

With ATR-FTIR the changes in the acyl chain conformation of di-14:0 PC during the gel to liquid crystalline phase transition were established without peptide and with the C12-WALP mono- and dimer. For these experiments a hydrated film of the sample was heated from 15ºC to 40ºC, the range in which the phase transition of di-14:0 PC occurs. Conformational changes can be derived from the position of the lipid $v(CH_2)$, which shifts to higher wavelengths upon gauche bond formation of the chains.[40] In Figure 10 the values for the CH_2-stretching vibrations of the sn-1 chain are shown as a function of temperature. When no peptide was present, a shift from 2917.6 cm^{-1} till 2921.9 cm^{-1} was observed. A sharp increase of the amount of gauche bonds occurred, starting at the phase transition temperature of 23ºC. This is as expected, since the amount of disorder increases when going from the gel to the liquid crystalline phase. The C12-WALP monomer shows a 'flattening effect' on the di-14:0 PC phase transition curve. The di-14:0 PC is more disordered in the gel phase and more ordered in the liquid crystalline phase. In comparison to the monomer, the C12-WALP dimer shows slightly less ordering in the gel phase, but significantly more ordering in the liquid crystalline phase. Since the helix/lipid ratios were the same for monomers and dimers, the data imply that the dimer has a more pronounced effect than the monomer on di-14:0 PC in the liquid crystalline phase. Because it was estimated that there are less lipids surrounding the dimer than the monomer (see previous paragraph), the effect per lipid is even larger for the dimer.

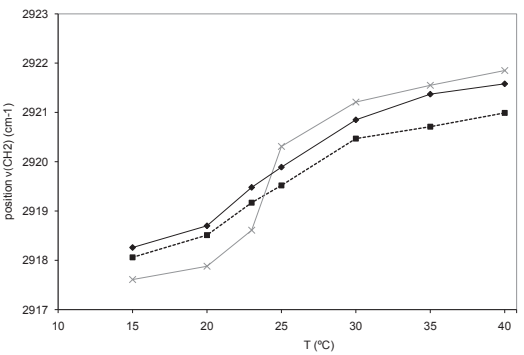

Figure 10. Positions of v(CH2) of di-14:0 PC without peptide (grey, crosses) or with C12-WALP monomers (solid black, diamonds) and dimers (broken black, squares) incorporated at a helix/lipid ratio of 1:30

Concluding discussion

In the results section, we presented the first step towards a systematic investigation of the influence of cross sectional diameter and helix-helix interactions on the properties and lipid interactions of transmembrane helices. Here, first the structural characterization of all peptides will be summarized. Then, the comparison of monomers and dimers and the comparison of the different dimers are considered. Finally, the new insights that were obtained on the behaviour and properties of natural membrane proteins, by comparing monomeric and dimeric WALP model peptides, will be evaluated.

Characterization of monomers and dimers

All monomers and dimers employed in this chapter consisted of highly defined and stable α-helices in di-14:0 PC. This was derived from the positions and narrow band shape of the amide I and II vibrations and the minimal degree (20-30%) of deuterium exchange, as measured by ATR-FTIR. Exchange of membrane proteins may also be low, such as for bacteriorhodopsin, which levels at 30% exchange, indicating that the model peptides mimic the behaviour of more complex assemblies quite well. In addition, the incorporation properties of all peptides were similar, as was shown by their similar amide I band intensities with respect to the lipid ν(C=O) band. These results are in agreement with the results obtained by CD in chapter 2. The CD-spectra showed that the WALP monomers and dimers were α-helical in DDM, SDS and di-14:0 PC, even under denaturing conditions and that the peptides had comparable incorporation properties. Apparently, the three different forced interfaces of the dimers do not affect their stability differently.

Comparison of properties and lipid effects of monomers and dimers

In the following paragraphs, we will discuss the similarities and differences in the properties and lipid effects of monomers and dimers. Since most data was obtained with the C12-WALP mono- and dimers, these results will be used in the comparison of monomers and dimers. The results obtained with the employed techniques will be further discussed and the outcomes of the different approaches combined.

The tilt of monomers and dimers is similar

For the three WALP analogues and their dimeric assemblies similar tilt angles of ~20º were calculated from dichroic ATR-FTIR spectra. This value is analogous to tilt angles recently obtained for WALP23 in di-14:0 PC by the high resolution solid state NMR technique, MACADAM.[41] Additionally, this tilt angle is similar to the average tilt of 21º, found for a set of membrane proteins.[42] The tilt with respect to the membrane normal is not necessarily the tilt with respect to the lipid acyl chains, since these may also tilt. However, above the phase transition temperature it is not likely that the acyl chain tilt is considerable. It is striking that no significant differences in tilt angle were observed for dimers in comparison to monomers. However, changes in tilt have to be relatively radical to be detected by ATR-FTIR. Therefore, it is possible that systematic changes do occur, but are too subtle to detect. Indeed, the simulation data show an increase in tilt of 2-6° for the C12-WALP dimer, which is a significant but not major difference.

It has to be noted that the angle of 20º determined by ATR-FTIR represents the maximum tilt angle. Deviations in the applied parameters can result in a decrease of the calculated tilt. First, the order of the system was assumed to be 1. Any disorder will decrease this value and thereby the tilt. The assumption of complete order of the system was validated by the high dichroic ra-

tios of the $\gamma_w(CH_2)$ bands. With this well-defined lipid orientation, it is unlikely that the peptides will show disorder. Second, for the orientation of the amide I dipole with respect to the helix long axis the widely used value of 27º, determined for bacteriorhodopsin in purple membrane was applied in our calculation.[34] In literature, angles ranging from 20-40º are reported.[43] When using the most recent value of 38º, determined for the water soluble polyglutamate α-helix oriented on silicon substrates,[44] the dichroic ratios were out of range, implying that the helices should be tilted less than parallel to the membrane normal. Since this is physically not possible, the value of 27º appeared to be the most appropriate to determine the tilt angles of the WALP peptides in di-14:0 PC and it certainly is appropriate for the sake of the comparison.

The tilt angle of 10º, found in coarse grained simulations of a WALP monomer in di-18:1 PC, is lower than that determined for the current monomers in di-14:0 PC by ATR-FTIR and than that determined by fluorescence for a WALP in di-18:1 PC[45]. However, it is in line with recently published CG simulations on a WALP23, which predict a residual tilt around 10° for the peptide under negative mismatch or in matching conditions.[46] As mentioned above, the MACADAM method has recently been applied to WALP23 in di-14:0 PC. A tilt of 21° was determined.[41] It would be interesting to apply a similar approach to a peptide in negative mismatching conditions such as WALP23 in di-18:1 PC.

Dimers are less dynamic than monomers

With ^{2}H-NMR measurements of the Ala17-d_3 in the C12-WALP analogue, differences between monomers and dimers were clearly observed. Most evident is the increase in $\Delta\nu_q$ of the dimer with respect to the monomer spectra. In the matching di-18:1 PC an increased splitting of 2 kHz was found. This increase can be due to a change in tilt and/or rotation or a decrease of the dynamics of the dimer. A combination is also possible. With dichroic ATR-FTIR no change in tilt could be detected and with simulations a significant but only small difference in tilt angles was found. This suggests that the increase in splitting cannot be explained exclusively by a change in tilt. With simulation, also information on the rotational differences between monomers and dimers was obtained. However, on basis of the present results, it is difficult to draw conclusions about the possibility of rotational differences underlying the differences in the ^{2}H-NMR spectra. Regarding dynamics, the slight broadening of the outer peaks in the dimer-spectrum in comparison to that in the monomer-spectrum may also indicate a decrease in motional averaging. To obtain further information specifically on the dynamics of WALP monomers and dimers, coarse-grained simulation were performed.

A restriction of dynamics for dimers with parallel and antiparallel topology in comparison to a WALP monomer was found in coarse-grained simulations. The lateral diffusion of the dimers was 1.4 times lower than that of the monomers, as shown by the mean squared displacements of the peptides. In addition, the narrow distributions of the azimuthal rotations and the rotational autocorrelation function of the helices in the dimer indicated that they were motionally more restricted than the monomers. This makes sense, since the helices in the dimer are restrained by the disulfide bond. Finally, the decay of the rotational autocorrelation function also showed slower relaxation dynamics for the dimers in comparison the monomer. Thus, next to tilt, dynamics may have an important influence on the ^{2}H-NMR splittings of the dimers. The changes in tilt and dynamics might be related. From results in a recent article, it may be derived that the small increase in tilt of the dimer is partly responsibly for the decrease in lateral diffusion.[47]

To further investigate the differences between monomers and dimers, ^{2}H-NMR spectra were recorded of the mono- and dimers in a series of lipids. The results showed that the lipid de-

pendence of the splittings was different for the monomers and dimers. The $\Delta\nu_q$ of Ala17d_3 of the monomer was constant, regardless of the lipids employed, with values around 6 kHz in both positive and negative mismatch. In previous ^{2}H-NMR experiments with a WALP23-A17d_3 in di-18:1, -14:0, -13:0 and -12:0 PC the splitting increased 1.5 kHz with increasing positive mismatch.[29] This was presumably caused by an increase in tilt as a response to mismatch. The difference in lipid-dependence of the C12-WALP monomer in comparison to the WALP23 seems strange. However, regarding the responses to positive mismatch it has to be noted that in the published study saturated lipids were employed while we used lipids with mono unsaturated acyl chains. Furthermore, the results obtained with di-20:1 and 22:1 PC cannot be compared to previous research since the response to negative mismatch has not been addressed before in ^{2}H-NMR investigations. Contrasting the monomers, the $\Delta\nu_q$ of the C12-WALP dimers varied with the hydrophobic length of the lipids. A minimum in splitting was found for the lipids that match the hydrophobic length of the dimer most; di-16:1 and -18:1 PC. If $\Delta\nu_q$ would mainly depend on tilt, a more or less linear relationship between splitting and acyl chain length would be expected, because in positive mismatch the peptide will be tilted most and in negative mismatch least. An increase in splitting due to a decrease in motion would more probably be lowest in matching and highest in mismatching lipids, because it is expected that with both positive and negative mismatch the peptide is more restricted in its motions. Thus, the splittings of the dimers in the series of di-14:1 PC to di-22:1 PC supports our hypothesis that not only tilt, but also dynamics is a determinant of the splitting in the C12-WALP dimer spectrum.

The topology of the C12-WALP dimer is most probably antiparallel

Coarse-grained simulations showed differences in orientation for the dimers with the helices in parallel or antiparallel topology. The helices in the antiparallel dimer showed the same tilt and rotation angles. Conversely, for the parallel dimer different angles were found for both helices. The dependence of the orientation of the helices on their topology is related to the packing in the dimers and may be explained by the preference to arrange the helices antiparallel. This explanation is supported by previous research in which it was suggested that WALP peptides favour an antiparallel topology.[28, 46] The fact that a single splitting was found for the C12-WALP dimer in ^{2}H-NMR also indicates that both helices have a similar orientation. The NMR results seem consistent with the simulations of the antiparallel dimer. In the simulations of the parallel dimer different tilt and rotation angles are found for the two helices, which would result in two splittings. In the antiparallel dimer, simulations show a similar tilt for both helices. However, the rotational data show that, though both helices have the same rotation in the two simulations, there are two different possible values for this rotation. This should give rise to two different quadrupolar splittings, unless the conformation exchange is fast as compared to the NMR time scale. It has been shown recently that fast (within hundreds of nanoseconds) exchange occurs for WALP dimers that are not covalently coupled.[46] For the C12-WALP dimer the exchange is probably more difficult, because of the disulfide bridge, but likely not impossible. Work to assess this possibility of exchange is currently ongoing

Dimers have a stronger influence on surrounding lipids than monomers

In ATR-FTIR measurements, the mono- and dimers show a 'flattening' of the lipid phase transition curve. In the gel phase the peptides order the lipid acyl chains, while in the liquid phase they have a disordering effect on. Similar effects were shown for gramicidin in lipid bilayers, though with this protein the effect in the liquid crystalline phase depends on its concentration.[48-53] The broadening of the phase transition

was also found in previous differential scanning calorimetry measurements of WALP peptides of several lengths in di-16:0 PC.[19]

Dimers had a more pronounced ordering effect on the acyl chains of mismatching lipids than monomers. This was shown by ^{2}H-NMR and ATR-FTIR measurements of di-14:0 PC vesicles with monomers or dimers incorporated. In the experiments, the number of helices was kept constant. Consequently, there were fewer lipids in direct contact with the dimers than with the monomers. Since the dimers had a similar or increased effect on the acyl chains in comparison to the monomers, it can be concluded that the effect per adjacent lipid is more pronounced for the dimers. The increased effect of a dimer on the lipids may be related to the suggested decrease in dynamics.

Comparison of properties and lipid effects of dimers with different interfaces

There are not only differences between the monomers and dimers, but also between the three dimers. These differences are probably related to the varying interfaces of the covalent assemblies, resulting from the positions of the cysteine residues in the C11-, C12- and C13-WALP analogues (Figure 11). The forced helix-helix interfaces will result in different packing and helix-lipid interfaces. Thus, the hydrophobic profiles and the interactions of the tryptophan residues with the lipids will be different for the three dimers. Besides this, in the C11- and C13-WALP dimer there are two tryptophan residues pointing towards the helix-helix interface, which may cause steric hindrance. In the C12-WALP dimer this will not be the case, since all the tryptophan residues face outward.

The ^{2}H-NMR measurements of the Ala17-d_3 in the peptides showed an isotropic-like peak for the C11- and C13 WALP dimers and a clear splitting for the C12-WALP dimer. It is likely that this difference is related to the differences in helix-helix interface. The shape of the ^{2}H-NMR spectra of the C11- and C13-WALP dimers in comparison to that of the C12-WALP dimer can have numerous causes. One option is a difference in tilt or rotational angle of the C11- and C13-WALP dimers with respect to all monomers and the C12-WALP dimers. As was observed in previous research, Ala-d_3 residues at some position along the helix axis show isotropic-like signals, related to the peptide tilt and rotation.[29, 54] Asymmetry in the tilt and rotation of the two helices in a dimer may also generate an isotropic-like signal. It can be envisaged that a distribution of orientations and some motional averaging, together with the peak of residual deuterium in the water, add up to a spectrum that appears

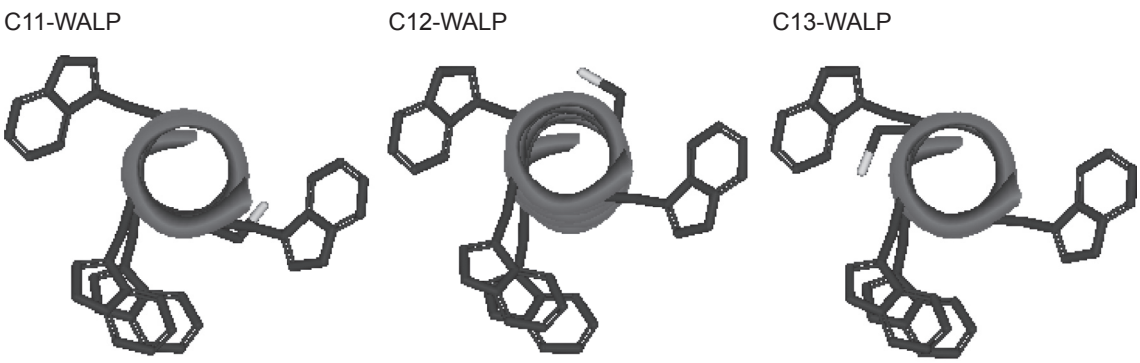

Figure 11. The orientation of the tryptophan residues with respect to the cysteine residue. The tryptophan residues are depicted in stick representation in dark grey and the cysteine residue in stick representation with the SH in light grey. In this view the tryptophan residues at the right- and right bottom-side of each helix are in one leaflet of the bilayer and the tryptophan residues at the left- and left bottom-side in the opposite leaflet.

isotropic. Indeed, the ^{2}H-NMR spectrum of the C11- and C13-WALP dimers could be fitted better with one Lorentzian and two relatively small quadrupolar splittings than with one Lorentzian. Related to this, it is possible that fast rotation of the peptide around the bilayer normal is restricted in the C11- and C13-WALP dimers, because of their potentially other conformation than that of the C12-WALP dimer. Another ground for an isotropic like peak could be aggregation. However, ATR-FTIR and CD strongly indicate similar incorporation properties of all dimers and monomers. Also, non-covalent oligomeric assemblies would more probably result in an increase in $\Delta\nu_q$ of the spectrum due to decreased dynamics. To determine the exact origin of the isotropic shape of the C11- and C13-WALP dimer spectra, extra experiments will be necessary, which are beyond the scope of this study.

For the C13-WALP, the dimer had a significantly more pronounced ordering effect on the lipid acyl chains than the monomer, as shown by solid state ^{2}H-NMR measurements with di-14:0 PC-d_{54}. For the C12-WALP the effects of mono- and dimer were rather similar. It is possible that this difference is related to differences in helix-helix and helix-lipid interactions. Alternatively, the point of connection may influence the hydrophobic length of the dimer. The coupling point in the C11- and C13-WALP dimers is slightly of centre. In case the dimers have an antiparallel arrangement, the connection may result in a somewhat larger hydrophobic length, hence causing a larger ordering effect. Furthermore, it is plausible that the difference in ^{2}H-NMR spectra of the peptides is related to the difference in ordering effect. The observed effects may have the same underlying mechanism or the different behaviour of the peptide might affect the lipids differently.

Implications of the results for membrane protein behaviour

In the introduction it was stated that there are several indications that differences in cross sectional diameter of membrane spanning segments can result in differences in lateral diffusion, incorporation properties, effect on lipid phase transition, lipid stretching effects and facilitating lipid translocation. By the comparison of monomeric and dimeric WALP model peptides, described in this chapter, a first step was taken towards a more systematic approach to investigate the influence of size. It was shown that dimerization affects the properties and lipid interactions of the model peptides. The results obtained with the mono- and dimers indicate that the cross sectional diameter and/or the corresponding helix-helix interactions of a membrane protein will significantly influence its behaviour and lipid interactions. Below, the data on orientation, dynamics and influence on lipids will be discussed in light of membrane protein structure and properties.

The ability to tilt did not seem to be largely increased for the dimers of all three WALP analogues. It is likely that the position of the disulfide bond between the helices restricts the assembly from tilting more easily than a monomer. With the covalent bond in the middle of the helix the two helices are considerably impaired in their movement with respect to each other and optimal helix-helix packing may not be possible. The current results indicate that it is likely that the flexibility to slightly change the interface is a requirement for easier tilting. Indeed, in polytopic membrane proteins the helices are usually either connected via relatively flexible extracellular loops or not connected, providing for this flexibility. For example in the mechanosensitive channel MscL, in which the helices have a large degree of freedom, the gating mechanism involves an iris-like expansion in which all helices rearrange.[55] In this process the interface between all the helices with respect to each other changes. Besides the point of connection, it is likely that the tryptophan flanking residues influence the tilting of the dimers, just as they do in the monomers.[45, 56] Thus, in case of the WALP peptides, the preferred orientation of the tryp-

tophan residues may have a larger influence than the oligomerization. This may again relate to the behaviour of the mechanosenstive channel, which does not contain tryptophan residues at the interface.

The, per adjacent lipid, more pronounced effect on acyl chain order of dimers is a clear illustration of the influence of size on protein-lipid interactions. As was stated in the introduction, WALP peptides only slightly increase the bilayer thickness in comparison to the rigid, β-helical gramicidin. The results in this chapter indicate that cross sectional diameter is at least part of the cause of this difference, either by itself or via an increase in rigidity. Since dimers appear to be the first step towards multispan membrane proteins, it is likely that the lipid effects of membrane proteins are also increased with regard to WALP monomers. This may have relevant implications for our understanding of membrane protein localization. It is known for a number of membrane proteins that they tend to localize to micro domains with matching lipids,[15, 23, 24, 57] while this has never been shown for WALP peptides.[25, 26] From the current results it is suggested that this difference may be related to the larger cross sectional diameter of membrane proteins via the increase of the penalty to reside in mismatching domains. However, the systems in which the localization studies have been performed are considerably different. To validate the above suggested underlying mechanism and size-dependence of localization, a systemic comparison in bilayers with the same lipid composition should be performed. It would be interesting to investigate whether dimers have a stronger tendency to localize to micro domains than monomers.

The differences in behaviour of the three dimers suggest that the helix interface is another determinant of membrane protein function. Factors like flexibility and packing of the helices will dictate the possibility to adapt to and interact with the surrounding lipids. It can be envisaged that if the membrane proteins are more tightly packed or have a forced configuration, the ability to adapt decreases. This may in turn cause an altered influence on lipids.

Experimental section

General

All chemicals were obtained from commercial sources, unless stated otherwise. Absorption spectroscopy was performed on a Perkin Elmer Lambda 18 spectrometer. ATR-FTIR spectra were recorded on a Bruker IFS 55 equipped with a liquid nitrogen cooled mercury cadmium telluride detector. Solid state NMR measurements were performed on a Bruker Avance 500 MHz NMR spectrometer.

Sample preparation

Stock solutions

Stock solutions of lipids were prepared by dissolving an appropriate amount of lipid in chloroform. The concentration of lipid stocks was determined according to the method of Rouser.[58] Typical lipid concentrations varied for the various biophysical measurements and will be specified in the following sections. For peptide stock solutions, peptides were dissolved in TFE and the exact concentration of the solution was determined with absorption spectroscopy, using the tryptophan absorption at 280 nm. For monomers an extinction coefficient of 22400 was used in the calculations[30] and for dimers a value of 44800 was taken. Typical concentrations of peptide stocks were 1-2 mM.

ATR-FTIR

Since weight and weight-ratio's are important in ATR-FTIR, the weight of peptide was held constant at 20 µg and the final concentration of peptide plus lipid in H_2O was 2 $\mu g \cdot \mu l^{-1}$ in all samples. Peptide (~8 nmol monomer, ~4 nmol dimer, 20 µg) in TFE was mixed with the appropriate amount of a 1.3 mM di-14:0 PC stock (~0.23 µmol) in chloroform to obtain a molar monomer/lipid ratio of 1:30 or a molar dimer/lipid ratio of 1:60. The sample was mixed vigorously on a vortex, the solvents evaporated under a N_2-flow and the film left under vacuum overnight. The samples were hydrated with H_2O (~90 µl) and freeze-thawed ten times by subsequently freezing in ethanol/dry ice and thawing in a 50ºC water bath.

^{2}H-NMR peptides and ^{31}P-NMR lipids

Typically a stock of lipids in chloroform with a concentration of around 100 mM was used. Lipid (18.75 µmol) in chloroform was mixed with the appropriate amount of peptide in TFE to obtain P/L ratio's of 1:100 or 1:200 for the dimers and 1:100 for the monomers. The solvents were evaporated under a N_2-flow and the films further dried under vacuum, usually overnight. The films were hydrated with 100 µl deuterium depleted H_2O and ten times subsequently frozen in ethanol/dry-ice and thawed in a water bath of 40°C. The samples were kept at -20ºC until use.

^{2}H-NMR lipids

In all samples 3 µmol of di-14:0 PC-d54 from a 29 mM stock in chloroform was mixed with the appropriate amount of peptide in TFE. In the pure lipid sample no peptide was added, but only some TFE. Helix/lipid ratio's of either 1:30 or 1:60 were used, resulting in 4 samples; monomer/lipid 1:30 (0.1 µmol monomer), monomer/lipid 1:60 (0.05 µmol monomer), dimer/lipid 1:60 (0.05 µmol dimer) and dimer/lipid 1:120 (0.025 µmol dimer). Chloroform and TFE were evaporated under a N_2-flow. The resulting films were further dried, usually overnight, under vacuum and hydrated with 100 µl of deuterium-depleted water. The lipid suspensions were subjected to ten cycles of subsequently freezing in ethanol/dry ice and thawing in a water bath of 50ºC.

Biophysical analysis

ATR-FTIR measurements

The internal reflection element was a trapezoidal germanium plate. The crystal was cleaned and made more hydrophilic by washing with water and a soap with basic pH, distilled water, methanol and chloroform successively. For the regular, dichroic and H/D-exchange spectra 10 µl of samples was applied on the surface. Excess water was slowly evaporated under a N_2-flow while spreading the sample with a pipette-tip. The plate was placed in a sample holder to achieve the appropriate incident angle of the IR light into the IRE, yielding 25 reflections. For the non-polarized spectra, first a background was measured on an area of the germanium plate without sample, averaging 128 scans. Next the sample was measured as an average of 128 scans with automatic subtraction of the background, a resolution of 2 cm^{-1}, in the range of 800-4000 cm^{-1}. The dichroic spectra were recorded similarly, except that the incident light was polarized parallel or perpendicular to the incident plane. For the H/D exchange measurements, one background spectrum was recorded which was used for automatic correction of all recorded spectra. The sample holder was attached to a D_2O-saturated nitrogen flow. A spectrum was recorded every minute for half an hour, where the first measurement was collected after three minutes. Furthermore, the same settings and corrections as described above were employed. All spectra mentioned above were recorded at room temperature. For the temperature dependent spectra a slightly different procedure was followed. Two 30 µl aliquots of two different samples were spread at two separate areas of the IRE and dried under a nitrogen flow. The compartment of the sample holder in touch with the samples was filled with H_2O. The temperature of the holder and thus of the H_2O was controlled by a water bath. At temperatures of 15, 20, 23, 25, 30, 35 and 40ºC, one background was recorded followed by measurement of both samples, which were automatically corrected with this background.

ATR-FTIR data analysis

All spectra analysis was performed by Kinetics, a software running under Matlab 7.5.0 (R2007b). All spectra were first corrected for contributions of water vapour and CO_2, a 4 cm^{-1} apodization was applied and a 1783-1598-1510-1401-1130-999 baseline was subtracted. Peak positions and areas were determined with respectively the peak picking and integration tools in Kinetics. The exchange of the peptides was established using the amide II band area (1760-1700 cm^{-1}). The exchange kinetics was calculated and fitted, considering the area of the first spectrum as 100% hydrogen and normalizing on the lipid ν(C=O) area (1590-1510 cm^{-1}). The tilt was computed with the homemade 'Stdwave' software, routinely used in the Goormaghtigh-group. The dichroic ratios, defined as $A_{\parallel}/A_{\perp}$, R^{ATR} and R^{iso} were calculated from the amide I and lipid ν(C=O) band areas respectively. In the standing wave program, the following parameters were specified: n_1=4.00 (germanium), n_2=1.44 (lipids), n_3=1.0 (air), real membrane thickness computed from the R^{iso} value, 27º angle between molecular axis and dipole of peptide ν(C=O). Subsequently, the tilt angle was determined from the dichroic ratio R^{ATR} with the 'angle' polarizer tool in the software.

Solid state NMR measurements

Solid state ^{31}P-NMR spectra were recorded of all samples. The measurements were performed at 202.5 MHz using a Hahn echo experiment with a 5 µs 90º pulse, a 10 µs 180º pulse, an echo delay of 50 µs and a relaxation delay of 2 s. A 100 kHz spectral width, 4096 data points and gated proton decoupling were applied. The measurements were performed at room temperature and 400-1000 scans were collected. The spectra were processed with zero-filling to 2048 data points and a 100 Hz exponential multiplica-

tion before Fourier transformation. The ^{2}H-NMR spectra of the peptides were performed essentially as described previously,[29] except that the 90º pulse was 2.5 µs, 2048 data points and no zero filling were used, 300k-800k scans were collected and a 200 Hz exponential multiplication was applied. The di-14:0 PC-d_{54} samples were equilibrated at 40ºC for at least 10 minutes before the ^{2}H-NMR measurements. The experiments were performed at 76.78 MHz with the same quadrupolar echo sequence as used for the peptide spectra, except that a recycling time of 600 ms was used. In some experiments a recycling delay of 1 second was applied. There were no differences found with the other spectra, which confirmed that the 600 ms delay was sufficient. The spectra were recorded with a spectral width of 500 kHz and 4096 data points, starting the acquisition at the echo maximum. Between 20k and 40k scans were collected. Processing was performed by zero-filling to 8192 data points, Fourier transformation and centering the spectrum on the CH_3 signals.

Coarse-grained simulations

Coarse-grained (CG) simulations of monomeric WALP23, antiparallel and parallel C12 dimer in DOPC were performed using GROMACS 4.0.5 [59] with the MARTINI force field[60, 61] Two trajectories of 10 µs of the WALP23 monomer were performed at a P/L ratio of 1:200. The setup and details of these simulations are described elsewhere.[46] To setup the C12-WALP parallel dimer simulations, a WALP23 helix was duplicated and both helices were positioned with the Cys12 residues facing each other. For the antiparallel dimer the duplicated monomer was first rotated upside down. For both dimers a bond was created to mimic the disulfide bridge. Then an energy minimization was performed followed by a short MD in vacuum, which allowed the system to relax and the helices to pack optimally on each other. Then each dimer was embedded in a hydrated bilayer of DOPC (P/L ratio of 1:200), and an equilibration of 50 ns was performed to allow the lipids to pack on the peptide. For each topology two separate simulations of 10 µs were run with different initial velocities which allowed an increase of sampling. All the other details of the simulations (thermostat, barostat, cut-off, etc) are described elsewhere.[46] Because of smoother interactions in the CG potential, the kinetics is faster with MARTINI than for all-atom simulations. As an indication, MARTINI water diffuses 4 times faster than regular SPC water, however other processes such as helix dynamics may raise a different increase in kinetics.[60]

References

1. van den Brink-van der Laan, E., Chupin, V., Killian, J. A., and de Kruijff, B., Stability of KcsA tetramer depends on membrane lateral pressure, *Biochemistry*, **2004**, 43(14), p. 4240-50
2. Beevers, A. J. and Kukol, A., Phospholemman transmembrane structure reveals potential interactions with Na+/K+-ATPase, *J Biol Chem*, **2007**, 282(45), p. 32742-8
3. Nyholm, T. K., Ozdirekcan, S., and Killian, J. A., How protein transmembrane segments sense the lipid environment, *Biochemistry*, **2007**, 46(6), p. 1457-65
4. Marsh, D., Protein modulation of lipids, and vice-versa, in membranes, *Biochim Biophys Acta*, **2008**, 1778(7-8), p. 1545-75
5. Zoonens, M., Reshetnyak, Y. K., and Engelman, D. M., Bilayer interactions of pHLIP, a peptide that can deliver drugs and target tumors, *Biophys J*, **2008**, 95(1), p. 225-35
6. Venturoli, M., Smit, B., and Sperotto, M. M., Simulation studies of protein-induced bilayer deformations, and lipid-induced protein tilting, on a mesoscopic model for lipid bilayers with embedded proteins, *Biophys J*, **2005**, 88(3), p. 1778-98
7. Powl, A. M., East, J. M., and Lee, A. G., Different effects of lipid chain length on the two sides of a membrane and the lipid annulus of MscL, *Biophys J*, **2007**, 93(1), p. 113-22
8. Demmers, J. A., van Dalen, A., de Kruijff, B., Heck, A. J., and Killian, J. A., Interaction of the K+ channel KcsA with membrane phospholipids as studied by ESI mass spectrometry, *FEBS Lett*, **2003**, 541(1-3), p. 28-32
9. Barrera, F. N., Renart, M. L., Poveda, J. A., de Kruijff, B., Killian, J. A., and Gonzalez-Ros, J. M., Protein self-assembly and lipid binding in the folding of the potassium channel KcsA, *Biochemistry*, **2008**, 47(7), p. 2123-33
10. John, K., Schreiber, S., Kubelt, J., Herrmann, A., and Muller, P., Transbilayer movement of phospholipids at the main phase transition of lipid membranes: implications for rapid flip-flop in biological membranes, *Biophys J*, **2002**, 83(6), p. 3315-23
11. Lee, A. G., Lipid-protein interactions in biological membranes: a structural perspective, *Biochim Biophys Acta*, **2003**, 1612(1), p. 1-40
12. Killian, J. A., Synthetic peptides as models for intrinsic membrane proteins, *FEBS Lett*, **2003**, 555(1), p. 134-8
13. de Planque, M. R. and Killian, J. A., Protein-lipid interactions studied with designed transmembrane peptides: role of hydrophobic matching and interfacial anchoring, *Mol Membr Biol*, **2003**, 20(4), p. 271-84
14. de Planque, M. R., Greathouse, D. V., Koeppe, R. E., 2nd, Schafer, H., Marsh, D., and Killian, J. A., Influence of lipid/peptide hydrophobic mismatch on the thickness of diacylphosphatidylcholine bilayers. A 2H NMR and ESR study using designed transmembrane alpha-helical peptides and gramicidin A, *Biochemistry*, **1998**, 37(26), p. 9333-45
15. Dumas, F., Sperotto, M. M., Lebrun, M. C., Tocanne, J. F., and Mouritsen, O. G., Molecular sorting of lipids by bacteriorhodopsin in dilauroylphosphatidylcholine/distearoylphosphatidylcholine lipid bilayers, *Biophys J*, **1997**, 73(4), p. 1940-53
16. Sperotto, M. M., Ipsen, J. H., and Mouritsen, O. G., Theory of protein-induced lateral phase separation in lipid membranes, *Cell Biophys*, **1989**, 14(1), p. 79-95
17. Riegler, J. and Mohwald, H., Elastic Interactions of Photosynthetic Reaction Center Proteins Affecting Phase Transitions and Protein Distributions, *Biophys J*, **1986**, 49(6), p. 1111-1118
18. Piknova, B., Perochon, E., and Tocanne, J. F., Hydrophobic mismatch and long-range protein/lipid interactions in bacteriorhodopsin/phosphatidylcholine vesicles, *Eur J Biochem*, **1993**, 218(2), p. 385-96
19. Morein, S., Killian, J. A., and Sperotto, M. M., Characterization of the thermotropic behavior and lateral organization of lipid-peptide mixtures by a combined experimental and theoretical approach: effects of hydrophobic mismatch and role of flanking residues, *Biophys J*, **2002**, 82(3), p. 1405-17
20. Saffman, P. G. and Delbruck, M., Brownian motion in biological membranes, *Proc Natl Acad Sci U S A*, **1975**, 72(8), p. 3111-3
21. Ramadurai, S., Holt, A., Krasnikov, V., van den Bogaart, G., Killian, J. A., and Poolman, B., Lateral diffusion of membrane proteins, *J Am Chem Soc*, **2009**, 131(35), p. 12650-6
22. Gambin, Y., Lopez-Esparza, R., Reffay, M., Sierecki, E., Gov, N. S., Genest, M., Hodges, R. S., and Urbach, W., Lateral mobility of proteins in liquid membranes revisited, *Proc Natl Acad Sci U S A*, **2006**, 103(7), p. 2098-102
23. Martens, J. R., O'Connell, K., and Tamkun, M., Targeting of ion channels to membrane microdomains: localization of KV channels to lipid rafts, *Trends Pharmacol Sci*, **2004**, 25(1), p. 16-21
24. Briggs, J. A., Wilk, T., and Fuller, S. D., Do lipid rafts mediate virus assembly and pseudotyping?, *J Gen Virol*, **2003**, 84(Pt 4), p. 757-68
25. Ridder, A. N., Spelbrink, R. E., Demmers, J. A., Rijkers, D. T. S., Liskamp, R. M. J., Brunner, J., Heck, A. J., de Kruijff, B., and Killian, J. A., Photo-crosslinking analysis of preferential interactions between a transmembrane peptide and matching lipids, *Biochemistry*, **2004**, 43(15), p. 4482-9
26. van Duyl, B. Y., Rijkers, D. T. S., de Kruijff, B., and Killian, J. A., Influence of hydrophobic mismatch and palmitoylation on the association of transmembrane alpha-helical peptides with detergent-resistant membranes, *FEBS Lett*, **2002**, 523(1-3), p. 79-84
27. Kol, M. A., van Dalen, A., de Kroon, A. I., and de Kruijff, B., Translocation of phospholipids is facilitated by a subset of membrane-spanning proteins of the bacterial cytoplasmic membrane, *J Biol Chem*, **2003**, 278(27), p. 24586-93
28. Sparr, E., Ash, W. L., Nazarov, P. V., Rijkers, D. T. S., Hemminga, M. A., Tieleman, D. P., and Killian, J. A., Self-association of Transmembrane {alpha}-Helices in Model Membranes: IMPORTANCE OF HELIX ORIENTATION AND ROLE OF HYDROPHOBIC MISMATCH, *J Biol Chem*, **2005**, 280(47), p. 39324-39331
29. Strandberg, E., Ozdirekcan, S., Rijkers, D. T. S., van der Wel, P. C., Koeppe, R. E., 2nd, Liskamp, R. M., and Killian, J. A., Tilt angles of transmembrane model peptides in oriented and non-oriented lipid bilayers as determined by 2H solid-state NMR, *Biophys J*, **2004**, 86(6), p. 3709-21
30. de Planque, M. R., Goormaghtigh, E., Greathouse, D. V., Koeppe, R. E., 2nd, Kruijtzer, J. A., Liskamp, R. M., de Kruijff, B., and Killian, J. A., Sensitivity of single membrane-spanning alpha-helical peptides to hydrophobic mismatch with a lipid bilayer: effects on backbone structure, orientation, and extent of membrane incorporation, *Biochemistry*, **2001**, 40(16), p. 5000-10
31. Goormaghtigh, E., Ruysschaert, J. M., and Raussens, V., Evaluation of the information content in infrared spectra for protein secondary structure determination, *Biophys J*, **2006**, 90(8), p. 2946-57
32. Vigano, C., Manciu, L., Buyse, F., Goormaghtigh, E., and Ruysschaert, J. M., Attenuated total reflection IR spectroscopy as a tool to investigate the structure, orientation and tertiary structure changes in peptides and membrane proteins, *Biopolymers*, **2000**, 55(5), p. 373-80
33. Bechinger, B., Ruysschaert, J. M., and Goormaghtigh, E., Membrane helix orientation from linear dichroism of infrared attenuated total reflection spectra, *Biophys J*, **1999**, 76(1 Pt 1), p. 552-63
34. Rothschild, K. J. and Clark, N. A., Polarized infrared spectroscopy of oriented purple membrane, *Biophys J*, **1979**, 25(3), p. 473-87
35. Monticelli, L., Kandasamy, S. K., Periole, X., Larson, R. G., Tieleman, D. P., and Marrink, S. J., The MARTINI Coarse-Grained Force Field: Extension to Proteins, *J Chem Theory*

and Comput, **2008**, 4(5), p. 819
36. Wohlert, J. and Edholm, O., Dynamics in atomistic simulations of phospholipid membranes: Nuclear magnetic resonance relaxation rates and lateral diffusion, *J Chem Phys*, **2006**, 125(20), p. 204703
37. Sparr, E., Ganchev, D. N., Snel, M. M., Ridder, A. N., Kroon-Batenburg, L. M., Chupin, V., Rijkers, D. T. S., Killian, J. A., and de Kruijff, B., Molecular organization in striated domains induced by transmembrane alpha-helical peptides in dipalmitoyl phosphatidylcholine bilayers, *Biochemistry*, **2005**, 44(1), p. 2-10
38. Petrache, H. I., Tristram-Nagle, S., and Nagle, J. F., Fluid phase structure of EPC and DMPC bilayers, *Chem Phys Lipids*, **1998**, 95(1), p. 83-94
39. Ozdirekcan, S., Nyholm, T. K., Raja, M., Rijkers, D. T. S., Liskamp, R. M. J., and Killian, J. A., Influence of trifluoroethanol on membrane interfacial anchoring interactions of transmembrane alpha-helical peptides, *Biophys J*, **2008**, 94(4), p. 1315-25
40. Casal, H. L. and Mantsch, H. H., Polymorphic phase behaviour of phospholipid membranes studied by infrared spectroscopy, *Biochim Biophys Acta*, **1984**, 779(4), p. 381-401
41. Holt, A., Rougier, L., Reat, V., Jolibois, F., Saurel, O., Czaplicki, J., Killian, J. A., and Milon, A., Order parameters of a transmembrane helix in a fluid bilayer: case study of a WALP peptide, *Biophys J*, **2010**, 98(9), p. 1864-72
42. Bowie, J. U., Helix packing in membrane proteins, *J Mol Biol*, **1997**, 272(5), p. 780-9
43. Goormaghtigh, E., Cabiaux, V., and Ruysschaert, J. M., Determination of soluble and membrane protein structure by Fourier transform infrared spectroscopy. I. Assignments and model compounds, *Subcell Biochem*, **1994**, 23, p. 329-62
44. Marsh, D., Muller, M., and Schmitt, F. J., Orientation of the infrared transition moments for an alpha-helix, *Biophys J*, **2000**, 78(5), p. 2499-510
45. Holt, A., Koehorst, R. B., Rutters-Meijneke, T., Gelb, M. H., Rijkers, D. T. S., Hemminga, M. A., and Killian, J. A., Tilt and rotation angles of a transmembrane model peptide as studied by fluorescence spectroscopy, *Biophys J*, **2009**, 97(8), p. 2258-66
46. Monticelli, L., Tieleman, D. P., and Fuchs, P. F. J., Interpretation of 2H-NMR experiments on the orientation of the transmembrane helix WALP23 by computer simulations, *Biophys J*, **2010**, in press
47. Gambin, Y., Reffay, M., Sierecki, E., Homble, F., Hodges, R. S., Gov, N. S., Taulier, N., and Urbach, W., Variation of the lateral mobility of transmembrane peptides with hydrophobic mismatch, *J Phys Chem B*, **2010**, 114(10), p. 3559-66
48. Castresana, J., Valpuesta, J. M., Arrondo, J. L., and Goni, F. M., An infrared spectroscopic study of specifically deuterated fatty-acyl methyl groups in phosphatidylcholine liposomes, *Biochim Biophys Acta*, **1991**, 1065(1), p. 29-34
49. Cortijo, M. and Chapman, D., A comparison of the interactions of cholesterol and gramicidin A with lipid bilayers using an infrared station, *FEBS*, **1981**, 131(2), p. 245-248
50. Chapman, D., Cornell, B. A., Ellasz, A. W., and Perry, A., Interactions of helical polypepetide segments which span the hydrocarbon region of lipid bilayers. Studies of the gramicidin A lipid-water system, *J Mol Biol*, **1977**, 113(3), p. 517-38
51. Lee, D. C., Durrani, A. A., and Chapman, D., A difference infrared spectroscopic study of gramicidin A, alamethicin and bacteriorhodopsin in perdeuterated dimyristoylphosphatidylcholine, *Biochim Biophys Acta*, **1984**, 769(1), p. 49-56
52. Davies, M. A., Brauner, J. W., Schuster, H. F., and Mendelsohn, R., A quantitative infrared determination of acyl chain conformation in gramicidin/dipalmitoylphosphatidylcholine mixtures, *Biochem Biophys Res Commun*, **1990**, 168(1), p. 85-90
53. Zein, M. and Winter, R., Effect of temperature, pressure and lipid acyl chain length on the structure and phase behaviour of phospholipid-gramicidin bilayers, *Phys Chem Chem Phys*, **2000**, 2, p. 4545-4551
54. van der Wel, P. C., Strandberg, E., Killian, J. A., and Koeppe, R. E., 2nd, Geometry and intrinsic tilt of a tryptophan-anchored transmembrane alpha-helix determined by (2)H NMR, *Biophys J*, **2002**, 83(3), p. 1479-88
55. Sukharev, S., Betanzos, M., Chiang, C. S., and Guy, H. R., The gating mechanism of the large mechanosensitive channel MscL, *Nature*, **2001**, 409(6821), p. 720-4
56. Holt, A. and Killian, J. A., Orientation and dynamics of transmembrane peptides: the power of simple models, *Eur Biophys J*, **2009**, 39(4), p. 609-621
57. Fernandes, F., Loura, L. M., Prieto, M., Koehorst, R., Spruijt, R. B., and Hemminga, M. A., Dependence of M13 major coat protein oligomerization and lateral segregation on bilayer composition, *Biophys J*, **2003**, 85(4), p. 2430-41
58. Rouser, G., Fkeischer, S., and Yamamoto, A., Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, *Lipids*, **1970**, 5(5), p. 494-6
59. Hess, B., GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation, *J Chem Theory Comput*, **2008**, 4, p. 435-447
60. Marrink, S. J., Risselada, H. J., Yefimov, S., Tieleman, D. P., and de Vries, A. H., The MARTINI force field: coarse grained model for biomolecular simulations, *J Phys Chem B*, **2007**, 111(27), p. 7812-24
61. Ozdirekcan, S., Etchebest, C., Killian, J. A., and Fuchs, P. F. J., On the orientation of a designed transmembrane peptide: toward the right tilt angle?, *J Am Chem Soc*, **2007**, 129(49), p. 15174-81

4

Parallel and antiparallel dimers of transmembrane model peptides; The effect of helix-helix interactions on protein-lipid interactions

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Abstract

In this chapter, the study on the influence of dimerization of transmembrane helices on their behaviour and lipid interactions is described. This investigation is approached systematically by comparing the orientation, lipid effects and ^{2}H-NMR fingerprints of monomers and covalent dimers of synthetic model peptides. The dimers are coupled at the termini and have a forced parallel or antiparallel arrangement of the helices. An increase in tilt is shown for the dimers with respect to the monomers. The parallel dimer may also be slightly more tilted than the antiparallel dimer. The current results, combined with the results obtained in the previous chapter, suggest that helix-helix interactions are a determining factor for the tilt angle of transmembrane segments with respect to the membrane normal. Rather surprisingly, the parallel and antiparallel dimers and their corresponding monomers do not show a lipid stretching effect. The results indicate that there is a correlation between tilt and lipid effects, with large increases in tilt angle corresponding to small lipid stretching effects.

Introduction

Membrane proteins are an abundant class of proteins[1], which have many functions that are essential for life. To better understand membrane proteins, knowledge has to be obtained on the factors that are important for their structure and function. A key factor in membranes is the interaction of the membrane spanning segments with the surrounding lipids.[2, 3] The lipid composition of the membrane can significantly influence protein features[4-8] and the membrane is in turn influenced by proteins[9-11]. Membrane protein properties and lipid interactions are quite complex and diverse and therefore difficult to study. The use of synthetic model peptides in vesicles of varying lipid content provides a possibility to systematically study protein-lipid interactions. Examples of such model peptides are the WALP peptides (Ac-GWW$(LA)_n$LWWA-NH_2), which mimic the features of natural membrane proteins.[12] One approach to obtain knowledge on the general principles of protein-lipid interactions is by studying the adaptations of the model system to a mismatch situation. For example, in positive mismatch the hydrophobic stretch of the peptide would exceed the hydrophobic thickness of the lipid bilayer. To relieve this energetically unfavourable situation, the peptides can tilt[13] or oligomerize[14] and the lipids can stretch the acyl chains[15]. Relevant insights into principles of membrane protein behaviour have been obtained by studying the responses described above.[16-19]

A limitation of the WALP peptide is that it spans the membrane only once. To better mimic and understand multispan membrane proteins, the aim of the research described in this thesis is to synthesize and study covalent oligomers of WALP peptides. In the previous two chapters, dimers covalently coupled in the middle of the helix were prepared and studied. The so-called C11-, C12- and C13-WALP analogues (Ac-GWWLALALALXXXLALALALWWA-NH_2, XXX= CLA, ACA or ALC respectively) were compared to their dimeric assemblies. It was shown that the orientation of the dimers with respect to the membrane normal was hardly altered in comparison to that of momomers, but that their dynamics decreased and their ability to stretch the lipids increased. The results formed the first systematic evidence for the influence of cross sectional diameter and helix-helix interactions on membrane protein behaviour. Hence, the studies on C11-, C12- and C13-WALP dimers can be considered as a first step towards systematic studies on multispan membrane proteins. However, it may be questioned if these dimers, which were covalently coupled in the middle of the helices, are adequate models for helices in polytopic membrane proteins, because such proteins are typically coupled at the termini, allowing the helices a larger degree of flexibility with respect to each other.

To further improve the protein mimicking features of the transmembrane model peptides, we designed another type of dimers. The dimers that will be discussed in this chapter are coupled either at the N- or C-terminus via a relatively flexible loop. In addition, the helices in these newly designed dimers have a forced parallel or antiparallel topology. In previous research the WALP peptides appeared to have a preferentially antiparallel arrangement.[14, 20] Simulations indicated that this was caused by the dipole-dipole interactions of the helices. Also the results with the cysteine containing dimers pointed towards an antiparallel orientation of the helices. However, the design of these dimers did not allow to fully investigate this feature. The forced topology in the design of the current dimers allows us to further investigate the influence of parallel or antiparallel arrangement of the helices.

In membrane proteins parallel and antiparallel topologies may both occur. In multispan membrane proteins the arrangement is usually restricted by the extracellular loops that connect the transmembrane segments. Many mem-

brane proteins contain recognition motifs[21], such as the leucine zipper or the GXXXG motif[22-25], that usually occur in conjunction with a parallel orientation of the interacting helices. Such favourable helix-helix packing interactions are especially important in proteins or complexes that consist of multiple units that have to assemble to form a functional entity. If no recognition motif is present, the dipole moments of the helices may cause a preference for an antiparallel topology.[19] Finally, also lipid interactions may influence helix-helix interactions[23, 26-28] and thereby play an important role in membrane structure and function.

In this chapter the synthesis, purification, characterization, and biophysical analysis of parallel and antiparallel dimers and their monomeric building blocks will be described. The secondary structure and stability of the helices was characterized by circular dichroism (CD) and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). The orientation, the conformational and dynamical fingerprint and the lipid effects of the monomers and dimers in (mis)matching bilayers were studied by ATR-FTIR and solid state ^{2}H-NMR. The results for the monomers, antiparallel and parallel dimers and the dimers studied in chapter 3 will be compared and the differences discussed in light of understanding natural membrane proteins.

Results

Design, synthesis and purification of monomers and dimers

To selectively synthesize parallel or antiparallel dimers three WALP analogues were designed with either alkyne or azide functionalities (Table 1). An alkyne- and an azide group can be covalently coupled via the highly selective click-reaction under formation of a triazole.[29, 30] Thus, parallel dimers can be synthesized from the Alk-WALP-K and the N_3-WALP-K. For the antiparallel dimers Alk-WALP-K and K-WALP-N_3 can be coupled. To prevent steric hindrance to be an issue in dimerization, a short spacer was placed between the regular WALP23 peptide sequence and the functional alkyne- or azide groups. In addition, to facilitate characterization and handling, a lysine was placed at the terminus opposite to the coupling site.

To equip the WALP analogues with an alkyne or an azide moiety, first two lysine derivatives (Figure 1) were synthesized. According to HPLC and NMR the building blocks were apposite to use in solid phase peptide synthesis. (See for a more elaborate description of synthesis and characterization Chapter 5)

The N_3-WALP-K, Alk-WALP-K and K-WALP-N_3 were successfully synthesized by automated solid phase peptide synthesis, using the Fmoc-strategy. All three peptides were characterized with MALDI-TOF and HPLC. With MALDI-TOF mass spectrometry the sodium and potassium adducts were found of all products (Table 2). In MALDI-TOF analysis an azide can be reduced[31, 32], explaining the mass-shift of 27 Da for both azide-containing peptides. Lower masses were also detected, corresponding to a small amount of truncated peptides. The purity of the peptides according to HPLC was more than 80%. Only a small portion of the N_3-WALP-K and Alk-WALP-K was purified to use as monomeric references. For dimerization the crude forms of the two peptides were used, because the only impurities were the shorter peptides that will not be coupled since these miss the N-terminal azide or alkyne. The K-WALP-N_3 was purified both to use as a monomer reference and to be used in dimerization.

Table 1. WALP analogues designed to synthesize covalent dimers, with specifically parallel or antiparallel topology

Peptide	Sequence
N_3-WALP-K	Ac-K(N_3)GA-GWW$(LA)_8$LWWA-GAGAK-NH_2
K-WALP-N_3	Ac-KGAGA-GWW$(LA)_8$LWWA-GAK(N_3)-NH_2
Alk-WALP-K	Ac-K(alkyne)GA-GWW$(LA)_8$LWWA-GAGAK-NH_2

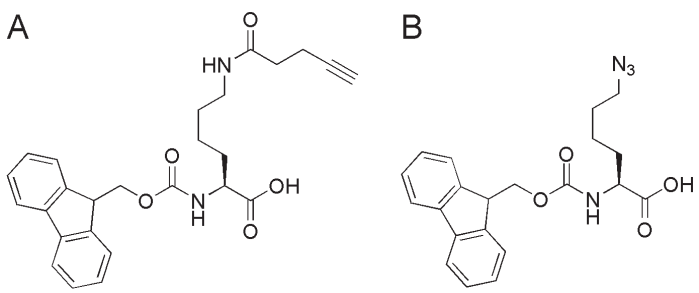


Figure 1. Building blocks to be used in the solid phase peptide synthesis of the three WALP analogues to be able to incorporate an alkyne (A) or azide (B) functionality

Table 2. MALDI-TOF analysis of the three WALP analogues

Peptide	Exact mass (calculated)	$[M+Na]^+$, $[M+K]^+$ (found)
N_3-WALP-K	3186.84	3182.77, 3209.79
K-WALP-N_3	3186.84	3182.73, 3209.74
Alk-WALP-K	3240.91	3263.82, 3279.83

Dimerization of the azide and alkyne-containing WALP analogues was achieved by click-chemistry, using copper(I)acetate as a catalyst and microwave heating. In these dimers two positively charged lysine residues are present to facilitate ionization and a broad peak at a position corresponding to the molecular weight of the dimers could be detected with MALDI-TOF mass spectrometry (data not shown). This indicates the formation of covalent dimers. Also with tris-tricine gel electrophoresis (Figure 2A) it was shown that dimers (lane 2 and 3, MW≈6400) were formed. As references, a monomer (lane 1, MW≈3200) as well as a dimer from a cysteine containing WALP (lane 4, MW≈5000, chapter 2) are depicted. As stated in chapter 2, the additional band above the bands of the dimers is probably due to aggregation and was not found systematically. Purification could be achieved by using size exclusion chromatography in organic solvents. A successful purification of the parallel dimer over a Sephadex LH 20 column is shown in Figure 2B. On a small scale (max. 10 mg), the peptides could be purified using methanol as mobile phase. However, on a larger scale the solubility of the peptides in methanol was not sufficient and the peptides had to be purified with a column in DMF. In both solvents, the resolution of the separation over Sephadex LH 20 was low, which caused only low yields, around 3%, of isolated dimers per column. From gel electrophoresis, the purity of the dimers after purification was estimated to be more than 95% (data not shown).

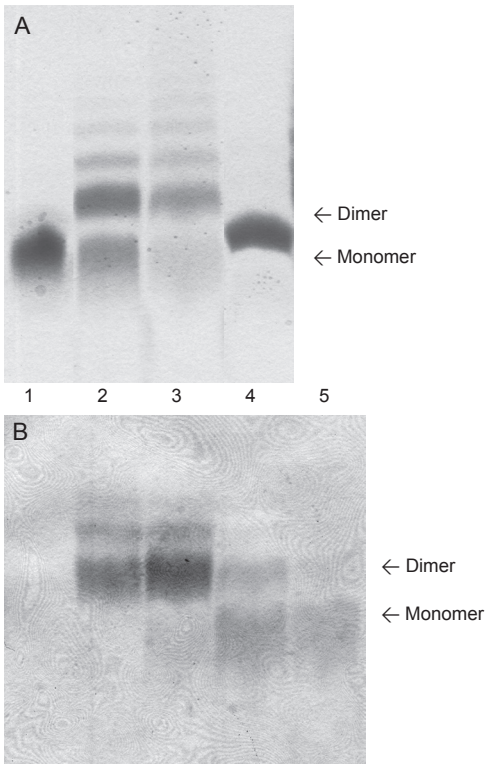


Figure 2. Tricine-SDS-PAGE of dimer synthesis (A) with Alk-WALP-K monomer (lane 1), parallel dimer (lane 2), antiparallel dimer (lane 3) and dimer of C13-WALP (lane 4) and of purification of parallel dimer (B) with fraction 6-10 (lane 1-5)

Structural characterization of monomers and dimers

CD experiments

The secondary structure and stability of the parallel and antiparallel dimers and the three WALP analogues were established by circular dichroism (CD). In Figure 3A the CD spectra of the two dimers and one monomer in SDS are shown. The spectra demonstrate that all peptides are α-helical, with two minima at 222 and 208 nm and a maximum at 192 nm. Similar spectra were obtained for the other two monomers (data not shown). To investigate the stability of the α-helices, all peptides were treated with the denaturing agent urea. It was found

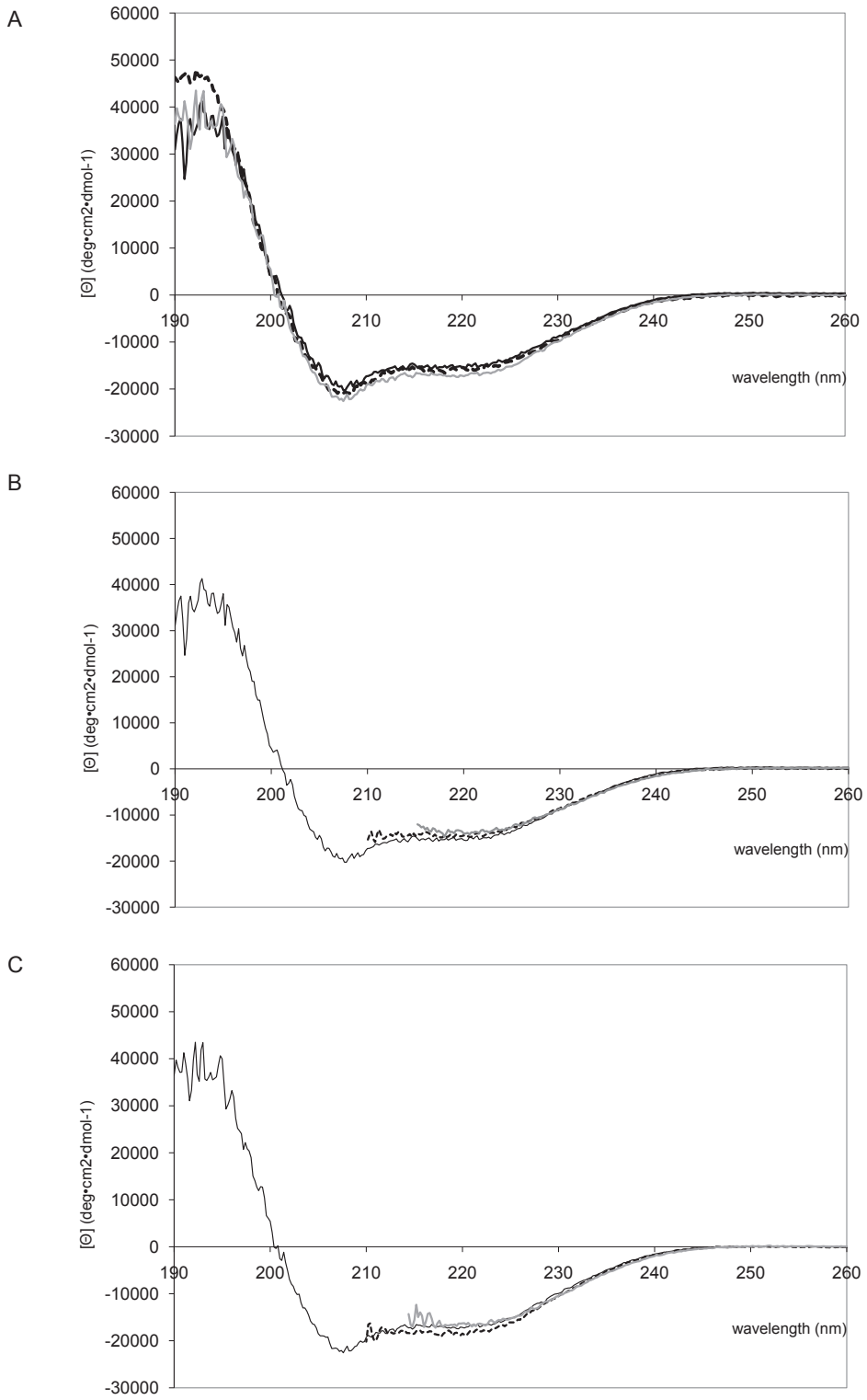


Figure 3. CD spectra of K-WALP-N3 (dotted black line), antiparallel dimer (black line) and parallel dimer (grey line) in SDS (A) and spectra of antiparallel (B) and parallel (C) dimer in SDS without denaturant (solid black lines), with 5M urea (dotted black lines) and incubated with 5M urea at 90ºC (solid grey lines)

that the secondary structure of the monomers and dimers was not altered after incubation with 5M urea, not even when heating the sample at 90ºC for one hour. This is illustrated for the parallel and antiparallel dimer in Figure 3B and 3C respectively. Though part of the spectra could not be analyzed due to absorbance of urea, the similarity in the 210-260 nm wavelength region shows that both dimers stayed α-helical under these rather extreme denaturing conditions. Identical results were obtained for the monomers (not shown).

The secondary structure of all peptides was also measured in unilamellar vesicles of di-14:0 PC. In Figure 4 the spectra of the antiparallel dimer and the K-WALP-N_3 monomer are shown. The spectra illustrate that the peptides are well-defined α-helices. These spectra were representative for all peptides (data not shown). Furthermore, all spectra had similar intensities. As shown in previous research[33] and in chapter 2, this provides a first indication of similar incorporation properties for all peptides. Finally, as in SDS, also in lipid vesicles all monomers and dimers proved to be extremely stable, staying α-helical upon incubation with 5M urea, with and without heating (data not shown).

ATR-FTIR experiments

The secondary structure of the monomers and dimers in di-14:0 PC was also derived from ATR-FTIR spectra of the peptides. In Figure 5 the 1800-1500 cm^{-1} region of the spectra of the N_3-WALP-K monomer (solid grey line) and the parallel (dotted black line) and antiparallel (solid black line) dimers are shown. The spectra of K-WALP-N_3 and Alk-WALP-K were similar to that of the N_3-WALP-K (not shown). In the depicted region of Figure 5 the amide I (1700-1600 cm^{-1}) and II (1600-1500 cm^{-1}) bands are present, resulting from respectively the ν(C=O) and δ(N-H) vibrations of the helix backbone. The position and full width at the half-height (FWHH) of these bands denote the secondary structure of the peptides. In chapter 3 it was demonstrated that the cysteine containing WALP analogues are well defined α-helices. Therefore, the spectrum of the C11-WALP analogue (dotted grey line) is shown in Figure 5 for comparison. From the similar position and narrowness of the amide I and II bands of all peptides, it can be concluded that the N_3-WALP-K and parallel and antiparallel dimers are equally well-defined α-helices. The positions of the amide I bands were between 1658.3 and 1659 cm^{-1} and the FWHH values

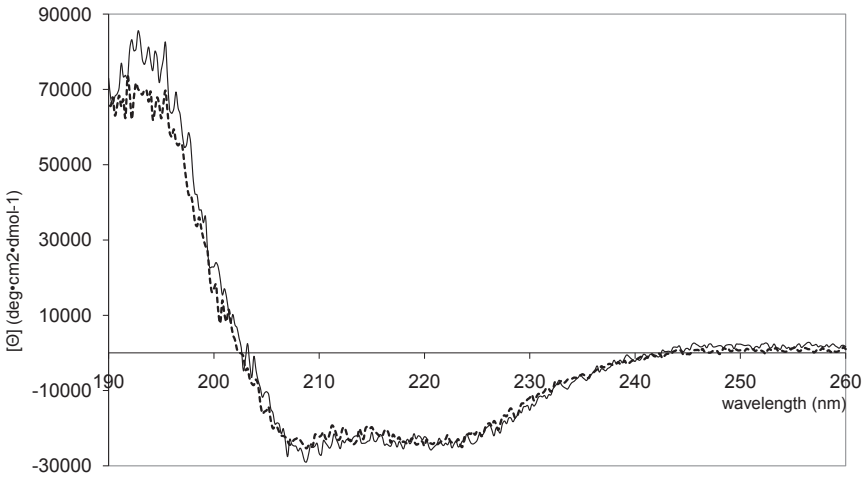


Figure 4. CD spectrum of K-WALP-N3 (dotted black line) and antiparallel dimer (solid black line) in di-14:0 PC

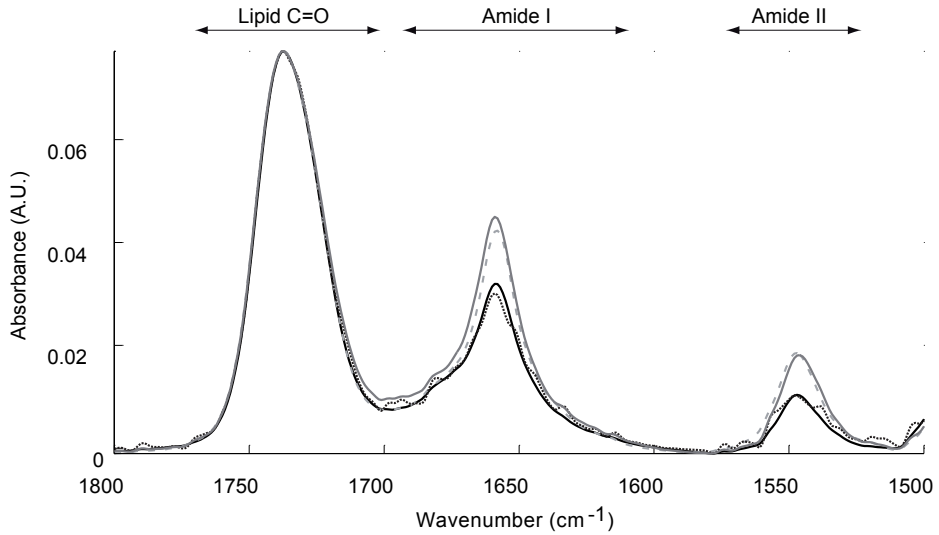


Figure 5. ATR-FTIR spectra of C11-WALP (dotted grey), N3-WALP-K (grey), parallel dimer (dotted black) and antiparallel dimer (solid black) in di-14:0 PC

around 20 cm^{-1}. Since the peptide spectra are normalized to the lipid ν(C=O) band (1780-1700 cm^{-1}), the area of the amide I band reflects the helix/lipid ratio and hence the extent of incorporation of the peptides.[33] From the measured intensities, it can be concluded that the three monomers all incorporated to a similar extent. However, of the antiparallel and parallel dimers, slightly less seemed to incorporate in the di-14:0 PC, which is inconsistent with the CD-results. The reason for this discrepancy is not clear, but may be related to the difference in peptide/lipid ratios applied in the two methods. In the ATR-FTIR samples the dimers may be more prone to aggregation due to the high concentration and such aggregates would not be detected on the germanium plate.

Also the stability of the secondary structure of the peptides was determined by ATR-FTIR, using H/D exchange as an indicator of stability. Spectra of the peptides in di-14:0 PC were recorded, while exposing the sample to a D_2O saturated N_2-flow. The decrease of the area under de amide II band was used as a measure of exchange, since this band is especially sensitive to the replacement of hydrogen for deuterium. The exchange kinetics of both dimers and the Alk-WALP-K monomer as a control, is shown in Figure 6. Of the Alk-WALP-K and parallel dimer,

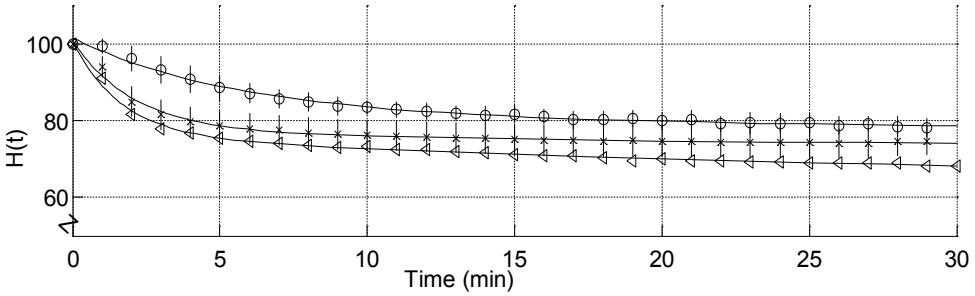


Figure 6. Exchange kinetics of Alk-WALP-K (x), parallel dimer (o) and antiparallel dimer (Δ) in di-14:0 PC. The data points of the Alk-WALP-K and antiparallel dimer are mean values and error bars are given in the graphs

three measurements with different aliquots of the same sample were performed that showed that these measurements were quite reproducible. The exchange rate is relatively low; the amount of hydrogen left in the peptide amides after 30 minutes levels at 70-80%, indicating that the α-helices in the monomer and dimers are highly stable.

Comparison of orientation and dynamics of monomers and dimers

Determination of the tilt of the peptides by ATR-FTIR

The orientation of the peptides was established by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). From the so-called dichroic difference spectra information on the orientation of peptides and lipids can be derived. The underlying principle of this method is described in the general introduction and in chapter 3. The orientation of the lipids can be derived from the lipid CH_2 stretching and wagging vibrations. The symmetric and antisymmetric lipid CH_2 stretching vibrations (2915, 2850 cm^{-1}) showed a negative deviation, where that of the CH_3 (2960, 2870 cm^{-1}) were positive (data not shown). This indicates an orientation of the lipid acyl chains normal to the germanium surface on which the sample is applied. The well defined orientation of the lipids was confirmed by the positive values for the series of bands at 1180, 1203, 1228, 1258 1280, 1304, and 1328 cm^{-1}, resulting from the CH_2 wagging vibrations. In Figure 7 this band progression is shown for the antiparallel dimer and one of the monomers. The lipid signals were comparable for all peptides and showed that the bilayers were well-oriented. The orientation of the peptides can be derived from the amide I and II bands. In figure 7, the dichroic N_3-WALP-K (grey line) spectrum is representative for all three monomers and the dichroic spectrum of the antiparallel dimer (black line) for both dimers. For both peptides, the amide I band is positive, indicating a transmembrane orientation. However, in the dimer spectrum the perpendicular component has gained in relative intensity, yielding a lower amide I signal in the dichroic difference spectrum. This

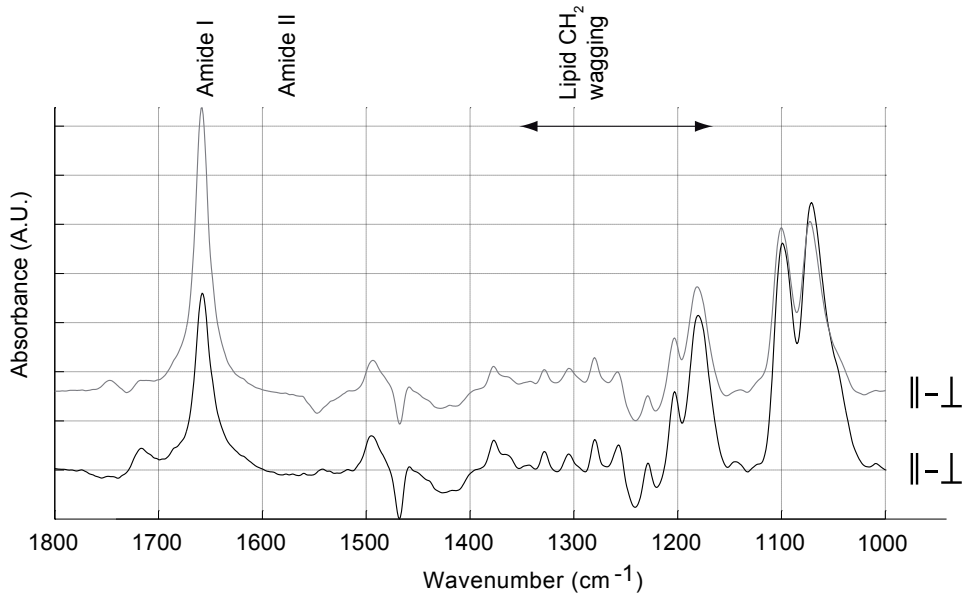


Figure 7. The 1800-1000 cm-1 region of the dichroic difference ATR-FTIR spectra of the N3-WALP-K monomer (grey) and the antiparallel dimer (black) in di-14:0 PC

would correspond to a larger tilt of the dimer. An increased tilt of the dimer in comparison to the monomer is also indicated by the amide II bands of both peptides. The δ(N-H) in the dichroic difference spectrum of the monomer is negative and that of the dimer has no deviation. As explained in chapter 3, this is likely to be caused by the peptide being less parallel to the membrane normal.

The so-called maximum tilt angles were calculated for all WALP analogues and for the dimeric assemblies, using the same method and parameters as described in chapter 3. Shortly, the dichroic ratios R^{ATR} and R^{iso} were derived by $A_{//}/A_{\perp}$ using the areas under the peptide and lipid v(C=O) respectively. From these values the maximum tilt angle θ was calculated, assuming a 27º angle between the v(C=O) dipole and the helix. The dichroic ratios and tilt angles of the measured samples are depicted in Table 3. The monomers all had a tilt of around 20º with respect to the bilayer normal. The dimers were clearly tilted more than the monomers, with angles of 30-34º. There may also be a slight but significant difference in tilt between the dimers, with the parallel having a slightly larger tilt angle than the antiparallel dimer. The duplicate experiments, executed with another aliquot of the same sample, suggest that the experimental error is around 2º, which is comparable to the error found in chapter 3.

Fingerprinting the properties of the peptides by solid state ^{2}H-NMR

In all peptides, an Ala-d_3 was incorporated at the 17th position. Solid state ^{2}H-NMR spectra of the monomers and dimers in di-18:1 PC were measured as a fingerprint of their orientational and motional behaviour. Remarkably, the splittings in the three monomeric WALP analogue spectra were significantly different (Table 4). The differences between the N_3-WALP-K and Alk-WALP-K might fall within the experimental error, but the increase of 2.8-3.2 kHz for the K-WALP-N_3 does not. This divergence may be related to the lysine tag (see concluding discussion).

Table 4. 2H-NMR splittings of WALP analogues in di-18:1 PC, with peptide/lipid ratio of 1:100

Peptide	$\Delta\nu_q$ (kHz)
N_3-WALP-K	5.2
K-WALP-N_3	8.4
Alk-WALP-K	5.6

The spectra of the dimers in di-18:1 PC showed isotropic-like peaks (Figure 8). Apparently, the dimers behave differently than the monomers. The shape did not seem to be exclusively isotropic, since the spectrum could not be fitted

Table 3 Dichroic ratios and orientations of all monomers and dimers in di-14:0 PC with respect to ATR-surface

Peptide	$R^{ATR, a}$	$R^{iso, b}$	θ (º) [c]	θ (º) [d]
N_3-WALP-K	2.72	1.26	18	18
K-WALP-N_3	2.54, 2.45[e]	1.23, 1.26[e]	18, 23[e]	21
Alk-WALP-K	2.62	1.25	19	19
Antiparallel dimer	2.20	1.30	30	30
Parallel dimer	2.07, 1.96[e]	1.31, 1.32[e]	33, 35[e]	34

a. dichroic ratio of amide I bands
b. dichroic ratio of lipid v(C=O) bands
c. maximum tilt angles
d. average
e. duplicate measurement

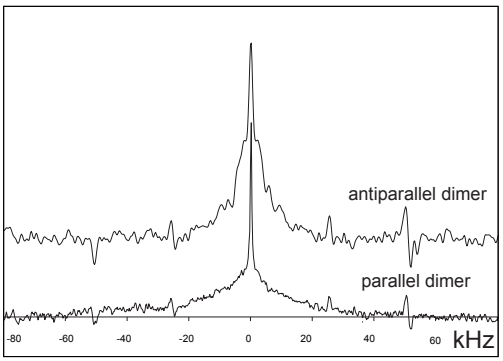


Figure 8. Solid state NMR spectra of the dimers denoted at the spectra in di-18:1 PC at a P/L ratio of 1:200

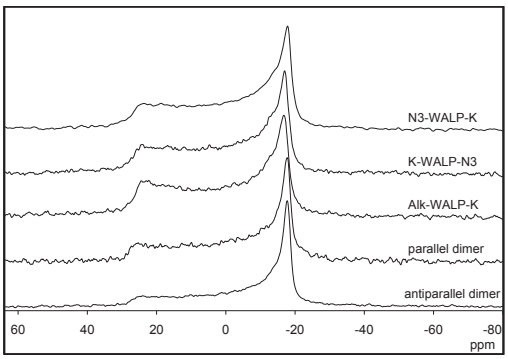


Figure 9. 31P-NMR spectra of multilamellar di-18:1 PC vesicles containing the peptides denoted at the spectra

with a Lorentzian. However, fitting with one or two quadrupolar splittings was also difficult. We were able to fit the antiparallel dimer with a Lorentzian and two components of similar intensity with Δv_q values of 6.7 and 20.4. If the fit is correct, this would indicate that the two helices in the antiparallel dimer behave differently. No fit could be found for the parallel dimers. The differences between monomers and dimers may be caused by changes in orientation and/or dynamics or by aggregation of the dimers. Alternatively, the lipid organization may be affected.

To check the properties of the vesicles, ^{31}P-NMR was performed (Figure 9). The shape of the spectra with a low field shoulder and a high field peak is characteristic for bilayers in the liquid crystalline phase. In addition, all spectra were comparable, regardless of the peptides that were incorporated. This indicates that the differences between the ^{2}H-NMR spectra were not related to a discrepancy in vesicle properties.

Comparison of influence on lipids of monomers and dimers

Determination of ordering effect of peptides by solid state ^{2}H-NMR

The influence of the monomers and dimers on the order of lipid acyl chains was studied by solid state ^{2}H-NMR on di-14:0 PC-d_{54} vesicles. Spectra were recorded of the perdeuterated chains of this lipid, with and without peptide. An increase of width of the spectrum signifies an increase of order.[15] In Figure 10A, spectra of di-14:0 PC-d_{54} without peptide, with the three WALP analogues described in this chapter and with the C12-WALP analogue described in chapter 3, are shown. The spectra illustrate that the monomers synthesized to construct selectively parallel or antiparallel dimers had a rather small effect on the lipid acyl chains. This is surprising, especially when considering the relatively large effect of the C12-WALP monomer, which is comparable to the effect of WALP peptides shown in previous research[34]. The dimers have a similarly small effect as is evident from the spectra in Figure 10B. In the spectra with the monomers and dimers from the current study, a slight loss of resolution is observed, which is possibly due to an increased transverse relaxation time (T_2), as generally observed in the presence of transmembrane peptides.[35, 36]

Determination of ordering effect of peptides by ATR-FTIR

The effect of monomers and dimers on lipid acyl chains was also investigated by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). The acyl chain conformation was derived from the shift in the position of the antisymmetric $\nu(CH_2)$ vibration of the lipids upon heating the sample from 15 till 40ºC. A shift to higher wavelengths is related to an increase in disorder. With the N_3-WALP-K monomer incorporated, the di-14:0 PC is more disordered in

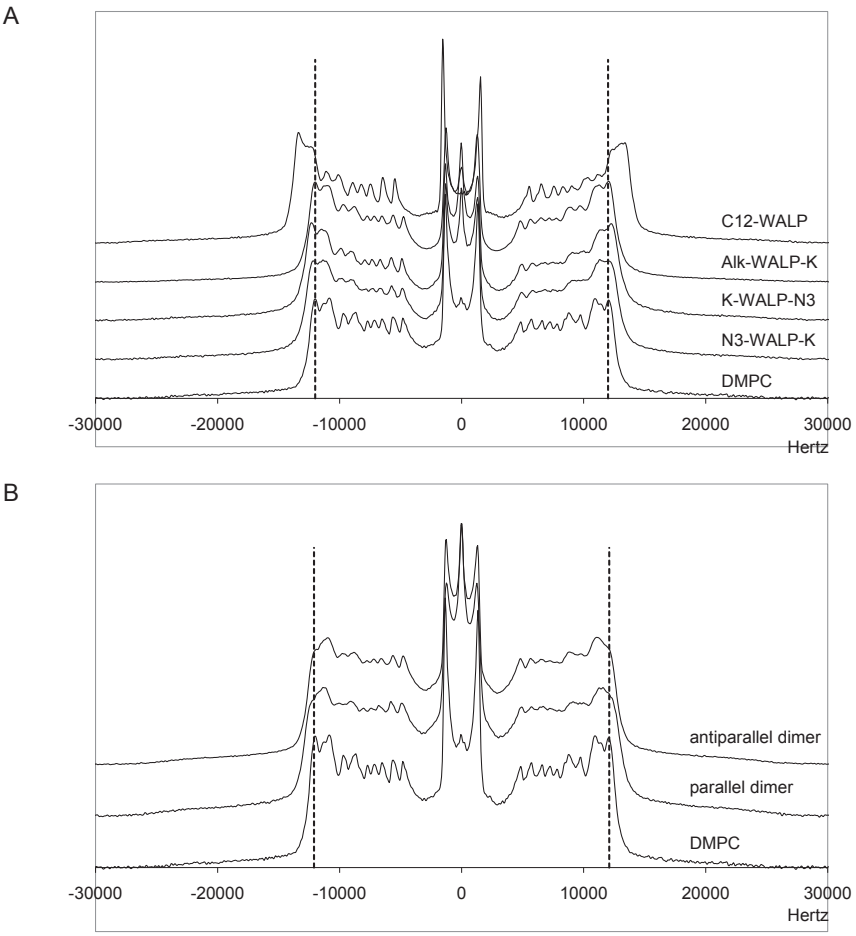


Figure 10. 2H-NMR spectra of di-14:0 PC-d54, at 40ºC, without peptide (DMPC) or with the peptides denoted at the spectra, at helix/lipid ratios of 1:30. Spectra to compare the C12-WALP monomer (see chapter 3) with the monomers in this chapter (A) or to compare the dimers (B) are shown.

the gel phase and possibly slightly more ordered in the liquid crystalline phase (Figure 11). Both dimers show even less effect than the monomer, having only a slight disordering effect in the gel phase and a curve similar to the pure di-14:0 PC above the phase transition and no significant differences between the two dimers. The absence of an effect on bilayers in the liquid crystalline phase is in agreement with the ^{2}H-NMR results. However, it is in contrast to results from previous research[10] and from chapter 3, which showed that above the phase transition temperature the WALP monomers and in particular the dimers have a pronounced effect on lipids.

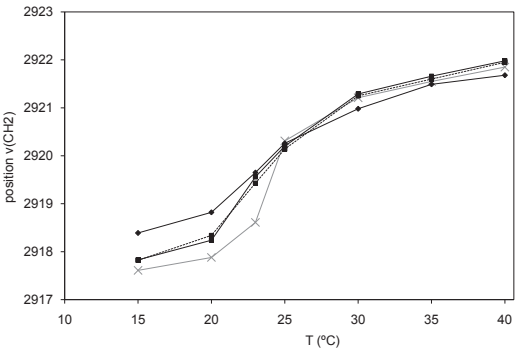


Figure 11. Positions of ν(CH2) of di-14:0 PC without peptide (grey crosses), with N3-WALP-K (black diamonds), with antiparallel dimers (black squares, solid line) and parallel dimers (black squares, dotted line) incorporated at a helix/lipid ratio of 1:30

Concluding discussion

In this chapter the synthesis and biophysical study of covalent dimers of the WALP model peptides with a forced parallel or antiparallel arrangement is described. Below, we will first discuss the synthesis and purification of the monomers and dimers and the characterization of the model system. We will then consider the behaviour and lipid interactions of the monomeric WALP analogues, followed by a discussion on the influence of dimerization, helix-helix packing and topology on protein-lipid interactions. Finally, a possible correlation between tilt and lipid interactions will be discussed.

Synthesis of model peptides and characterization of the model system

Three monomeric WALP analogues, designed to selectively construct parallel or antiparallel dimers, were synthesized, characterized and purified successfully. Via a click reaction with these analogues, parallel and antiparallel dimers were obtained, as indicated by MALDI-TOF and gel electrophoresis. Purification of the dimers was achieved, but with low (3%) yields. Hence, for a more extensive study that requires several milligrams of the dimers, optimization would be necessary, for example via other choices of solvents.[37]

The monomers and dimers proved to be highly defined and stable α-helices, as shown by CD and ATR-FTIR. With CD it was demonstrated that the peptides, when incorporated in DDM, SDS or di-14:0 PC were unaffected by the denaturant urea, even at high temperature. This indicates that they are stable. This stability was also found by H/D exchange kinetics, as measured by ATR-FTIR. Only around 20-30% of the hydrogen atoms were replaced by deuterium after 30 minutes. This amount is not as low as for the cysteine-containing WALP peptides in chapter 3, in which only 10% of the hydrogen atoms was replaced. This is most likely caused by exchange of the amide hydrogen atoms in the N- and C-terminal lysine tails of the peptides described in this chapter, which the peptides from chapter 3 lack. The equal stability of the parallel and antiparallel dimers may be surprising in view of previous research, which indicated that helices that do not contain a recognition motif, assemble preferentially antiparallel.[14, 38] It can thus be envisaged that dimers with a forced parallel arrangement would have a less stable secondary structure. However, the current results do not show any evidence for this.

When reconstituted in lipid bilayers, it may in principle be possible that the two helices of the (anti)parallel dimers reside in an opposing bilayer. This arrangement would be feasible because of the flexibility of the transmembrane peptides to freely move with respect to each other. However, it can be argued that this arrangement of the helices would give deformations in the bilayer stacks of the multilamellar vesicles. From the interbilayer repeat distance (D=62.7 Å) and the hydrophobic thickness ($2D_c$=26.2 Å) of a fully hydrated di-14:0 PC bilayers[39] it can be calculated that the spacing between the hydrophobic parts of adjacent bilayers, consisting of the lipid head groups plus the water layer of opposing bilayers is 36.5 Å. The two helices in the dimers are spaced by six amino acids (backbone-backbone distance of peptide in extended conformation≈3.5 Å per amino acid[40]), two lysine-side chains and a triazole bond. From this, it can be estimated that the maximum length of the spacer, when completely 'stretched', will be around 29 Å (6x3.5Å+8Å), which is not sufficient to bridge the interbilayer distance. Hence, it may now be estimated that the situation where the helices are positioned in two opposing bilayers is unlikely. In addition, resulting deformations in the bilayers are likely to be visible in the ^{31}P-NMR spectra, but no evidence for this is found in the spectra of the dimer containing vesicles. Also a dynamic

light scattering[41] measurement on the antiparallel dimers in large unilamellar vesicles was consistent with the peptide having both helices in one bilayer (data not shown), since it showed a uniform size distribution. If the helices would reside in different bilayers, two or more unilamellar vesicles would be 'tied together' and large sizes and size distributions would be found.

Properties of monomeric WALP analogues in lipid bilayers

Before comparing the properties of dimers and monomers, it is useful to first consider the properties and lipid interactions of the monomeric building blocks. The fingerprints obtained by solid state ^{2}H-NMR spectra show a significant difference for the K-WALP-N_3 as compared to the other two monomeric analogues. The splitting in the spectra of the N_3-WALP-K and Alk-WALP-K analogues is similar to the $\Delta\nu_q$ value of 5.6 found in previous research for a WALP23-A17d_3 in di-18:1 PC.[13] Also the cysteine containing WALP monomers in di-18:1 PC, described in chapter 3, showed a comparable splitting. In contrast, the K-WALP-N_3 shows a much larger splitting. This inconsistency between the three WALP monomers will complicate interpretation of the dimeric fingerprints. The difference in splitting can in principle be caused by a change in rotation, tilt angle and/or or dynamics. However, with ATR-FTIR no significant divergence of tilt was found among the monomers and thus the altered splitting will more probably be caused by a change in rotational angle and/or dynamics. It is possible that the position of the lysine tag influences the behaviour of the monomers. The K-WALP-N_3 has a positively charged N-terminus, while the N_3-WALP-K and Alk-WALP-K have the positive charge at the C-terminus. The resulting effect on the dipole moments and anchoring of the helices may be influential in a technique as sensitive as NMR.

A tilt angle of around 20º with respect to the membrane normal was found for all monomers by ATR-FTIR. This is in agreement with tilt angles of WALP peptides in similar systems, found in previous research.[42, 43] For three cysteine containing WALP analogues (chapter 3), equal tilt angles were determined. The calculated tilt angles are the maximum tilt angles. As discussed extensively in chapter 3, deviations from the assumed parameters may decrease the calculated values. Additionally, the tilt angles of the monomers and dimers studied in the current chapter might be slightly overestimated, since no correction was applied for the probable absence of a defined orientation in the tails at the N- and C-termini.

Most surprisingly, the three monomeric WALP analogues did not show ordering of the bilayer. This contrasts results from previous research[15, 44, 45] and from chapter 3, where the monomeric WALP peptides did show ordering of the acyl chains. Because of this different behaviour, the comparison of the corresponding dimers should be considered cautiously. It is likely that the lack of ordering effect of the N_3-WALP-K, K-WALP-N_3 and Alk-WALP-K is caused by their positively charged tail. A possible scenario might be that the tails recline on the surface of the membrane with the lysine residues positioned between the lipid head groups. It can be envisaged that such an insertion may cause a change in the packing of the lipid head groups, generating room for the lipid tails to attain a more disordered conformation.

Comparison of two types of dimers and their monomeric counterparts in lipid bilayers

In contrast to the monomers, the solid state ^{2}H-NMR spectra of the parallel and antiparallel dimer both show isotropic like peaks, albeit with a different line shape. The results signify that there are differences in tilt angle, rotation and/or dynamics for the monomers and dimers, as well as between the two dimers. However, with the current results, it is virtually impossible to determine the exact origin of the spectral shapes and differences in the various spectra.

By ATR-FTIR it is possible to specifically monitor the tilt angle. The tilt of the parallel and antiparallel dimers with respect to the bilayer normal was significantly larger than that of the monomers, as was calculated from dichroic ATR-FTIR spectra. The monomers had a tilt of around 20º, where the dimers showed tilts of 30-34º. This implies that dimerization can influence the orientation of transmembrane segments. The topology of the helices with respect to each other might also affect the orientation of the helices. The antiparallel dimer had a tilt of 30º and the parallel a tilt of 34º. This may be a significant difference, but more experiments would be required to confirm this.

The current results contrast the results on the tilt of dimers that were covalently coupled in the middle of the helices. (Chapter 3) Here, no differences in tilt between the monomers and dimers were detected. All peptides had a tilt of roughly 20º. The coupling position in the dimers of chapter 3 may result in a more rigid complex that tilts less easily than the more flexible (anti)parallel dimers. This influence of rigidity also correlates to the possible increased tilt of the parallel dimer with respect to the antiparallel dimer. In previous research it was shown that the WALP model peptides favour an antiparallel topology.[14] Thus, the antiparallel dimer might be more tightly packed and consequently form a more rigid complex, causing a restriction of the tilt. Alternatively, it is likely that in the cysteine containing WALP dimers, optimal packing could not be achieved, which might decrease the ability to tilt. Similarly, the differences in tilt angle between the parallel and antiparallel dimers might simply be a direct consequence of differences in the mode of helix-helix packing interactions in the two forms.

The results mentioned are interesting in view of understanding what features influence tilt in multispan membrane proteins. Membrane proteins may show huge tilts of the helices, as in the mechanosensitive channel MscL.[4, 46] In other cases, only minor tilts are found to occur.[5, 47] Also the tilt angle of single span WALP peptides is relatively minor.[42] The results, obtained in this and the previous chapter, indicate that oligomerization facilitates tilt, provided that the helices are able to achieve a favourable packing and/or are flexible with respect to each other.

Dimerization may not only affect properties of the helices, but also the interaction of the helices with the lipid bilayer. However, the ordering effect of the peptides studied in this chapter is minor and the differences between the monomers and parallel and antiparallel dimers are non-systematic. This was shown by solid state ^{2}H-NMR and ATR-FTIR measurements of monomer and dimer containing di-14:0 PC vesicles. It has to be noted that, in contrast, the cysteine containing dimers did show significant lipid effects. This may be related to differences in helix packing between the two helices. Alternatively, it is possible that the positively charged tail in the dimers investigated in the current chapter influence the peptide-lipid interactions.

Correlation between tilt and lipid effects in different types of dimers

The tilt and lipid effects of the dimers covalently coupled in the middle (chapter 3) with respect to the dimers coupled at the termini (this chapter) show remarkable differences. The C12 and C13-WALP dimers do not show a large difference in tilt with respect to the monomers, but do show a significant influence on the lipid order (see chapter 3). For the (anti)parallel dimers an increase in tilt is observed as compared to the monomers, but no effect on acyl chain order. Thus, a correlation between the two events is suggested for the dimeric assemblies of transmembrane model peptides. A similar link is observed when comparing tilt and lipid effects of the tryptophan flanked WALP peptides and the lysine flanked KALP peptides. WALP peptides show smaller tilts, but larger lipid effects than the KALP peptides.

The potential correlation between tilt and acyl chain ordering makes sense, since both are responses to relieve the mismatch situation that is induced by incorporation of the WALP units into bilayers of di-14:0 PC. A correlation between these response was shown before with simulations.[48] In the cited article, it was hypothesized that for smaller, more flexible proteins the main mechanism to compensate for a hydrophobic mismatch is the tilt, whereas large completely rigid proteins cause an increase of the hydrophobic thickness of the bilayer. A similar reasoning could apply in our case. The dimers with a flexible structure, thus the (anti)parallel dimers, are able to compensate by tilting. The dimers that are more rigid, thus the dimers coupled in the middle of the helix, stretch the bilayer. These results thus suggest that in membrane proteins, tilting of helices, if possible, is energetically more favourable than acyl chain ordering. However, more systematic experiments are required to confirm this hypothesis, because the nature of the helix-helix packing interactions may be a dominant factor in determining the behaviour of the peptides studied here.

Experimental section

General

All chemicals were obtained from commercial sources, unless stated otherwise. Peptides were synthesized on an Applied Biosystems 433A peptide synthesizer. For click reactions a Biotage Initiator microwave reactor was used. A Christ alpha RVC speedvac vacuum concentrator was used to remove DMF (at 40°C and 1 mbar). MALDI-TOF was performed on an Applied Biosystems Voyager-DE PRO spectrometer or on an Applied Biosystems 4700 proteomics analyzer. The gel electrophoresis setup was purchased from BioRad Laboratories B.V. Analytical HPLC was performed on a Shimadzu Class-VP HPLC, equipped with a Shimadzu SPD-10AVP UV-vis spectrometer. Preparative HPLC was performed on an automated Gilson preparative HPLC system. Sephadex LH 20 was obtained from GE Healthcare. Spots of the Sephadex LH 20 fractions were applied on Merck precoated silicagel 60 F-254. Absorption spectroscopy was performed on a Perkin Elmer Lambda 18 spectrometer. CD spectra were recorded on a Jasco J-810 spectropolarimeter with Jasco CDF-426S temperature control unit. ATR-FTIR spectra were recorded on a Bruker IFS 55 equipped with a liquid nitrogen cooled mercury cadmium telluride detector. Solid state NMR measurements were performed on a Bruker Avance 500 MHz NMR spectrometer. DLS experiments were performed on a Malvern Instruments Zetasizer 3000

Synthesis, characterization and purification of monomers and dimers

Synthesis N_3-WALP-K, K-WALP-N_3 and Alk-WALP-K (Table 1)

The K-WALP-N_3 was synthesized automatically on a 0.25 mmol scale on a Tentagel S-RAM resin. The azide at the C-terminus was incorporated via an Fmoc-Lys(N_3)-OH building block. For the N_3-WALP-K and Alk-WALP-K, the sequence GA-GWW$(LA)_8$LWWA-GAGAK was synthesized automatically on a 0.25 mmol scale on a Tentagel S-RAM resin. To half of the resin the Fmoc-Lys(N_3)-OH building block and to the other half the Fmoc-Lys(N^{ε}-4-pentynoyl)-OH building block was coupled manually. (See for structure and synthesis building blocks Figure 1 and chapter 5) In all three WALP analogues an Fmoc-Ala(d_3)-OH was coupled instead of a regular Fmoc-Ala-OH on the 17th position of the WALP unit (not including the tails). Deprotection during the automated synthesis was followed with the UV signal of the Fmoc group at 305 nm. A special program was used with conditional extra deprotection and prolonged coupling cycles and standard capping after each coupling. Couplings were performed with HBTU/HOBt and DiPEA in NMP and deprotections with piperidine in NMP. After the final deprotection the N-terminus was capped with an acetyl using acetic anhydride. Detachment from the resin and deprotection of the side-chains was performed by treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) for three hours. The peptides were precipitated in MTBE/n-hexane (1:1 v/v, 4°C, 40 ml) and spun down. The pellet was dissolved in tert-butanol/H_2O and lyophilized to obtain fluffy white powders. Crude yields of N_3-WALP-K, K-WALP-N_3 and Alk-WALP-K were 61, 64 and 61 % respectively with purities of more than 80%. N_3-WALP-K: Rt: 48.7 min, MALDI-TOF: calculated; 3186.84 ($C_{156}H_{235}D_3N_{40}O_{32}$, exact mass), found; 3182.78 $[M\text{-}N_2+2H+Na]^+$, 3198.76 $[M\text{-}N_2+2H+K]^+$, 3209.79 $[M+Na]^+$, 3225.76 $[M+K]^+$. K-WALP-N_3:

Rt: 49.7 min, MALDO-TOF: calculated; 3186.84 ($C_{156}H_{235}D_3N_{40}O_{32}$, exact mass), found; 3182.73 $[M-N_2+2H+Na]^+$, 3198.72 $[M-N_2+2H+K]^+$. Alk-WALP-K: Rt: 47.9 min, MALDI-TOF: calculated; 3240.88 ($C_{161}H_{241}D_3N_{38}O_{33}$, exact mass), found; 3263.82 $[M+Na]^+$, 3279.83 $[M+K]^+$

General procedure for the click-reactions

In a typical reaction 1 equiv of Alk-WALP-K (6.3 µmol, 20 mg) and 1.2 equiv of N_3-WALP-K or K-WALP-N_3 (7.5 µmol, 24.4 mg) were dissolved in DMF/H_2O (95/5 v/v, 0.5 ml). To the clear solution, approx 0.1 equiv copper(I)acetate (0.64 µmol, 80 µg) was added and the reaction mixture was heated in the microwave (10 minutes at 100°C and 20 minutes at 120°C). After the reaction time the solvents were evaporated in a speedvac concentrator. A concentrated solution in TFA (max. 1 ml) was made and dropped into a vortex of H_2O (40 ml, 4°C). The suspension was centrifuged and the pellet dissolved in tert-butanol/H_2O and lyophilized.

MALDI-TOF analysis

As a matrix α-cyano hydroxy cinnamic acid was used. A 5 $mgml^{-1}$ solution of matrix in acetonitrile/H_2O (1:1) + 0.1%TFA was mixed with a 1 $mgml^{-1}$ solution of peptide in TFE at a 1:1 ratio. A spot of 0.5 µl of this solution was applied on the MALDI plate. For calibration either a mix of angiotensin I, neurotensin, ACTH(1-17), ACTH(18-39) and ACTH(7-38) was used, or only ACTH(7-38).

Gel electrophoresis

The samples were dissolved in a loading buffer of 0.1 M TRIS-Cl, pH 6.8, 24% glycerol (v/v), 8% SDS (w/v) and bromophenol blue. Tricine-SDS-PAGE to separate molecules with molecular weights of 1-100 kDa was used.[49] As separating gel the composition described in the article as 16.5%T, 6%C was applied. An exception on the protocol was that for the stacking gel a 3M TRIS solution of pH 6.8 was used. The gel was run on a voltage of 30V for 30 minutes, followed by a voltage of 100V for 3 hours. Gels were fixated in destaining solution (H_2O/methanol/acetic acid, 315/150/35 v/v/v) for 10-30 minutes, stained with Coomassie brilliant blue G-250 for minimally one hour and destained overnight.

HPLC

Analysis and purification of monomers was performed and analysis of dimerization attempted by HPLC, using the following conditions. The peptide was eluted of a Prosphere C4 column (pore size 300Å, particle size 5 µm, 250x4.6mm) with a gradient of 10 to 100% buffer B in 40 minutes, where buffer A was H_2O/acetonitrile (80/20 v/v) + 0.1% TFA and buffer B was acetonitrile/iso-propanol/H_2O (50/45/5 v/v/v) + 0.1% TFA. A flow rate of 0.75 $ml \cdot min^{-1}$ was used and UV-detection at 220 and 280 nm. Preparative HPLC was performed with the same buffers. For the N_3-WALP-K and Alk-WALP-K analogues a semi preparative Prosphere C4 column (pore size 300Å, particle size 10 µm, 250x10mm) was used, at flow rate of 5 $ml \cdot min^{-1}$ and a gradient of 10 to 100% buffer B in 90 minutes. For the K-WALP-N_3 purification was performed with elution of a preparative Prosphere C4 column (pore size 300Å, particle size 10 µm, 250x22mm), applying a gradient of 10 to 100% buffer B in 100 minutes followed by maintaining at 100% buffer B for 15 minutes, with a flow of 9 $ml \cdot min^{-1}$.

Sephadex LH20

The parallel and antiparallel dimers were purified over Sephadex LH 20. On smaller scales (max. 8 mg) methanol was used as eluent and on larger scales DMF. Columns of the Sephadex medium in DMF of approximately 2.3 cm in diameter and a height of approximately 60 cm were poured in a glass column. With a glass wool cloth in the bottom opening the flow rate was too low for appropriate separation. Therefore a column with a glass frit was used. The elution of peptide

was followed by checking if a spot of a collected fraction on a TLC plate could be visualized by UV (254 nm). The first 5-8 peptide-containing fractions were analyzed by gel electrophoresis. To obtain samples suitable to apply on gel, a 50 µl aliquot was taken, the solvent was evaporated under a hot air stream and N_2-flow and the sample left under vacuum overnight.

Structural and biophysical analysis

Stock solutions

Stock solutions of lipids were prepared by dissolving an appropriate amount of lipid in chloroform. The concentration of lipid stocks was determined according to the method of Rouser.[50] Typical lipid concentrations varied for the various biophysical measurements and will be specified in the appropriate sections. Peptides were dissolved in TFE. The concentration of peptide stocks and CD-samples was determined by absorption spectroscopy, using the tryptophan absorption at 280 nm with an extinction coefficient of 22400.[33] For the dimers an extinction coefficient of 44800 was applied. Typical concentrations of peptide stock solutions were 0.5-2 mM

Circular dichroism

For the unilamellar vesicle samples the appropriate amount of peptide (from a 0.5-2 mM stock in TFE) were mixed with the appropriate amount of di-14:0 PC (from a 10 mM stock in chloroform) to obtain a P/L ratio of 1:100. Solvents were evaporated under a N_2-flow and the resulting films further dried under vacuum overnight. The film was hydrated with a 10 mM phosphate buffer of pH 7 (500 µl) to obtain a peptide concentration of 50 µM, freeze-thawed ten times and extruded ten times over 200 nm Whatman membrane filters to obtain large unilamellar vesicles (LUVs). CD-spectra were measured with the sample in a 1 mm cuvet, in the range 260-190 nm, with a scan speed of 20 nm·min^{-1}, averaging 10 scans, in continuous mode, with a data pitch of 0.2 nm, 1 nm bandwidth and 1 second response time, keeping the temperature at 30ºC. The molar ellipticity per residue was calculated using the following formula:

$$[\theta] = \frac{\theta}{l * AAn * c}$$

where θ is the ellipticity (in degrees), l is the path length of the cuvet (in cm), AAn is the number of amino acids in the peptide and c is the concentration of the peptide (in dmol·cm^{-3}), which was determined after the CD-measurement using absorption spectroscopy, to correct for possible loss of peptide during extrusion. For the concentration determination (see also 'stock solutions') an aliquot of the sample was added to 1 ml TFE, which disrupts the vesicles.
The SDS and DDM samples were prepared as described previously.[51] In a typical preparation 20 µl of a 0.4 M detergent solution in H_2O was added to the appropriate amount of peptide in 20 µl TFE. The peptide concentration was held constant at 50 µM and a P/detergent ratio of 1:400 was used. The sample was vortexed, diluted with 450 µl H_2O and vortexed again, yielding a clear solution which was frozen and lyophilized. The fluffy white powder was dissolved in either 400 µl 10 mM phosphate buffer, pH 7 or in a mixture of 10 mM phosphate buffer, pH 7 and 10 M urea in buffer (1:1 v/v) to obtain a clear sample. CD-spectra were measured with the same settings as used for the di-14:0 PC samples and normalized using the same formula, except that the samples with denaturant were measured in the range of 260-210 nm and when appropriate at 90ºC.

ATR-FTIR measurements

Since weight and weight-ratio's are important in ATR-FTIR, the weight of peptide was held constant at 20 µg and the final concentration of peptide plus lipid in H_2O was 2 µg·µl^{-1} in

all samples. The molar helix/lipid ratio was kept constant at 1:30. Thus, 20 µg of peptide (~6.3 nmol monomer, ~3.1 nmol dimer) in TFE was mixed with the appropriate amount of a 1.3 mM di-14:0 PC stock (~0.19 µmol) in chloroform to obtain a monomer/lipid ratio of 1:30 or a dimer/lipid ratio of 1:60. The sample was mixed vigorously on a vortex, the solvents evaporated under a N_2-flow and the film left under vacuum overnight. The samples were hydrated with H_2O (~90 µl) and freeze-thawed ten times by subsequently freezing in ethanol/dry ice and thawing in a 50ºC water bath. The internal reflection element for the ATR-FTIR measurements was a trapezoidal germanium plate. The crystal was cleaned and made more hydrophilic by washing with water and a soap with basic pH, distilled water, methanol and chloroform successively. For the regular, dichroic and H/D-exchange spectra 15 µl of samples was applied on the surface. Excess water was slowly evaporated under a N_2-flow while spreading the sample with a pipette-tip. The plate was placed in a sample holder to achieve the appropriate incident angle of the IR light into the IRE, yielding 25 reflections. For the non-polarized spectra, first a background was measured on an area of the germanium plate without sample, averaging 128 scans. Next the sample was measured as an average of 128 scans with automatic subtraction of the background, a resolution of 2 cm^{-1}, in the range of 800-4000 cm^{-1}. The dichroic spectra were recorded similarly, except that the incident light was polarized parallel or perpendicular to the incident plane. For the H/D exchange measurements, one background spectrum was recorded which was used for automatic correction of all recorded spectra. The sample holder was attached to a D_2O-saturated nitrogen flow. A spectrum was recorded every minute for half an hour, where the first measurement was collected after three minutes. Furthermore, the same settings and corrections as described above were employed. For the temperature dependent spectra a slightly different procedure was followed. Two 30 µl aliquots of two different samples were spread at two separate areas of the IRE and dried under a nitrogen flow. The compartment of the sample holder in touch with the samples was filled with H_2O. The temperature of the holder and thus the H_2O was controlled by a water bath. At temperatures of 15, 20, 23, 25, 30, 35 and 40ºC, one background was recorded followed by measurement of both samples, which were automatically corrected with this background.

ATR-FTIR data analysis

All spectra analysis was performed by Kinetics, a software running under Matlab 7.5.0 (R2007b). All spectra were first corrected for contributions of water vapour and CO_2, a 4 cm^{-1} apodization was applied and a 1783-1598-1510-1401-1130-999 baseline was subtracted. Peak positions and areas were determined with respectively the peak picking and integration tools in Kinetics. The exchange of the peptides was established using the amide II band area (1760-1700 cm^{-1}). The exchange kinetics was calculated and fitted, considering the area of the first spectrum as 100% hydrogen and normalizing on the lipid ν(C=O) area (1590-1510 cm^{-1}). The tilt was computed with the homemade 'Stdwave' software, routinely used in the Goormaghtigh-group. The dichroic ratios, defined as $A_{||}/A_{\perp}$, R^{ATR} and R^{iso} were calculated from the amide I and lipid ν(C=O) band areas respectively. In the standing wave program, the following parameters were specified: n_1=4.00 (germanium), n_2=1.44 (lipids), n_3=1.0 (air), real membrane thickness computed from the R^{iso} value, 27º angle between molecular axis and dipole of peptide ν(C=O). Subsequently, the tilt angle was determined from the dichroic ratio R^{ATR} with the 'angle' polarizer tool in the software.

Sample preparation solid state ^{2}H-NMR peptides and ^{31}P-NMR lipids

A stock of di-18:1 PC in chloroform with a concentration of 98 mM was used. Lipid

(18.75 µmol) in chloroform was mixed with the appropriate amount of peptide in TFE to obtain P/L ratio's of 1:200 for the dimers and 1:100 for the monomers. The solvents were evaporated under a N_2-flow and the films further dried under vacuum, usually overnight. The films were hydrated with 100 µl deuterium depleted H_2O and ten times subsequently frozen in ethanol/dry-ice and thawed in a water bath of 40°C. The samples were kept at -20ºC until use and then transferred to a 4 mm outer diameter, ZiO_2 sample tube for both ^{2}H-NMR and ^{31}P-NMR.

Sample preparation solid state ^{2}H-NMR lipids

In all samples 3 µmol of di-14:0 PC-d54 from a 29 mM stock in chloroform was mixed with the appropriate amount of 0.5-2 mM peptide in TFE. In the pure lipid sample no peptide was added, but only some TFE. Helix/lipid ratios of 1:30 were used, resulting in a monomer/lipid ratio of 1:30 (0.1 µmol monomer) and a dimer/lipid ratio of 1:60 (0.05 µmol dimer). Chloroform and TFE were evaporated under a N_2-flow. The resulting films were further dried, usually overnight, under vacuum and hydrated with 100 µl of deuterium-depleted water. The lipid suspensions were subjected to ten cycles of subsequently freezing in ethanol/dry ice and thawing in a water bath of 50ºC.

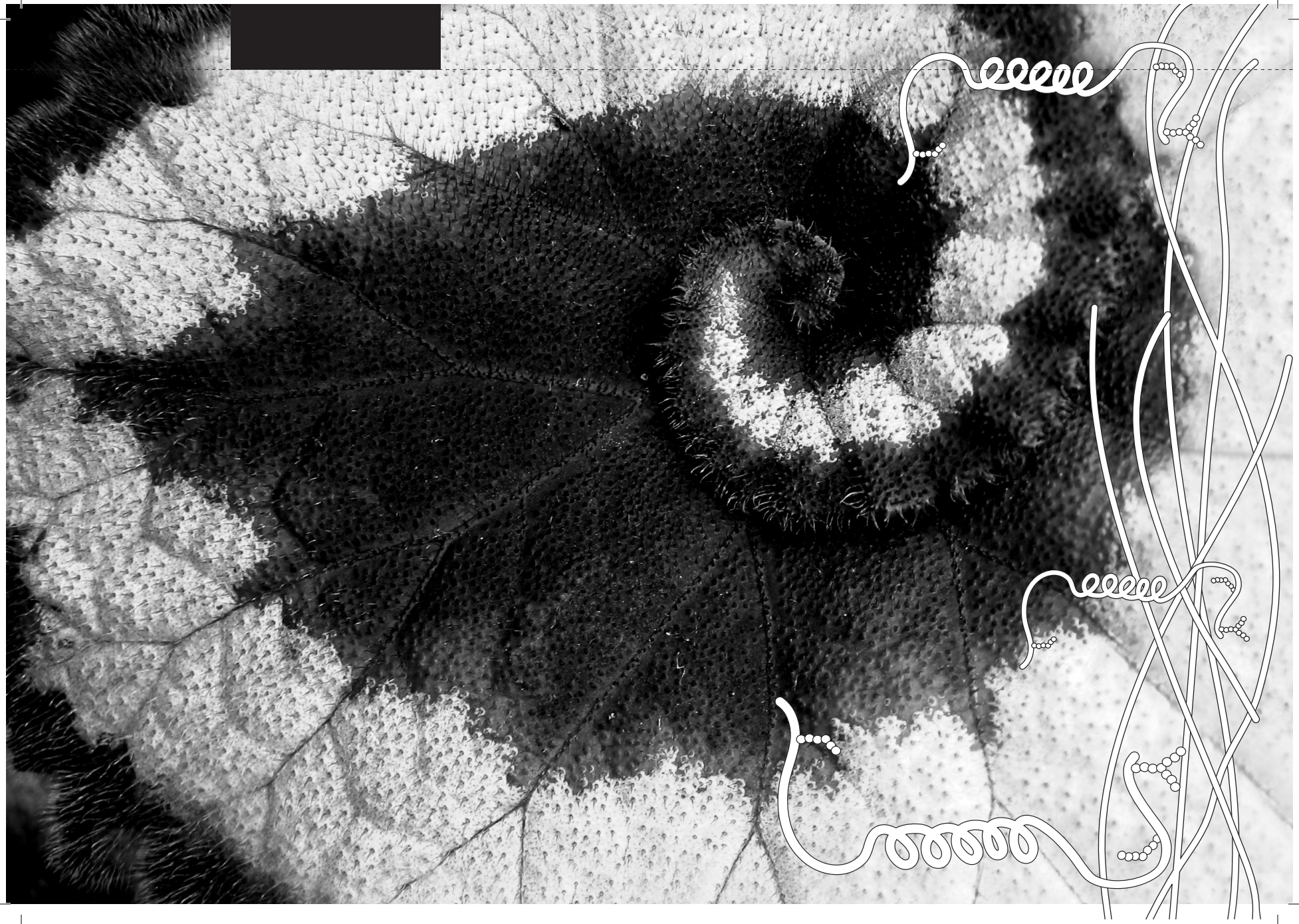
Solid state NMR measurements

Solid state ^{31}P-NMR spectra were recorded of all samples. The measurements were performed at 202.5 MHz using a Hahn echo experiment with a 5 µs 90º pulse, a 10 µs 180º pulse, an echo delay of 50 µs and a relaxation delay of 2 s. A 250 ppm spectral width, 4096 data points and gated proton decoupling were applied. The measurements were performed at room temperature and 400-1000 scans were collected. The spectra were processed with zero-filling to 2048 data points and a 100 Hz exponential multiplication before Fourier transformation. The ^{2}H-NMR spectra of the peptides were performed essentially as described previously,[13] except that the 90º pulse was 2.5 µs, 2048 data points and no zero filling were used, 300k-800k scans were collected and a 200 Hz exponential multiplication was applied. The di-14:0 PC-d_{54} samples were equilibrated at 40ºC for at least 10 minutes before the ^{2}H-NMR measurements. The experiments were performed at 76.78 MHz with the same quadrupolar echo sequence as used for the peptide spectra, except that a recycling time of 600 ms was used. In some experiments a recycling delay of 1 second was applied. There were no differences found with the other spectra, which confirmed that the 600 ms delay was sufficient. The spectra were recorded with a spectral width of 500 kHz and 4096 data points, starting the acquisition at the echo maximum. Between 20k and 40k scans were collected. Processing was performed by zero-filling to 8192 data points, Fourier transformation and centering the spectrum on the CH_3 signals.

References

1. Wallin, E. and von Heijne, G., Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms, *Protein Sci*, **1998**, 7(4), p. 1029-38
2. Marsh, D., Protein modulation of lipids, and vice-versa, in membranes, *Biochim Biophys Acta*, **2008**, 1778(7-8), p. 1545-75
3. Lee, A. G., How lipids affect the activities of integral membrane proteins, *Biochim Biophys Acta*, **2004**, 1666(1-2), p. 62-87
4. Sukharev, S., Betanzos, M., Chiang, C. S., and Guy, H. R., The gating mechanism of the large mechanosensitive channel MscL, *Nature*, **2001**, 409(6821), p. 720-4
5. Kusnetzow, A. K., Altenbach, C., and Hubbell, W. L., Conformational states and dynamics of rhodopsin in micelles and bilayers, *Biochemistry*, **2006**, 45(17), p. 5538-50
6. Molina, M. L., Encinar, J. A., Barrera, F. N., Fernandez-Ballester, G., Riquelme, G., and Gonzalez-Ros, J. M., Influence of C-terminal protein domains and protein-lipid interactions on tetramerization and stability of the potassium channel KcsA, *Biochemistry*, **2004**, 43(47), p. 14924-31
7. van den Brink-van der Laan, E., Killian, J. A., and de Kruijff, B., Nonbilayer lipids affect peripheral and integral membrane proteins via changes in the lateral pressure profile, *Biochim Biophys Acta*, **2004**, 1666(1-2), p. 275-88
8. Groemping, Y. and Rittinger, K., Activation and assembly of the NADPH oxidase: a structural perspective, *Biochem J*, **2005**, 386(Pt 3), p. 401-16
9. Blazyk, J. F. and Newman, J. L., Calorimetric studies of lipid phase transitions in native and heat-denatured membranes of beef heart submitochondrial particles, *Biochim Biophys Acta*, **1980**, 600(3), p. 1007-11
10. Morein, S., Killian, J. A., and Sperotto, M. M., Characterization of the thermotropic behavior and lateral organization of lipid-peptide mixtures by a combined experimental and theoretical approach: effects of hydrophobic mismatch and role of flanking residues, *Biophys J*, **2002**, 82(3), p. 1405-17
11. Dumas, F., Sperotto, M. M., Lebrun, M. C., Tocanne, J. F., and Mouritsen, O. G., Molecular sorting of lipids by bacteriorhodopsin in dilauroylphosphatidylcholine/distearoylphosphatidylcholine lipid bilayers, *Biophys J*, **1997**, 73(4), p. 1940-53
12. Killian, J. A., Synthetic peptides as models for intrinsic membrane proteins, *FEBS Lett*, **2003**, 555(1), p. 134-8
13. Strandberg, E., Ozdirekcan, S., Rijkers, D. T. S., van der Wel, P. C., Koeppe, R. E., 2nd, Liskamp, R. M., and Killian, J. A., Tilt angles of transmembrane model peptides in oriented and non-oriented lipid bilayers as determined by 2H solid-state NMR, *Biophys J*, **2004**, 86(6), p. 3709-21
14. Sparr, E., Ash, W. L., Nazarov, P. V., Rijkers, D. T. S., Hemminga, M. A., Tieleman, D. P., and Killian, J. A., Self-association of Transmembrane {alpha}-Helices in Model Membranes: IMPORTANCE OF HELIX ORIENTATION AND ROLE OF HYDROPHOBIC MISMATCH, *J Biol Chem*, **2005**, 280(47), p. 39324-39331
15. de Planque, M. R., Greathouse, D. V., Koeppe, R. E., 2nd, Schafer, H., Marsh, D., and Killian, J. A., Influence of lipid/peptide hydrophobic mismatch on the thickness of diacylphosphatidylcholine bilayers. A 2H NMR and ESR study using designed transmembrane alpha-helical peptides and gramicidin A, *Biochemistry*, **1998**, 37(26), p. 9333-45
16. Killian, J. A., Hydrophobic mismatch between proteins and lipids in membranes, *Biochim Biophys Acta*, **1998**, 1376(3), p. 401-15
17. Killian, J. A. and von Heijne, G., How proteins adapt to a membrane-water interface, *Trends Biochem Sci*, **2000**, 25(9), p. 429-34
18. Holt, A. and Killian, J. A., Orientation and dynamics of transmembrane peptides: the power of simple models, *Eur Biophys J*, **2009**, 39(4), p. 609-621
19. Nyholm, T. K., Ozdirekcan, S., and Killian, J. A., How protein transmembrane segments sense the lipid environment, *Biochemistry*, **2007**, 46(6), p. 1457-65
20. Sparr, E., Ganchev, D. N., Snel, M. M., Ridder, A. N., Kroon-Batenburg, L. M., Chupin, V., Rijkers, D. T. S., Killian, J. A., and de Kruijff, B., Molecular organization in striated domains induced by transmembrane alpha-helical peptides in dipalmitoyl phosphatidylcholine bilayers, *Biochemistry*, **2005**, 44(1), p. 2-10
21. Walters, R. F. and DeGrado, W. F., Helix-packing motifs in membrane proteins, *Proc Natl Acad Sci U S A*, **2006**, 103(37), p. 13658-63
22. Curran, A. R. and Engelman, D. M., Sequence motifs, polar interactions and conformational changes in helical membrane proteins, *Current opinion in structural biology*, **2003**, 13, p. 412-417
23. Johnson, R. M., Rath, A., Melnyk, R. A., and Deber, C. M., Lipid solvation effects contribute to the affinity of Gly-xxx-Gly motif-mediated helix-helix interactions, *Biochemistry*, **2006**, 45(28), p. 8507-15
24. Sal-Man, N., Gerber, D., Bloch, I., and Shai, Y., Specificity in transmembrane helix-helix interactions mediated by aromatic residues, *J Biol Chem*, **2007**, 282(27), p. 19753-61
25. Moore, D. T., Berger, B. W., and DeGrado, W. F., Protein-protein interactions in the membrane: sequence, structural, and biological motifs, *Structure*, **2008**, 16(7), p. 991-1001
26. Lee, J. and Im, W., Role of hydrogen bonding and helix-lipid interactions in transmembrane helix association, *J Am Chem Soc*, **2008**, 130(20), p. 6456-62
27. von Heijne, G., Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule, *J Mol Biol*, **1992**, 225(2), p. 487-94
28. Dowhan, W. and Bogdanov, M., Lipid-dependent membrane protein topogenesis, *Annu Rev Biochem*, **2009**, 78, p. 515-40
29. Tornoe, C. W., Christensen, C., and Meldal, M., Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides, *J Org Chem*, **2002**, 67(9), p. 3057-64
30. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B., A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes, *Angew Chem Int Ed Engl*, **2002**, 41(14), p. 2596-9
31. Peltier, J. M., Smith, R. W., MacLean, D. B., and Szarek, W. A., Reduction of azides under conditions of desorption-chemical ionization or fast-atom-bombardment mass-spectrometry, *Carbohydr Res*, **1990**, 207(1), p. 1-10
32. Kasper, P. T., Back, J. W., Vitale, M., Hartog, A. F., Roseboom, W., de Koning, L. J., van Maarseveen, J. H., Muijsers, A. O., de Koster, C. G., and de Jong, L., An aptly positioned azido group in the spacer of a protein cross-linker for facile mapping of lysines in close proximity, *Chembiochem*, **2007**, 8(11), p. 1281-92
33. de Planque, M. R., Goormaghtigh, E., Greathouse, D. V., Koeppe, R. E., 2nd, Kruijtzer, J. A., Liskamp, R. M., de Kruijff, B., and Killian, J. A., Sensitivity of single membrane-spanning alpha-helical peptides to hydrophobic mismatch with a lipid bilayer: effects on backbone structure, orientation, and extent of membrane incorporation, *Biochemistry*, **2001**, 40(16), p. 5000-10
34. de Planque, M. R., Kruijtzer, J. A., Liskamp, R. M., Marsh, D., Greathouse, D. V., Koeppe, R. E., 2nd, de Kruijff, B., and Killian, J. A., Different membrane anchoring positions of tryptophan and lysine in synthetic transmembrane alpha-helical peptides, *J Biol Chem*, **1999**, 274(30), p. 20839-46
35. Rice, D. and Oldfield, E., Deuterium nuclear magnetic resonance studies of the interaction between dimyristoylphosphatidylcholine and gramicidin A', *Biochemistry*, **1979**, 18(15), p. 3272-9
36. Rice, D. M., Hsung, J. C., King, T. E., and Oldfield, E., Protein-lipid interactions. High-field deuterium and

phosphorus nuclear magnetic resonance spectroscopic investigation of the cytochrome oxidase-phospholipid interaction and the effects of cholate, *Biochemistry*, **1979**, 18(26), p. 5885-92
37. Henke, H., *Preparative Gel Chromatography on Sephadex LH-20*. 1995, Heidelberg: Huthig. 617.
38. Yano, Y. and Matsuzaki, K., Measurement of thermodynamic parameters for hydrophobic mismatch 1: self-association of a transmembrane helix, *Biochemistry*, **2006**, 45(10), p. 3370-8
39. Nagle, J. F. and Tristram-Nagle, S., Structure of lipid bilayers, *Biochim Biophys Acta*, **2000**, 1469(3), p. 159-95
40. Monticelli, L., Kandasamy, S. K., Periole, X., Larson, R. G., Tieleman, D. P., and Marrink, S. J., The MARTINI Coarse-Grained Force Field: Extension to Proteins, *J Chem Theory and Comput*, **2008**, 4(5), p. 819
41. Berne, B. J. and Pecora, R., *Dynamic Light Scattering*. 1976, New York: John Wiley.
42. Holt, A., Koehorst, R. B., Rutters-Meijneke, T., Gelb, M. H., Rijkers, D. T. S., Hemminga, M. A., and Killian, J. A., Tilt and rotation angles of a transmembrane model peptide as studied by fluorescence spectroscopy, *Biophys J*, **2009**, 97(8), p. 2258-66
43. Holt, A., Rougier, L., Reat, V., Jolibois, F., Saurel, O., Czaplicki, J., Killian, J. A., and Milon, A., Order parameters of a transmembrane helix in a fluid bilayer: case study of a WALP peptide, *Biophys J*, **2010**, 98(9), p. 1864-72
44. Ozdirekcan, S., Nyholm, T. K., Raja, M., Rijkers, D. T. S., Liskamp, R. M. J., and Killian, J. A., Influence of trifluoroethanol on membrane interfacial anchoring interactions of transmembrane alpha-helical peptides, *Biophys J*, **2008**, 94(4), p. 1315-25
45. de Planque, M. R., Boots, J. W., Rijkers, D. T. S., Liskamp, R. M. J., Greathouse, D. V., and Killian, J. A., The effects of hydrophobic mismatch between phosphatidylcholine bilayers and transmembrane alpha-helical peptides depend on the nature of interfacially exposed aromatic and charged residues, *Biochemistry*, **2002**, 41(26), p. 8396-404
46. Biggin, P. C. and Sansom, M. S., Channel gating: twist to open, *Curr Biol*, **2001**, 11(9), p. R364-6
47. Wegener, A. A., Chizhov, I., Engelhard, M., and Steinhoff, H. J., Time-resolved detection of transient movement of helix F in spin-labelled pharaonis sensory rhodopsin II, *J Mol Biol*, **2000**, 301(4), p. 881-91
48. Venturoli, M., Smit, B., and Sperotto, M. M., Simulation studies of protein-induced bilayer deformations, and lipid-induced protein tilting, on a mesoscopic model for lipid bilayers with embedded proteins, *Biophys J*, **2005**, 88(3), p. 1778-98
49. Schagger, H. and von Jagow, G., Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal Biochem*, **1987**, 166(2), p. 368-79
50. Rouser, G., Fkeischer, S., and Yamamoto, A., Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots, *Lipids*, **1970**, 5(5), p. 494-6
51. Killian, J. A., Trouard, T. P., Greathouse, D. V., Chupin, V., and Lindblom, G., A general method for the preparation of mixed micelles of hydrophobic peptides and sodium dodecyl sulphate, *FEBS Lett*, **1994**, 348(2), p. 161-5



5

Tetrameric assemblies of transmembrane model peptides; Design, explorative synthesis and characterization

Tania Rutters Meijneke, Dirk T.S. Rijkers and J. Antoinette Killian

Abstract

In this thesis, the influence of cross sectional diameter of membrane proteins on protein-lipid interactions is under investigation. In order to vary the size of the membrane spanning segment systematically, oligomeric assemblies of the so-called WALP model peptide are synthesized. In previous chapters, two types of covalent dimers were constructed. Here, we will take one step further and explore the synthesis of a tetrameric assembly. For this purpose WALP analogues were synthesized to couple to a well-defined scaffold via 'click chemistry'. Three generations of WALP analogues were synthesized, to improve the characterization and purification of the assemblies. With all three peptides a tetramer (with a molecular weight in the range of 12-15 kDa) appeared to be formed, as indicated by SDS-PAGE, GPC and/or MALDI-TOF. However, the hydrophobicity of the tetrameric constructs was a limiting factor for their isolation.

Introduction

Membrane proteins play important roles in health and disease. It is therefore important to understand how they function. Unfortunately, membrane protein research is a problem-riddled field, due to the hydrophobic nature of this class of proteins. One of the approaches to obtain knowledge on membrane proteins is to start by determining which factors can be important for their behaviour. One of the factors that might be important for membrane protein behaviour is the cross sectional diameter. Though most membrane proteins have in common that they are multispan, there is a huge variation in sizes of these proteins. There have been studies into some of the potential consequences of differences in diameter. For example, the diffusion of proteins with different radii[1] or the influence of size on the translocation of lipids with a subset of membrane proteins[2] were studied. However, it is difficult to determine to what extent the different sizes truly play a role in behaviour, since the diameter is far from the only difference among the several membrane proteins.

For systematic studies on the effects of protein size on the behaviour of membrane proteins and protein-lipid interactions it would be useful to have a system in which size can be systematically varied. An possible way to achieve this is by using model systems of synthetic α-helices in lipid vesicles. Model systems of the so-called WALP peptides in vesicles of varying lipid content have already been studied extensively to obtain knowledge on the fundamentally important helix-lipid interactions.[3-6] The WALP peptide (Ac-GWW$(LA)_8$LWWA-NH_2) is an α-helical transmembrane peptide that mimics the features of natural membrane proteins. In a covalent assembly of multiple WALP peptides the cross sectional diameter can be systematically varied. The main difference of the oligomer with a monomer would be the diameter, because all monomeric units are equal, though also helix-helix interactions become a factor of influence.

In chapter 2 and 4 dimeric assemblies of WALP peptides were designed, synthesized and characterized. A tetrameric assembly would provide an additionally significant change in cross sectional diameter. With a covalent tetramer, more questions on the influence of diameter on membrane protein structure and function could be tackled.

The design of the covalent tetramer was based on the template assembled synthetic protein (TASP) concept of Mutter et al.[7, 8] This concept is used regularly for the *de novo* design of natural proteins, circumventing the so-called 'protein folding problem'.[9-11] Especially the synthesis of functional ion channels following the TASP approach has proven to be successful.[12-16] The scaffold applied in this chapter is a cyclic peptide of ten amino acids. The proline-glycine units at both ends are ß-turn inducers and provide for a rigid, well-defined structure.[17] Because of the preferential orientation of the units to which the peptides can be coupled, the favoured conformation of the WALP peptides will be with the helices oriented parallel to each other (Figure 1).

To make a covalent assembly of four WALP monomers, WALP analogues with an azide functionality were designed to couple to a TASP scaffold with four alkynes via click-chemistry[18, 19]. Similar

Figure 1. Tetrameric assembly of WALP peptides attached to a TASP-based scaffold

Table 1. WALP analogues designed to synthesize covalent tetramers

Peptide	Sequence
N_3-WALP	Ac-Lys(N_3)-GWW$(LA)_8$LWWA-NH_2
N_3-WALP-KK	Ac-Lys(N_3)-GWW$(LA)_8$LWWA-GAGAGKK-NH_2
N_3-WALP-SS	Ac-Lys(N_3)-GSGS-GWW$(LA)_8$LWWA-GSGSGSS-NH_2

approaches were recently applied successfully for the conjugation of sugars[20] and unprotected, water-soluble peptides[21] to the scaffold. Of the model peptides, three generations were designed (Table 1). In all designs, WALP peptides were equipped with an N-terminal azide via an Fmoc-Lys(N_3)-OH building block (Lys(N_3)). In the scaffold, four lysine residues functionalized with an alkyne (via coupling of 4-pentynoic acid) were incorporated. The choice of placing the azides on the WALP peptides and the alkynes on the scaffold was random and can be varied.

In the click-reaction, not only tetramers will be formed, which indicates that purification will be necessary. In chapter 2 and 4 it was demonstrated that the purification of dimers is challenging. Purification of the bigger and thus more hydrophobic tetramers will be even more difficult, especially since the WALP peptides do not dissolve in aqueous media. For this reason, solubility tags were attached. For isolation of the tetramers, methods conventionally used for peptides can be explored or methods used for natural membrane proteins may be adjusted and tested.

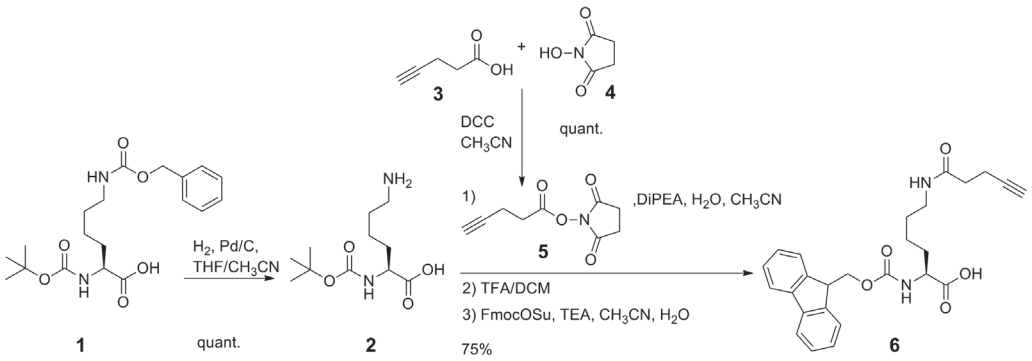
In this chapter the explorative synthesis and characterization of covalent tetramers will be presented. First, the synthesis, characterization and purification of all compounds that are necessary to obtain the tetrameric assemblies are described. Second, the construction and chemical analysis of three generations of tetramers is considered. In addition, the attempts to purify each generation are discussed.

Results

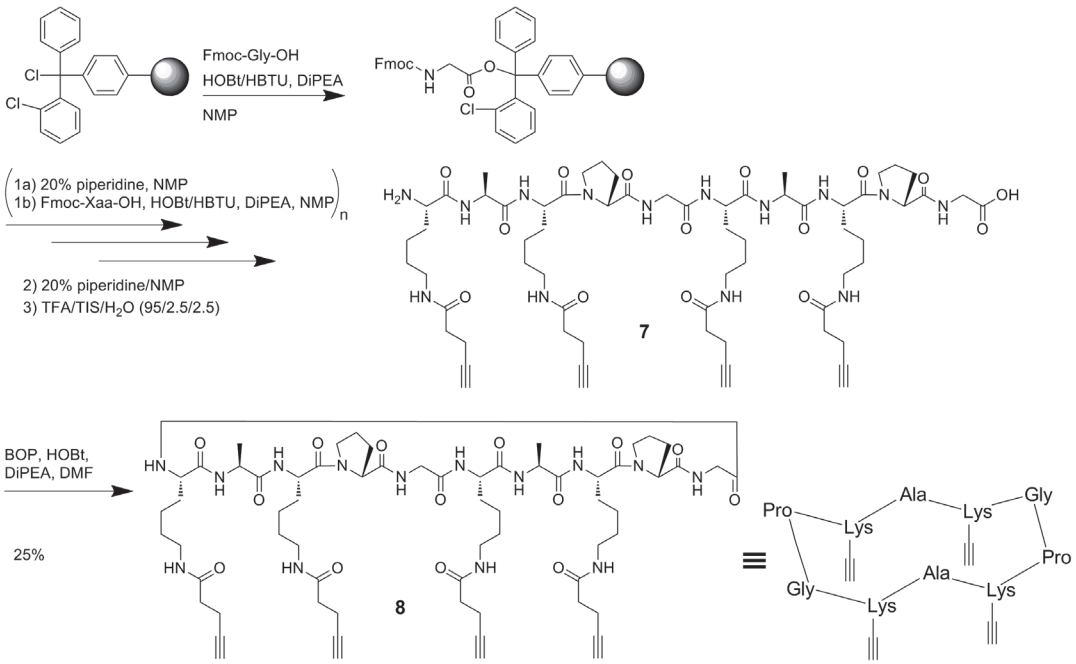
Synthesis of the scaffold

To obtain covalent tetramers, a cyclic scaffold had to be equipped with four alkynes. Therefore, an alkyne-functionalized lysine was synthesized (Scheme 1), which could be used in solid phase peptide synthesis. The ε-amine of Boc-Lys(Z)-OH **1** was deprotected by hydrogenation to obtain Boc-Lys-OH **2** in quantitative yield. Next, activated 4-pentynoic acid **5** was coupled to the free amine of **2**, after which the α-amine was deprotected and subsequently reprotected with an Fmoc functionality to obtain the Fmoc-Lys(N$^{\varepsilon}$-4-pentynoyl)-OH building block **6** in an overall yield of 75%. It was shown by ^{1}H-NMR and HPLC that compound **6** was apposite to use in the synthesis of the scaffold.

For the TASP-scaffold first the linear peptide **7** (Scheme 2) was synthesized by solid phase peptide synthesis on an 2-chlorotrityl chloride



Scheme 1. Synthesis of building block **6** to use in the solid phase peptide synthesis of the scaffold



Scheme 2. Synthesis of scaffold **8** to use in the synthesis of a tetrameric assembly of WALP model peptides

Table 2. ESI-MS analysis of linear peptide **7** and scaffold **8**

Peptide	MW (calculated)	$[M+H]^+$, $[M+Na]^+$ (found)
Linear peptide **7**	1300.72	1301.55, 1323.80
Scaffold **8**	1282.70	1283.90, 1306.35

Table 3. ESI-MS analysis of WALP analogues

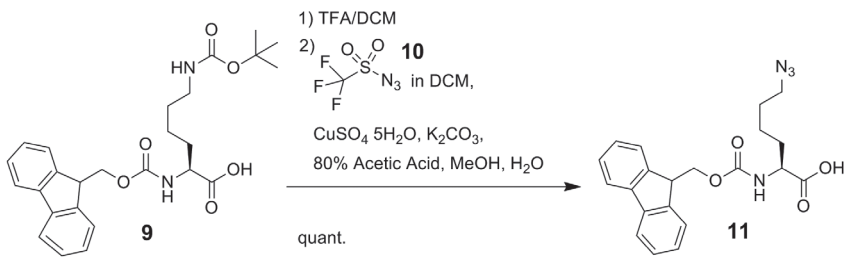
Peptide	MW (calculated)	$[M+2H]^{2+}$, $[M+2Na]^{2+}$ (found)
N_3-WALP	2676.27	1338.98, 1361.27
N_3-WALP-KK	3242.90	1622.07, 1643.80

resin using the Fmoc/tBu-strategy[22]. The first amino acid, glycine, was coupled manually. The rest of the peptide was synthesized automatically on a 0.25 mmol scale. The peptide was cleaved from the resin to obtain the linear peptide **7** with a free amine at the N-terminus and a free acid at the C-terminus. Analytical HPLC showed that product **7** was 60 % pure and the correct mass was found with ESI-MS (Table 2). Without further purification, linear peptide **7** was cyclised (Scheme 2). This was performed in a dilute solution to prevent intermolecular reactions. According to ESI-MS, cyclic peptide **8** was formed (Table 2) and pure scaffold **8** was obtained in 20% yield after purification by preparative HPLC.

Synthesis of the WALP analogues

To couple the WALP peptides to the scaffold they were equipped with an azide functionality. For this purpose, a lysine-based building block with an azide-functionality was synthesized (Scheme 3). First, the ε-amine of Fmoc-Lys(Boc)-OH **9** was deprotected. Then, a diazotransfer was performed on the free amine of Fmoc-Lys-OH following the method of Roice et al[23]. The building block Fmoc-Lys(N_3)-OH **11** was obtained in a near quantitative yield. According to ^{1}H-NMR and HPLC, the building block was suitable for usage in solid phase peptide synthesis.

The azide-containing WALP analogues (Table 1) were synthesized by automated solid phase peptide synthesis on a Tentagel S-RAM resin. The synthesis was done on a 0.25 mmol scale using the Fmoc/tBu-strategy.[22] All peptides were characterized with HPLC and mass spectrometry. The N_3-WALP, N_3-WALP-KK and N_3-WALP-SS had a purity of 82, 72 and 79%, respectively. The first two peptides were analyzed by ESI-MS and the correct molecular weight was found for each peptide (Table 3). The last peptide was analyzed by MALDI-TOF. With this technique the exact mass could not be measured, but a broad peak around the molecular weight of the peptide was found, indicating the formation of the peptide. Mostly, the crude peptides were used in the synthesis of the tetramers. Since the synthesis protocol made the presence of deletions unlikely, potential impurities would be the shorter peptides that do not contain an azide and thus cannot be coupled. To check the validity of this assumption, some of the N_3-WALP-KK was



Scheme 3. Synthesis of building block **11** to use in the solid phase peptide synthesis of the scaffold

purified. Purification was achieved by preparative HPLC with a system especially designed for these hydrophobic peptides (see experimental section). Yields between 30 and 40% were achieved.

Synthesis, characterization and purification of the first generation tetramers

In the initial design of the tetramers, the N_3-WALP analogues are coupled to the TASP scaffold. The structure of the azide-containing WALP peptide is shown in Figure 2, together with the molecular weights of the potential mono- till tetramers on the scaffold.

The first attempt to click the WALP analogues to the scaffold was performed at room temperature in DMF/H_2O (95/5) using $CuSO_4$/sodium ascorbate as Cu(I) source. Before characterization a pre-purification of the peptides had to be performed to remove the residual copper. For this purpose, the peptides were dissolved in TFA and precipitated in H_2O, leaving the copper in solution. After centrifugation, the pellet was dissolved in tert-butanol/H_2O (1:1) and lyophilized. Tris-tricine gel electrophoresis showed that under these conditions no tetramers were formed (Figure 3A, lane 2). Like for the monomer (Figure 3A, lane 1) a dimer band is observed, which can be covalent or non-covalent by aggregation phenomena. Fortunately, when performing the reaction via microwave irradiation and in the presence of copper(I) acetate, significant amounts of tetramer seemed to be formed according to tris-tricine gel electrophoresis (Figure 3A, lane 3). The assignment of the bands is simply based on the fact that there are four bands distinguishable, suggesting monomeric, dimeric, trimeric and tetrameric assemblies. There are some bands above the more intense tetramer-band, which are attributed to non-covalent aggregates, as was seen before with the dimers (chapter 2 and 4). The fact that using less equivalents of N_3-WALP in the click reaction resulted in a decrease in intensity of the highest band and increase in the dimeric and trimeric bands (Figure 3B, lane 1) reinforces the assignments.

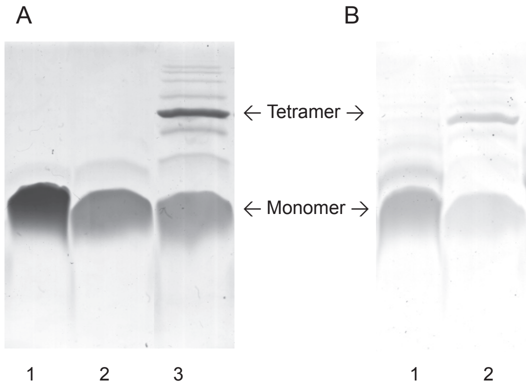
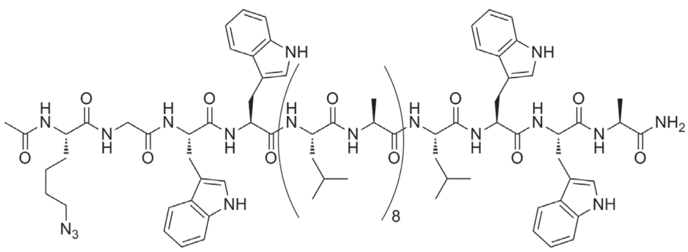


Figure 3. Tricine-SDS-PAGE of first tetramerization trials (A), showing N3-WALP monomer (lane 1), reaction with CuSO4/sodium ascorbate where no tetramers were formed (lane 2) and reaction with copper(I) acetate where tetramers were formed (lane 3) and tricine-SDS-PAGE of comparison of reactions (B) with shortage (lane 1) and excess (lane 2) of N3-WALP

In Figure 4, GPC traces of an N_3-WALP, a C12-WALP monomer/dimer mixture (see Chapter 2) and the click-reaction mixture, run with DMF/LiCl, are shown. With GPC, molecular weights can be determined, using an appropriate



N_3-WALP	2676
Monomer	3960
Dimer	6636
Trimer	9312
Tetramer	11989

Figure 2. Structure of the N_3-WALP and molecular weights of putative first generation mono-, di-, tri- and tetramers

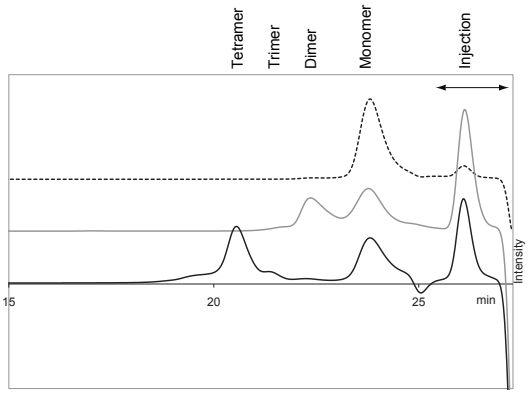


Figure 4. GPC traces of N_3-WALP (dotted black line), mixture of C12-WALP monomer and dimer (solid grey line) and tetramerization of N_3-WALP (solid black line) with peaks of injection, monomer, dimer and tetramer

series of standards with known molecular weights. Since there was no peptide-standard available, a polymer-standard was used. Therefore, molecular weights could not be determined exactly, yielding 1500 Da for the C12-WALP (MW≈2500) monomer and 3700 Da for the C12-WALP dimer (MW≈5000). However, the respective molecular weights supported the conclusion that tetramers were formed, with a molecular weight of 9900 for the supposed tetramer-peak. Additionally, it was shown with FTIR that the amount of azide in the reaction mixture was decreased and the amount of triazole was increased in comparison to the monomers (data not shown). In order to confirm the formation of tetramers MALDI-TOF analysis was performed on the reaction mixtures. Unfortunately, only peaks around the molecular weight of the mono- and di-substituted scaffold could be detected. This could have been expected, since dimers were already too difficult to ionize, as a result of their hydrophobicity and absence of charged side chains (see Chapter 2). Attempts to force the reaction to completion by prolonged reaction times, higher reaction temperatures, more equivalents of Cu(I) or more equivalents of N_3-WALP were not successful.

To isolate the tetrameric assemblies of the N_3-WALP analogues, different techniques were explored. First, HPLC with buffers and a gradient especially developed for hydrophobic peptides was performed.[24] Unfortunately, the analytical HPLC chromatogram of the reaction mixture showed only one small peak at the retention time of the N_3-WALP (data not shown). Subsequently, the purification was tried with size exclusion chromatography. First, the use of Sephadex LH 20 was explored. Sephadex LH 20 is well-compatible with organic solvents, but has a separation range of only 50-5000 Da. Pure tetramer could not be obtained by a run in dichloromethane/methanol, probably because the fractionation range was too low. As an alternative, a Superdex 75 prep grade is less compatible with organic solvents, but separates globular proteins of 3-70 kDa. Tetramers could not be isolated by runs in the solvent systems dichloromethane/methanol and DMF, illustrating that also the Superdex 75 stationary phase was not suitable to purify the assemblies. Next, dialysis was tried. Two membrane types were used. One standard dialysis tube with a molecular weight cut-off of 12 kDa hardened in organic solvent and consequently was not usable. The commercially available Pierce slide-a-lyzer dialysis cassette with a cut-off of 10 kDa, dissolved in the organic solvent and also the membrane did not seem to swell properly. Thus, dialysis could not be used to isolate the tetramers.

Synthesis, characterization and purification of the second generation tetramers

To improve the analysis by mass spectrometry and the solubility of the tetramers, the N_3-WALP-KK analogue was designed (Figure 5). Lysine is frequently employed as a solubility tag.[25] The positively charged amines should facilitate ionization of the peptides and may increase solubility. Because of the tail, this peptide had a higher molecular weight than the N_3-WALP. The molecular weights of the putative mono-, di-, tri- and tetramer are shown in the figure.

The microwave-assisted click reaction of this peptide in the presence of copper(I) acetate was equally efficient as in case of the N_3-WALP

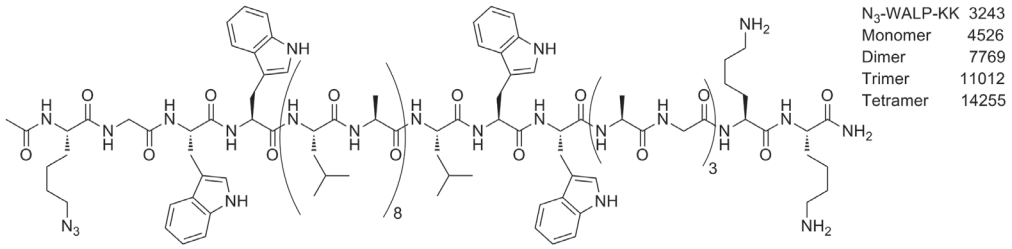


Figure 5. Structure of the N3-WALP-KK and molecular weights of putative second generation mono-, di-, tri- and tetramers

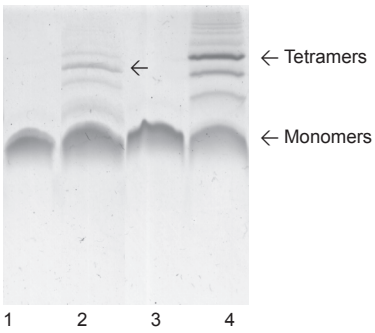


Figure 6. Tricine-SDS-PAGE of tetramerization reactions with N_3-WALP (monomer in lane 1, reaction in lane 2) and N_3-WALP-KK (monomer in lane 3, reaction in lane 4). Because the N_3-WALP-KK has a higher molecular weight than the N_3-WALP the tetramers run slower

analogues according to tris-tricine gel electrophoresis (Figure 6). Again, also other reaction condition were employed, such as addition of some DMSO or other modifications described previously to be successfull[26]. However, this did not result in an increase of the amount of the tetramers. Additionally, the use of purified instead of crude N_3-WALP-KK did not alter the tetramerization results. Strangely, GPC characterization was not straightforward with these assemblies. GPC traces of the monomeric N_3-WALP-KK, run in DMF/LiCl, showed three peaks and that of the tetramerization reaction more than four peaks (data not shown). This might be due to aggregation, which may be related to the slightly amphiphilic character that is caused by the solubility tag. GPC with tetrahydrofuran as eluent gave traces with only weak and broad signals. Fortunately, due to the solubility tag, with MALDI-TOF analysis the N_3-WALP-KK and the monomeric till tetrameric assemblies could be detected (Figure 7). It has to be noted that mass spectrometry is not quantitative. It is likely that the tetramers ionize to a lower extent than the monomers.

Several attempts were made to purify the tetrameric assembly of the N_3-WALP-KK analogues.

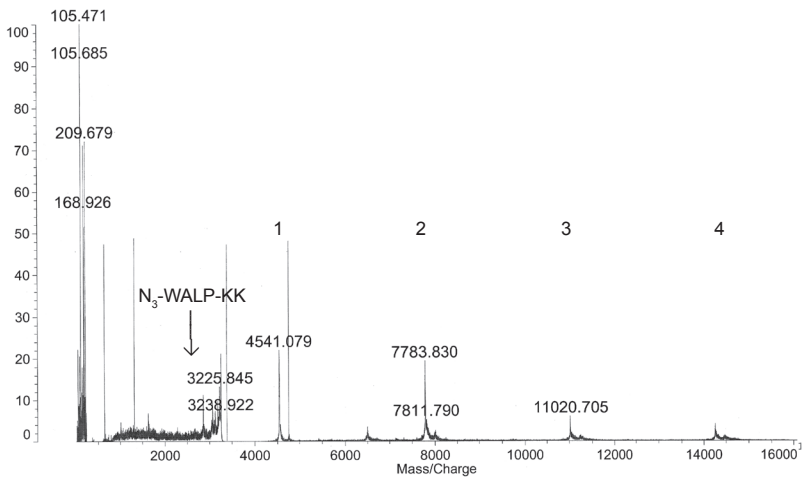


Figure 7. MALDI-TOF analysis of tetramerization reaction with N3-WALP-KK, showing the scaffold with 1, 2, 3, or 4 WALP analogues attached.

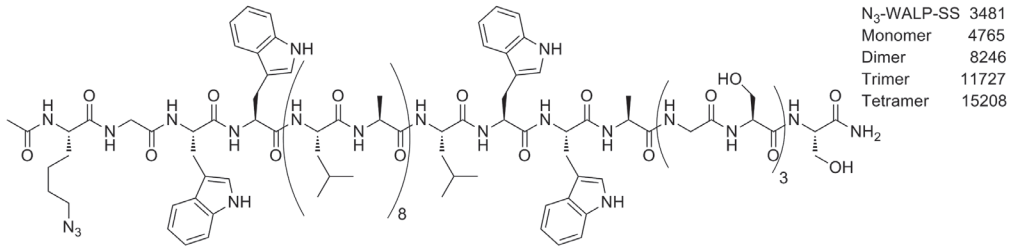


Figure 8. Structure of the N_3-WALP-SS and molecular weights of putative third generation mono-, di-, tri- and tetramers

The results with HPLC were similar to those with the first generation of tetramers. HPLC analysis with another buffer system did not improve the results. Also Superdex 75 prep grade runs did not show an improvement in purification potential. Since the tetrameric assemblies of N_3-WALP-KK were somewhat more water-soluble, Superdex with methanol/H_2O mixtures was applied, but without success. It was also attempted to recover the tetramer from the corresponding gel electrophoresis band. Usually retrieving proteins from a gel for proteomics approaches, is achieved by in-gel tryptic digestion of an excised spot and incubating alternatingly in buffer and acetonitrile. The tetramers are not soluble in aqueous buffers and not suitable to be cut by proteases, but they are smaller than globular proteins and soluble in acetonitrile. Therefore the gel bands of the tetramer and (as a reference) monomer, cut into small pieces, were incubated overnight in either methanol or acetonitrile. Unfortunately, with MALDI-TOF no monomers or tetramers could be detected. As a last attempt to purify this generation of tetramers, a 'fishing-experiment' was performed. A solution of the reaction mixture in DMF, together with TCEP, TBTA and $CuSO_4$, was incubated overnight with MagnaBind™ magnetic beads that were functionalized with azide groups. All monomeric, dimeric and trimeric assemblies have at least one alkyne left on the scaffold and thus should react with the azide groups on the magnetic beads. After overnight incubation the beads were pulled aside with a magnet and the solution was analyzed by tris-tricine gel electrophoresis. Unfortunately the gel-pattern looked the same as that of the original reaction mixture.

Synthesis, characterization and purification of the third generation tetramers

As a last attempt to improve the design of the WALP analogues, the N_3-WALP-SS peptides (Figure 8) were synthesized. The alcohols of the serine residues should have a similar effect, though not as effective, as the amines. However, where the amines could generate charge-repulsion effects or an amphiphilic character in the tetramer, the hydroxyl groups are less likely to have this effect.

Like with the other WALP analogues, coupling of the N_3-WALP-SS to the scaffold could be achieved by a click reaction with copper(I) acetate via microwave irradiation. This was shown by tris-tricine gel electrophoresis (Figure 9). Apparently, the serine residues were not as effective as the lysine residues to ionize the oligomeric assemblies, because no oligomers were detected with MALDI-TOF. Regrettably, also these tetramers could not be purified by HPLC. Furthermore, the assemblies did not have improved solubility properties. Therefore, (alternative) methods were not explored.

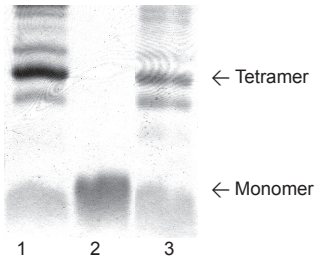


Figure 9. Tricine-SDS-PAGE of tetramerization reactions with N_3-WALP-KK (lane 1) and N_3-WALP-SS (monomer in lane 2, reaction in lane 3)

Concluding discussion

All compounds necessary to create a tetrameric assembly of the transmembrane WALP model peptide were synthesized successfully. Tetramers were obtained with all three WALP analogues. The first indication for formation of three generations of tetramers came from SDS-PAGE. Additionally, GPC traces indicated the presence of first generation tetramers. Moreover, second generation tetramers were detected by MALDI-TOF analysis. Detection of these tetramers was probably better achievable due to the lysine-tail, which facilitates ionization.

Several attempts were made to purify the tetramers. These covalent assemblies of WALP analogues are gradually growing towards the size of some natural membrane proteins. Thus, they may require purification methods that suit these sizes, instead of the conventional purification methods for regular peptides. However, for natural membrane proteins, usually purification methods are applied that require aqueous solutions. Unfortunately, the WALP peptides do not dissolve in any aqueous medium. Therefore purification methods are limited to methods that work with organic solvents. Both the methods used for more conventional peptides (HPLC, Sephadex LH 20) and some adjusted methods used for natural proteins (Superdex 75 prep grade, gel electrophoresis, dialysis, 'fishing') did not result in the isolation of the tetramers. Two solutions to this problem can be envisaged. First, there might still be some adjustments possible of the already explored purification methods. Second, the construct may be further optimized to obtain a 'workable' compound for purification.

Here, three prospects for purification by high performance liquid chromatography (HPLC) or size exclusion chromatography (SEC) will be discussed. First, HPLC has been used previously to purify transmembrane peptides and membrane proteins, with conditions similar to those applied in this chapter, but with other columns. Though the concerning compounds were less hydrophobic than the tetramers, the employed Vydac C4[27-32] (properties and retention times can vary significantly between different types of columns), cyanopropyl,[33] Lipidex-5000[34] or Nucleosil C2[35] columns may be suitable to isolate the tetramers. Second, it is possible that the purification by Sephadex LH 20 and Superdex 75 prep grade was not successful, because organic solvents may alter the properties of the medium.[36, 37] Tests will be needed to find a suitable medium-solvent combination, in which the altered properties in different solvents may even be used in our advantage.[37] Third, for the N_3-WALP tetramerization it seemed that with analytical GPC all assemblies were resolved. Therefore, though the resolution of a separation usually decreases when extending from analytical to preparative scale, the use of GPC for purification should be further explored. Possibly with another solvent, since the applied DMF/LiCl eluent would yield significant amounts of salts in the fractions on a preparative scale.

The most important problem in the purification of the tetrameric assemblies seems to be their hydrophobicity. Radical adaptations to either the model peptides or the scaffold will be necessary to make the construct somewhat more hydrophilic. Possible modification of the model peptides will be discussed in chapter 6. For the adaptation of the scaffold we will discuss several options here. Adjustment of the cyclic scaffold **8** by replacement of the alanine residue(s) would be the most desirable option, because of its well-defined three dimensional structure and ease of synthesis. An attractive possibility is the incorporation of a cysteine, as was done previously.[9] To the cysteine, a solubility tag, such as PEG[38, 39], could be attached reversibly via a disulfide bond. It has to be noted that the presence of free cysteine-residues is not compatible with the click reaction (risking precipitation), so an appropriate protection/synthesis strategy

would have to be designed. In addition, if we want to remove the tag after purification, there is another purification step necessary to remove the derivative. Alanine could also be replaced by lysine[8, 17, 20] and aspartic- or glutamic acid. The coupling of tags would not be reversible in these cases. If we do not remove the tag, it has to be made sure that the derivative does not influence the behaviour of the tetramers. Besides adaptation of the currently used scaffold, a completely different scaffold may be used. Though these require elaborate synthesis work, there are some scaffolds that, in comparison to scaffold **8**, have the same ability to orient the model peptides, though with an increased solubility.[40-42]

It is such an important objective to obtain pure tetramers, because of the possibilities for systematic studies with these assemblies. As stated in the introduction, with oligomers of the WALP peptides, the influence of the cross sectional diameter on membrane protein behaviour and protein-lipid interactions can be investigated. In chapter 3 and 4 it was already established that covalent dimers show small but systematic differences in properties and lipid effects compared to monomers. The covalent tetramers would be a next step towards multispan membrane proteins, enabling to extend the dimer results. Previously shown differences in diffusion[1], lipid translocation[2], bilayer thickness[43-45], localization properties[46-51] and lipid phase transition[52-57] between proteins with different sizes and WALP peptides can be further explored with the tetramers. In addition, the question if diameter is important in the fact that our model peptides show only small adaptations to mismatch, while natural membrane proteins can undergo huge structural changes, can also be addressed. It was for instance observed that a WALP27 peptide did not show the localization to matching microdomains (unpublished data), common for membrane proteins. With a tetramer of WALP27 peptides, it can be investigated if this lack of localization is due to the single spanning nature of the model peptide. Besides cross sectional diameter, other factors that might be important for properties of membrane proteins may be studied with tetrameric assemblies of WALP peptides. For example, the topology of the helices with respect to each other appears to be important.[5, 58-60] The topology of the helices can easily be varied, by varying the position of azide- and alkyne building blocks in the scaffold and WALP analogues, thus creating an opportunity to study the effect of topology on the behaviour and lipid interactions of transmembrane helices.

Experimental section

General

All chemicals were obtained from commercial sources, unless stated otherwise. ^{1}H-NMR spectra were recorded on a Varian G-300 (300 MHz). The chemical shifts are given in ppm relative to TMS. Determination of R_f values by thin layer chromatography (TLC) was performed on Merck precoated silicagel 60 F-254 plates. Spots were visualised by UV, ninhydrin and Cl_2/TDM.[61] For click reactions a Biotage Initiator microwave reactor was used. A Christ alpha RVC speedvac vacuum concentrator was used to remove DMF (at 40°C and 1 mbar). The gel electrophoresis setup was obtained from BioRad laboratories B.V. FTIR-spectra were measured on a Bio-Rad FTS 6000 spectrometer. Samples were mixed with KBr and a pellet was obtained using a Beckman press. GPC traces were measured on an Alliance C apparatus, with a Waters 2695 separation module. As detection method refractive index at 256 nm was used on a Waters 2414 detector. MALDI-TOF analysis was performed on a Kratos Axima CFR spectrometer. As the matrix α-cyano hydroxy cinnamic acid was used. ESI-MS spectra were measured on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer. Analytical HPLC was performed on a Shimadzu Class-VP HPLC, equipped with a Shimadzu SPD-10AVP UV-vis spectrometer. For some runs a Polymer Laboratories PL-ELS 1000 light scattering detector was used. Preparative HPLC was performed on an automated Gilson preparative HPLC system. Sephadex LH 20 and Superdex 75 prep grade were obtained from GE Healthcare. For dialysis experiments a standard dialysis tube with MWCO 12 kDa and a Pierce slide-a-lyzer 10K dialysis cassette with a MWCO of 10 kDa and a 0.5-3 ml sample volume were used.

Synthesis

*Synthesis Fmoc-Lys(N$^{\varepsilon}$-4-pentynoyl)-OH **6***

Boc-Lys(Z)-OH **1** (39.4 mmol, 15 g) was dissolved in tetrahydrofuran (100 ml). H_2O (25 ml) and Pd/C (210 mg), suspended in tert-butanol/H_2O (1:1 v/v), were added and the suspension was vigorously stirred under a H_2 atmosphere for 2 days. The Pd/C catalyst was filtered off over Hyflo and the solvents were removed in vacuo. Product **2** was obtained in quantitative yield and used with purification in the next synthesis step. Boc-Lys-OH **2** (15 mmol, 3.7 g) was dissolved in acetonitrile/H_2O (1:1 v/v, 80 ml) and to this solution small portions of active ester **5** (1.0 equiv, 15 mmol, 2.9 g) dissolved in acetonitrile (50 ml) and DiPEA (1.25 equiv, 18.75 mmol, 3.25 ml) were added in alternating order during approx 10 min. After stirring overnight, the pH of the reaction mixture was adjusted to approx 7 with 1N $KHSO_4$ and acetonitrile was evaporated in vacuo. The aqueous phase was acidified to pH 2 with 1N $KHSO_4$ under formation of a white solid, which was extracted with ethylacetate (3x). The combined ethylacetate extracts were washed with 1N $KHSO_4$ (1x) and brine (1x), dried on Na_2SO_4 and filtered. The ethylacetate was evaporated in vacuo. The product was obtained as a white foam and was used without further purification. Boc-Lys(N$^{\varepsilon}$-4-pentynoyl)-OH (10 mmol) was dissolved in dichloromethane (40 ml) and TFA (40 ml) was added. After 35 min of stirring the dichloromethane and TFA were evaporated in vacuo. After coevaporation with toluene (2x10 ml) and chloroform (2x10 ml) the residue was dried at high vacuum overnight and a yellow oil was obtained. This TFA salt of H-Lys(N$^{\varepsilon}$-4-pentynoyl)-OH was dissolved in acetonitrile/H_2O (1:1 v/v, 80 ml). The pH was brought to 8.5 with TEA. A solution of Fmoc-ONSu (10 mmol, 3.37 g) in acetonitrile (25 ml) was added drop wise, while the pH was kept between 8 and 8.5 with TEA. After 30 min at pH 8-8.5, the reaction mixture was neutralized to pH 7 with 1N $KHSO_4$. Acetonitrile was evaporated

in vacuo and the aqueous phase was acidified to pH 2 with 1N $KHSO_4$. The suspension was extracted with ethylacetate (3x70 ml). The combined extracts were washed with 1N $KHSO_4$ (2x50 ml) and brine (50 ml), dried on Na_2SO_4 and filtered. Ethylacetate was evaporated in vacuo. The product Fmoc-Lys(N^{ε}-4-pentynoyl)-OH **6** was purified over a silica flash P60 column using a gradient from ethylacetate/hexane (2:1 v/v) to ethylacetate/methanol (1:1 v/v), to give **6** in an overall yield of 75%. R_f: 0.66 (chloroform/methanol/acetic acid (95/20/3 v/v/v)), Rt: 25.2 min (RP-C8 column), ^{1}H-NMR (DMSO-d_6): δ = 7.30-7.91, (m, 8H, Fmoc-Ar-*H*), 7.62 (br s, 1H, N*H*), 7.06 (br s, 1H, N*H*), 4.19-4.30 (m, 3H, O-C*H*-CH_2), 3.79-3.81 (q, 1H, α-C*H*), 2.98-3.03 (q, 2H, ε-CH_2), 2.74-2.76 (t, 1H, ≡C*H*), 2.21-2.37 (dt, 4H, CH_2-CH_2), 1.20-1.72 (m, 6H, ß/γ/δ-CH_2)

*Synthesis ONSu ester of 4-pentynoic acid **5***

To a solution of 4-pentynoic acid **3** (50.97 mmol, 5 g) in acetonitrile (60 ml) N-hydroxysuccinimide **4** (1.1 equiv, 56.07 mmol, 6.45 g) was added. The solution was cooled on ice and dicyclohexylcarbodiimide (1.05 equiv, 53.52 mmol, 11 g) was added, resulting in a cream white suspension. After addition of 20 ml acetonitrile the suspension was stirred overnight. The suspension was filtered and the resulting clear solution was evaporated in vacuo. The crude solid was recrystallized from iso-propanol, giving salmon coloured crystals in near quantitative yield. ^{1}H-NMR ($CDCl_3$): δ = 2.86-2.91 (t, 2H, CH_2CO), 2.84 (s, 4H, N-(CO)-CH_2), 2.59-2.65 (m, 2H, ≡C-CH_2), 2.04-2.06 (t, 1H, ≡C*H*)

*Synthesis H-(Lys(N^{ε}-4-pentynoyl)-Ala-Lys (N^{ε}-4-pentynoyl)-Pro-Gly)$_2$-OH **7***

The first glycine was coupled manually to a 2-chlorotrityl chloride resin (1.1 mmol/g, 0.48 g). An Fmoc determination was performed to determine the loading. For this determination a small batch of known weight of the glycine-loaded resin was Fmoc-deprotected and the concentration of Fmoc was determined to be 0.55 mmol/g.[62] The synthesis was continued on an Applied Biosystems 433A peptide synthesizer on a 0.25 mmol scale using HBTU/HOBt and DiPEA as coupling reagents and piperidine for removal of the Fmoc-group. The Lys(N^{ε}-4-pentynoyl) units were incorporated via the Fmoc-Lys(N^{ε}-4-pentynoyl)-OH building block **6**. The resin was treated with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) for 75 minutes to obtain the linear peptide **7** by precipitation in MBTE/n-hexane (1:1 v/v, 4°C, 40 ml). The suspension was centrifuged and the pellet was dissolved in tert-butanol/H_2O and lyophilized. The linear peptide was used for cyclisation without further purification. Rt: 18.6 min, ESI-MS: calculated; 1300.72 ($C_{64}H_{96}N_{14}O_{15}$, exact mass), found; 1301.55 $[M+H]^+$, 1323.80 $[M+Na]^+$

*Synthesis cyclic scaffold **8***

For the cyclisation reaction, H-(Lys(N^{ε}-4-pentynoyl)-Ala-Lys(N^{ε}-4-pentynoyl)-Pro-Gly)$_2$-OH **7** (0.077 mmol, 100 mg) was dissolved in DMF (16 ml) and BOP (1 equiv, 0.077 mmol, 34 mg), HOBt (1.1 equiv, 0.085 mmol, 13 mg) and DiPEA to obtain a pH of 8 were added. The reaction mixture was stirred for 7 days, after which the DMF and DiPEA were evaporated in vacuo and the remaining film was dissolved in tert-butanol/H_2O and lyophilized. The cyclic peptide **8** was obtained in 20% yield after purification by HPLC. Rt: 18.9 min, ESI-MS: calculated; 1282.71 ($C_{64}H_{94}N_{14}O_{14}$, exact mass), found; 1283.90 $[M+H]^+$, 1306.35 $[M+Na]^+$

*Synthesis Fmoc-Lys(N_3)-OH **11***

Fmoc-Lys(Boc)-OH **9** (15 mmol, 4.1 g) was dissolved in dichloromethane (40 ml). TFA (40 ml) was added and the solution was stirred for 50 min. The dichloromethane and TFA were evaporated in vacuo and the residue was coevaporated with toluene (3x10 ml) and chloroform (2x10 ml), obtaining Fmoc-Lys-OH in quantitative yield. Diazotransfer was performed on the free amine according to the method of

Roice et al.[23] Fmoc-Lys-OH (15 mmol, 5.5 g) was dissolved in 80% acetic acid and $CuSO_4 \cdot 5H_2O$ (75 mg) in H_2O (3 ml) was added. The pH was adjusted to 7 with K_2CO_3, and methanol (160 ml) and H_2O (75 ml) were added. After addition of the triflic azide (2 equiv, 30 mmol) in dichloromethane (125 ml) the pH was adjusted to 8.5 with K_2CO_3. The solution was vigorously stirred overnight. The dichloromethane phase was separated from the aqueous layer and washed twice with H_2O. The pH of the aqueous phase was adjusted to 2 with 3M HCl and extracted twice with dichloromethane. The dichloromethane fractions were combined, the solvent was removed in vacuo and the residue was coevaporated with toluene (4x20 ml) and chloroform (3x20 ml). The Fmoc-Lys(N3)-OH **11** was obtained in near quantitative yield and of appropriate purity to be used in solid phase peptide synthesis. R_f: 0.38 (dichloromethane/methanol, 9:1 v/v), Rt: 22.3 min, ^{1}H-NMR ($CDCl_3$): δ = 7.25-7.77 (m, 8H, Fmoc-Ar-*H*), 5.27-5.30 (d, 1H, N*H*), 4.42-4.44 (m, 3H, O-CH_2, α-C*H*), 4.20-4.24 (t, 1H, O-CH_2-C*H*), 3.26-3.30 (t, 2H, ε-CH_2), 1.70-1.95 (dm, 2H, ß-CH_2), 1.60-1.62 (m, 2H, γ-CH_2), 1.47-1.48 (m, 2H, δ-CH_2)

*Synthesis triflic azide **10*** [63]

Sodium azide (185 mmol, 12g) was dissolved in H_2O at 0°C, dichloromethane was added (20 ml) and under vigorous stirring trifluoromethane sulfonic anhydride (35 mmol, 10 g) was added drop wise during two hours. After one hour of stirring, the H_2O layer was separated from the dichloromethane layer. The aqueous phase was extracted twice with dichloromethane. All dichloromethane fractions (125 ml) were combined, washed with 5% Na_2CO_3 and used immediately in the next step.

Synthesis N_3-WALP, N_3-WALP-KK and N_3-WALP-SS (Table 1)

All three WALP analogues were synthesized automatically on an Applied Biosystems 433A peptide synthesizer on a 0.25 mmol scale using a Tentagel S-RAM resin to obtain the C-terminally amidated peptides. Fmoc/group removal was monitored by the UV signal of the dibenzofulvene-piperidine adduct at 305 nm. A special program was used with conditional extra deprotection and prolonged coupling cycles and standard capping after each amino acid coupling. Coupling reactions were performed with HBTU/HOBt and DiPEA in NMP and the Fmoc-group was removed with piperidine in NMP. On the 17th position of the WALP unit an Fmoc-Ala(d3)-OH was coupled instead of a regular Fmoc-Ala-OH. At the N-termini of the peptides, the Fmoc-Lys(N3)-OH building block **11** was incorporated. After the final Fmoc removal, the N-terminus was capped with acetic anhydride. Cleavage from the resin and deprotection of the side-chains was performed by treatment with TFA/TIS/H_2O (95/2.5/2.5 v/v/v) for two hours. The crude peptides were precipitated in MTBE/n-hexane (1:1 v/v, 4°C, 40 ml) and the suspension was centrifuged. The pellet was dissolved in tert-butanol/H_2O and lyophilized to obtain fluffy white powders. The yields of crude N_3-WALP, N_3-WALP-KK and N_3-WALP-SS were 70, 56 and 68% respectively with a peptide purity of 82, 72 and 79% respectively. N_3-WALP: Rt: 50.5 min (60 min gradient), ESI-MS: calculated; 2674.57 ($C_{135}H_{199}D_3N_{32}O_{25}$, exact mass), found; 1338.29 $[M+2H]^{2+}$, 1361.27 $[M+2Na]^{2+}$. N_3-WALP-KK: Rt: 39.8 min (40 min gradient), ESI-MS: calculated; 3240.88 ($C_{159}H_{245}N_{41}O_{32}$, exact mass), found; 1622.07 $[M+2H]^{2+}$, 1643.80 $[M+2Na]^{2+}$. N_3-WALP SS: Rt: 44.8 min (40 min gradient), MALDI-TOF: calculated; 3481.85 ($C_{163}H_{244}D_3N_{43}O_{42}$, exact mass), found; broad peaks around 3485 and 3503.

General procedure for the click-reactions

The cyclic scaffold was dissolved in DMF/H_2O (95/5 v/v) at a concentration of 2 mgml^{-1}. Either 6 equivalents of a non-purified azide containing WALP analogue (1.5 equivalent of azide per alkyne) or 4.8 equivalents of a purified azide containing WALP analogue (1.2 equivalent of azide per alkyne) and 0.5 equivalents of

copper(I)acetate were added and the reaction mixture was heated in the microwave reactor (15 min at 100°C and 10 minutes at 120°C). After the reaction time, the solvents were evaporated in a speedvac concentrator. A concentrated solution in TFA (max. 1 ml) was prepared and the solution was dropped into a vortex of H_2O (45 ml, 4°C). The suspension was centrifuged and the pellet was dissolved in tert-butanol/H_2O and lyophilized.

Characterization and purification

HPLC N_3-WALP and tetramerization of N_3-WALP and N_3-WALP-KK

Analytical HPLC was performed using TEAP buffers and an Adsorbosphere XL butyl column as described previously.[24]

HPLC (tetramerization of) N3-WALP-KK, N3-WALP-SS

Analytical HPLC was performed with an Adsorbosphere XL butyl column (pore size 300Å, particle size 5 µm, 250x4.6mm). The peptide was eluted with a gradient of 10 to 100% buffer B in 40 or 60 min, in which buffer A was H_2O/acetonitrile (80/20 v/v) + 0.1% TFA and buffer B was acetonitrile/iso-propanol/H_2O (50/45/5 v/v/v) + 0.1% TFA. A flow rate of 0.75 $mlmin^{-1}$ was used and UV-detection at 220 and 280 nm. Preparative HPLC was performed in a similar way, though in this case a Prosphere C4 column (pore size 300Å, particle size 10 µm, 250x22mm) and an 80 min gradient was used.

*HPLC scaffold **8***

Analytical and preparative HPLC were performed over an Alltech Adsorbosphere XL C8 90Å column (particle size 5 µm, 250x4.6mm and particle size 10 µm, 250x22mm respectively) eluting with a gradient of H_2O/acetonitrile (95/5 v/v) + 0.1% TFA to H_2O/acetonitrile (5/95 v/v) + 0.1% TFA. For the analytical runs a gradient of 20 min was applied and detection was performed by measuring the UV-signal at 220 and 254 nm and ELSD. For the preparative runs a gradient of 55 min was used and the UV-signal at 220 nm. Pure fractions were pooled and lyophilized.

Gel electrophoresis

The samples were dissolved in a loading buffer of 0.1M TRIS-Cl, pH 6.8, 24% glycerol (v/v), 8% SDS (w/v) and bromophenol blue at a concentration of 2 $mg \cdot ml^{-1}$. Tricine-SDS-PAGE to separate molecules with molecular weights of 1-100 kDa was used.[64] As separating gel the composition described in the article as 16.5%T, 6%C was applied. An exception on the protocol was that for the stacking gel a 3M TRIS solution of pH 6.8 was used. The gel was run on a voltage of 30V for 30 min, followed by a voltage of 100V for 3 hours. Gels were fixated in destaining solution (H_2O/methanol/acetic acid, 315/150/35 v/v/v) for 10-30 min, stained with Coomassie Brilliant Blue G-250 for minimally 1 hour and destained overnight.

GPC

Samples were dissolved in DMF/LiCl, shaken overnight and filtered over Alltech pre-cut membranes of 0.45 µm. Samples were run on a Mesopore column of Polymer Laboratories, using filtered DMF/LiCl, DMF or tetrahydrofuran as mobile phase and a runtime of 45 or 60 min.

Sephadex LH20

An attempt to purify the tetramers of the N_3-WALP was performed with Sephadex LH20 in dichloromethane/methanol (1:1 v/v). A LKB Biochrom Ultrospec 4050 was attached to follow the elution of peptide at a wavelength of 280 nm.

Superdex 75 prep grade

Purification of the tetramers was attempted using Superdex 75 prep grade. Columns of

approximately 2.3 cm in diameter and a height of approximately 60 cm were poured in a glass column on a glass wool cloth at the bottom. As mobile phase dichloromethane/methanol (1:1 v/v), methanol/dichloromethane (8:2 v/v) and DMF were tried for the tetramers with N_3-WALP and H_2O/methanol (8:2 v/v) and methanol/H_2O (3:1 v/v) for those with N_3-WALP-KK. A UV-detector was attached to the column, using the absorbance of either the tryptophan-residues or of the backbone of the peptides, to monitor elution of the peptides.

References

1. Ramadurai, S., Holt, A., Krasnikov, V., van den Bogaart, G., Killian, J. A., and Poolman, B., Lateral diffusion of membrane proteins, *J Am Chem Soc*, **2009**, 131(35), p. 12650-6
2. Kol, M. A., van Dalen, A., de Kroon, A. I., and de Kruijff, B., Translocation of phospholipids is facilitated by a subset of membrane-spanning proteins of the bacterial cytoplasmic membrane, *J Biol Chem*, **2003**, 278(27), p. 24586-93
3. Killian, J. A. and von Heijne, G., How proteins adapt to a membrane-water interface, *Trends Biochem Sci*, **2000**, 25(9), p. 429-34
4. Killian, J. A., Synthetic peptides as models for intrinsic membrane proteins, *FEBS Lett*, **2003**, 555(1), p. 134-8
5. Nyholm, T. K., Ozdirekcan, S., and Killian, J. A., How protein transmembrane segments sense the lipid environment, *Biochemistry*, **2007**, 46(6), p. 1457-65
6. Holt, A. and Killian, J. A., Orientation and dynamics of transmembrane peptides: the power of simple models, *Eur Biophys J*, **2009**, 39(4), p. 609-621
7. Mutter, M. and Tuchscherer, G., Non-native architectures in protein design and mimicry, *Cell Mol Life Sci*, **1997**, 53(11-12), p. 851-63
8. Dumy, P., Eggleston, I. M., Cervigni, S., Sila, U., Sun, X., and Mutter, M., A convenient synthesis of cyclic peptides as regioselectively addressable functionalized templates (RAFT), *Tetrahedron Letters*, **1995**, 36(8), p. 1255-1258
9. Haehnel, W., Chemical synthesis of TASP arrays and their application in protein design, *Mol Divers*, **2004**, 8(3), p. 219-29
10. Tuchscherer, G., Scheibler, L., Dumy, P., and Mutter, M., Protein design: on the threshold of functional properties, *Biopolymers*, **1998**, 47(1), p. 63-73
11. Kochendoerfer, G. G., Tack, J. M., and Cressman, S., Total chemical synthesis of a 27 kDa TASP protein derived from the MscL ion channel of M. tuberculosis by ketoxime-forming ligation, *Bioconjug Chem*, **2002**, 13(3), p. 474-80
12. Becker, C. F., Oblatt-Montal, M., Kochendoerfer, G. G., and Montal, M., Chemical synthesis and single channel properties of tetrameric and pentameric TASPs (template-assembled synthetic proteins) derived from the transmembrane domain of HIV virus protein u (Vpu), *J Biol Chem*, **2004**, 279(17), p. 17483-9
13. Bayley, H., Designed membrane channels and pores, *Curr Opin Biotechnol*, **1999**, 10(1), p. 94-103
14. Grove, A., Mutter, M., Rivier, J. E., and Montal, M., Template-assembled synthetic proteins designed to adopt a globular, four-helix bundle conformation form ionic channels in lipid bilayers, *J Am Chem Soc*, **1993**, 115, p. 5919-5924
15. Pawlak, M., Meseth, U., Dhanapal, B., Mutter, M., and Vogel, H., Template-assembled melittin: structural and functional characterization of a designed, synthetic channel-forming protein, *Protein Sci*, **1994**, 3(10), p. 1788-805
16. Futaki, S., Aoki, M., Fukuda, M., Kondo, F., Niwa, M., Kitagawa, K., and Nakaya, Y., Assembling of the four individual helixes corresponding to the transmembrane segments (S4 in repeat I-IV) of the sodium channel, *Tetrahedron Letters*, **1997**, 38, p. 7071-7075
17. Dumy, P., Eggleston, I. M., Esposito, G., Nicula, S., and Mutter, M., Solution structure of regioselectively addressable functionalized templates: an NMR and restrained molecular dynamics investigation, *Biopolymers*, **1996**, 39(3), p. 297-308
18. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B., A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes, *Angew Chem Int Ed Engl*, **2002**, 41(14), p. 2596-9
19. Tornoe, C. W., Christensen, C., and Meldal, M., Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides, *J Org Chem*, **2002**, 67(9), p. 3057-64
20. Wang, J., Li, H., Zou, G., and Wang, L. X., Novel template-assembled oligosaccharide clusters as epitope mimics for HIV-neutralizing antibody 2G12. Design, synthesis, and antibody binding study, *Org Biomol Chem*, **2007**, 5(10), p. 1529-40
21. Avrutina, O., Empting, M., Fabritz, S., Daneschdar, M., Frauendorf, H., Diederichsen, U., and Kolmar, H., Application of copper(I) catalyzed azide-alkyne [3+2] cycloaddition to the synthesis of template-assembled multivalent peptide conjugates, *Org Biomol Chem*, **2009**, 7(20), p. 4177-85
22. Jones, J., *Amino Acid and Peptide Synthesis*. 2nd ed. 2002, New York: Oxford University Press.
23. Roice, M., Johannsen, I., and Meldal, M., High Capacity Poly(ethylene glycol) Based Amino Polymers for Peptide and Organic Synthesis, *QSAR Comb. Sci.*, **2004**, 23, p. 662-673
24. Rijkers, D. T. S., Kruijtzer, J. A., Killian, J. A., and Liskamp, R. M. J., A convenient solid phase synthesis of S-palmitoyl transmembrane peptides, *Tetrahedron Letters*, **2005**, 46, p. 3341-3345
25. Melnyk, R. A., Partridge, A. W., Yip, J., Wu, Y., Goto, N. K., and Deber, C. M., Polar residue tagging of transmembrane peptides, *Biopolymers*, **2003**, 71(6), p. 675-85
26. Arnusch, C. J., Branderhorst, H., de Kruijff, B., Liskamp, R. M., Breukink, E., and Pieters, R. J., Enhanced membrane pore formation by multimeric/oligomeric antimicrobial peptides, *Biochemistry*, **2007**, 46(46), p. 13437-42
27. Graddis, T. J., Myszka, D. G., and Chaiken, I. M., Controlled formation of model homo- and heterodimer coiled coil polypeptides, *Biochemistry*, **1993**, 32(47), p. 12664-71
28. Kassim, S. Y., Restrepo, I. M., and Kalivretenos, A. G., Synthesis and purification of hydrophobic peptides for use in biomimetic ion channels, *J Chromatogr A*, **1998**, 816(1), p. 11-20
29. Khemtemourian, L., Lavielle, S., Bathany, K., Schmitter, J. M., and Dufourc, E. J., Revisited and large-scale synthesis and purification of the mutated and wild type neu/erbB-2 membrane-spanning segment, *J Pept Sci*, **2006**, 12(5), p. 361-8
30. Sussman, M. R., Purification of integral plasma membrane proteins by reverse-phase high performance liquid chromatography, *Anal Biochem*, **1988**, 169(2), p. 395-9
31. Tiburu, E. K., Dave, P. C., Vanlerberghe, J. F., Cardon, T. B., Minto, R. E., and Lorigan, G. A., An improved synthetic and purification procedure for the hydrophobic segment of the transmembrane peptide phospholamban, *Anal Biochem*, **2003**, 318(1), p. 146-51
32. Lee, R. P., Doughty, S. W., Ashman, K., and Walker, J., Purification of hydrophobic integral membrane proteins from Mycoplasma hyopneumoniae by reversed-phase high-performance liquid chromatography, *J Chromatogr A*, **1996**, 737(2), p. 273-9
33. Fisher, L. E. and Engelman, D. M., High-yield synthesis and purification of an alpha-helical transmembrane domain, *Anal Biochem*, **2001**, 293(1), p. 102-8
34. Stark, M., Jornvall, H., and Johansson, J., Isolation and characterization of hydrophobic polypeptides in human bile, *Eur J Biochem*, **1999**, 266(1), p. 209-14
35. Thiaudiere, E., Soekarjo, M., Kuchinka, E., Kuhn, A., and Vogel, H., Structural characterization of membrane insertion of M13 procoat, M13 coat, and Pf3 coat proteins, *Biochemistry*, **1993**, 32(45), p. 12186-96
36. Garcia, R., Recalde, I. B., Figueruelo, J. E., and Campus, A., Quantitative evaluation of the swelling and crosslinking degrees in two organic gel packings for SEC, *Macromol Chem Phys*, **2001**, 202(17), p. 3352-3362
37. Henke, H., *Preparative Gel Chromatography on Sephadex LH-20*. 1995, Heidelberg: Huthig. 617.
38. Fee, C. J. and Alstine, J. M. V., PEG-proteins: Reaction engineering and separation issues, *Chemical Engineering Science*, **2006**, 61, p. 924-939
39. Roberts, M. J., Bentley, M. D., and Harris, J. M., Chemistry for peptide and protein PEGylation, *Adv Drug Deliv Rev*, **2002**, 54(4), p. 459-76
40. van den Heuvel, M., van den Berg, T. A., Kellogg, R. M.,

Choma, C. T., and Feringa, B. L., Synthesis of a non-heme template for attaching four peptides: an approach to artificial iron(II)-containing peroxidases, *J Org Chem*, **2004**, 69(2), p. 250-62
41. Akerfeldt, K. S., Kim, R. M., Camac, D., Groves, J. T., Lear, J. D., and DeGrado, W. F., Tetraphilin: A four-helix proton channel built on a tetraphenylporphyrin framework, *J Am Chem Soc*, **1992**, 114, p. 9656
42. Jensen, K. J. and Barany, G., Carbopeptides: carbohydrates as potential templates for de novo design of protein models, *J. Peptide Res.*, **2000**, 56(1), p. 3-11
43. Harroun, T. A., Heller, W. T., Weiss, T. M., Yang, L., and Huang, H. W., Experimental evidence for hydrophobic matching and membrane-mediated interactions in lipid bilayers containing gramicidin, *Biophys J*, **1999**, 76(2), p. 937-45
44. Weiss, T. M., van der Wel, P. C., Killian, J. A., Koeppe, R. E., 2nd, and Huang, H. W., Hydrophobic mismatch between helices and lipid bilayers, *Biophys J*, **2003**, 84(1), p. 379-85
45. de Planque, M. R., Greathouse, D. V., Koeppe, R. E., 2nd, Schafer, H., Marsh, D., and Killian, J. A., Influence of lipid/peptide hydrophobic mismatch on the thickness of diacylphosphatidylcholine bilayers. A 2H NMR and ESR study using designed transmembrane alpha-helical peptides and gramicidin A, *Biochemistry*, **1998**, 37(26), p. 9333-45
46. Fernandes, F., Loura, L. M., Prieto, M., Koehorst, R., Spruijt, R. B., and Hemminga, M. A., Dependence of M13 major coat protein oligomerization and lateral segregation on bilayer composition, *Biophys J*, **2003**, 85(4), p. 2430-41
47. Martens, J. R., O'Connell, K., and Tamkun, M., Targeting of ion channels to membrane microdomains: localization of KV channels to lipid rafts, *Trends Pharmacol Sci*, **2004**, 25(1), p. 16-21
48. Briggs, J. A., Wilk, T., and Fuller, S. D., Do lipid rafts mediate virus assembly and pseudotyping?, *J Gen Virol*, **2003**, 84(Pt 4), p. 757-68
49. Dumas, F., Sperotto, M. M., Lebrun, M. C., Tocanne, J. F., and Mouritsen, O. G., Molecular sorting of lipids by bacteriorhodopsin in dilauroylphosphatidylcholine/distearoylphosphatidylcholine lipid bilayers, *Biophys J*, **1997**, 73(4), p. 1940-53
50. van Duyl, B. Y., Rijkers, D. T. S., de Kruijff, B., and Killian, J. A., Influence of hydrophobic mismatch and palmitoylation on the association of transmembrane alpha-helical peptides with detergent-resistant membranes, *FEBS Lett*, **2002**, 523(1-3), p. 79-84
51. Ridder, A. N., Spelbrink, R. E., Demmers, J. A., Rijkers, D. T. S., Liskamp, R. M. J., Brunner, J., Heck, A. J., de Kruijff, B., and Killian, J. A., Photo-crosslinking analysis of preferential interactions between a transmembrane peptide and matching lipids, *Biochemistry*, **2004**, 43(15), p. 4482-9
52. Killian, J. A., Hydrophobic mismatch between proteins and lipids in membranes, *Biochim Biophys Acta*, **1998**, 1376(3), p. 401-15
53. Sperotto, M. M., Ipsen, J. H., and Mouritsen, O. G., Theory of protein-induced lateral phase separation in lipid membranes, *Cell Biophys*, **1989**, 14(1), p. 79-95
54. Morein, S., Killian, J. A., and Sperotto, M. M., Characterization of the thermotropic behavior and lateral organization of lipid-peptide mixtures by a combined experimental and theoretical approach: effects of hydrophobic mismatch and role of flanking residues, *Biophys J*, **2002**, 82(3), p. 1405-17
55. George, R., Lewis, R. N., and McElhaney, R. N., Studies on the purified Na+,Mg(2+)-ATPase from Acholeplasma laidlawii B membranes: a differential scanning calorimetric study of the protein-phospholipid interactions, *Biochem Cell Biol*, **1990**, 68(1), p. 161-8
56. Riegler, J. and Mohwald, H., Elastic Interactions of Photosynthetic Reaction Center Proteins Affecting Phase Transitions and Protein Distributions, *Biophys J*, **1986**, 49(6), p. 1111-1118
57. Piknova, B., Perochon, E., and Tocanne, J. F., Hydrophobic mismatch and long-range protein/lipid interactions in bacteriorhodopsin/phosphatidylcholine vesicles, *Eur J Biochem*, **1993**, 218(2), p. 385-96
58. Rath, A., Melnyk, R. A., and Deber, C. M., Evidence for assembly of small multidrug resistance proteins by a "two-faced" transmembrane helix, *J Biol Chem*, **2006**, 281(22), p. 15546-53
59. Schuldiner, S., When biochemistry meets structural biology: the cautionary tale of EmrE, *Trends Biochem Sci*, **2007**, 32(6), p. 252-8
60. Aller, P., Garnier, N., and Genest, M., Transmembrane Helix Packing of ErbB/Neu Receptor in Membrane Environment: A Molecular Dynamics Study, *J Biomol Struct Dyn*, **2006**, 24(3), p. 209-28
61. von Arx, E., Faupel, M., and Brugger, M., Das 4,4'-tetramethyldiamino-diphenylmethan reagens (TDM): Eine modifikation der chlor-o-tolidin farbereaktion fur die dunnschicht-chromatographie, *J Chromatogr A*, **1976**, 120(1), p. 224-228
62. Chan, W. C. and White, P. D., *Fmoc Solid Phase Peptide Synthesis: a practical approach*. 2000, New York: Oxford University Press.
63. Lundquist, J. T. and Pelletier, J. C., Improved solid-phase peptide synthesis method utilizing alpha-azide-protected amino acids, *Org Lett*, **2001**, 3(5), p. 781-793
64. Schagger, H. and von Jagow, G., Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal Biochem*, **1987**, 166(2), p. 368-79

6

Summarizing discussion

Aim and approach of the research

The research described in this thesis was performed to obtain a better understanding of the basic principles of protein-protein and protein-lipid interactions in membranes. Since membrane proteins are complex, diverse and difficult to handle, model systems of transmembrane peptides in lipid vesicles were employed. One advantage of this approach is that properties of peptides and lipids can be systematically varied, which allows for methodical studies of factors that influence protein-lipid interactions. In addition, the relatively easy incorporation of labels, together with the simplification of the system, makes biophysical studies well achievable. The so-called WALP peptides (Ac-GWW$(LA)_n$LWWA-NH_2) mimic the features of protein transmembrane helices. Important insights were obtained by biophysical studies of these single spanning α-helices on the general principles of membrane protein behaviour and lipid interactions.[1-3] However, most membrane proteins are multispan. To better mimic this situation, three types of covalent assemblies of the WALP peptides were designed. With these peptides a first step towards a systematic study was performed on the influence of cross sectional diameter and helix-helix interactions on protein-lipid interactions. The results will be reviewed in this chapter. First, the synthesis and purification of the different types of oligomers is discussed. The pitfalls in obtaining the covalent assemblies will be considered and some possible solutions will be discussed. Second, the biophysical studies that were performed with two types of covalent dimers are considered. The comparison of the behaviour and lipid interactions of monomers and dimers and the implications of the results for our knowledge on multispan membrane proteins will be discussed. Finally, some future prospectives on possible studies with oligomeric model peptides are provided.

Oligomerization of WALP model peptides

To be able to compare protein-lipid interactions of monomeric and oligomeric WALP peptides, several types of covalent assemblies of WALP model peptides were designed. In the following paragraphs, the results of the synthesis, characterization and purification of the oligomers will be discussed. Then, some potential solutions to overcome the difficulties in purification of the covalent assemblies will be considered.

First, three covalent dimers were synthesized that were coupled via a cysteine in the middle of the helix (Chapter 2). The sequences of the monomeric WALP starting compounds and a schematic representation of a dimer are depicted in Figure 1A. The cysteine positions were chosen to obtain varying helix-helix interfaces in the dimers and to allow the helices to adopt the most favourable orientation with respect to each other (parallel or antiparallel). It was shown that dimers could be obtained in a solution of the WALP analogues in an organic solvent, under basic conditions, with oxygen from the air as catalyst. Dimerization was characterized by gel electrophoresis and quantified by HPLC. Purification of the assemblies was achieved by HPLC, using modified conditions to facilitate elution of the hydrophobic peptides. Starting from monomer, the yield of pure dimer was typically around 20%.

To further improve the mimicking of multispan membrane proteins, dimers connected via a loop at the termini were constructed (Chapter 4). The sequences of the monomeric WALP analogues were designed to allow synthesis of specifically parallel and antiparallel dimers (Figure 1B) through the selective reaction between the incorporated azide and alkyne functionalities. Dimers were obtained in solution via the so-called click-reaction[4, 5], by using microwave irradiation and a Cu(I) catalyst. Formation of dimers was visualised by gel electrophoresis. HPLC purification of these constructs was not successful. The dimers did not elute from the C4 column, which is most likely caused by their aggregation on the column due to the aqueous conditions. Since the (anti)parallel dimers are attached at the termini, they may form a long hydrophobic helix that is more prone to aggregation than the above mentioned more compact dimers coupled in the middle of the helix. However, it was pos-

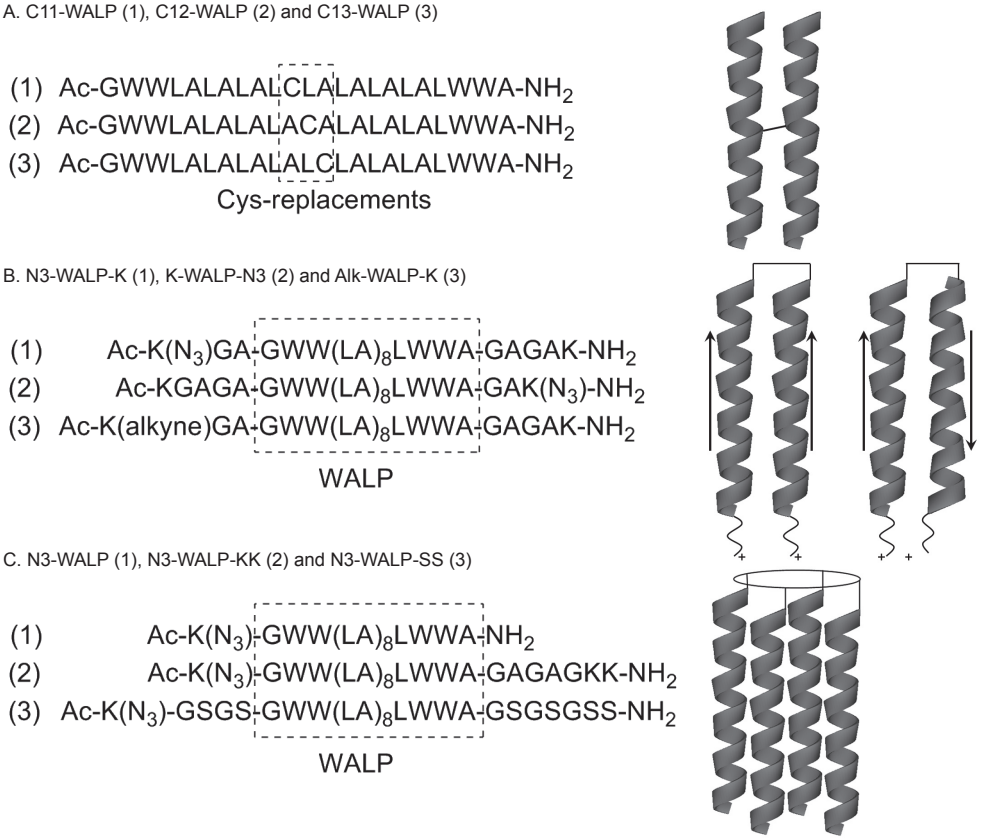


Figure 1. Monomeric WALP building blocks (names and sequences on the left) for the three types of oligomeric assemblies (schematically depicted on the right)

sible to purify the dimers over the size exclusion medium Sephadex LH 20. Though yields were low (~3%), sufficient amounts could be obtained to perform biophysical studies.

Finally, to increase the size of the oligomeric assemblies, tetramers were constructed by coupling four WALP analogues to a scaffold (Chapter 5). For the scaffold, the template-assembled synthetic protein (TASP)-approach was followed[6-8] and alkyne-functionalized lysine residues were incorporated. For the monomeric model peptides, three generations of azide-containing WALP analogues (Figure 1C) were synthesized. The tails were added with the aim to improve ionization and purification potential. The highly selective click-reaction between the alkyne and azide functionalities was performed in dimethylformamide with copper(I)acetate under microwave irradiation. Gel electrophoresis, MALDI-TOF and GPC results indicated that it was possible to synthesize tetramers (schematically depicted in Figure 1C) with all three monomeric WALP analogues. However, none of the tetrameric assemblies could be isolated.

As illustrated above, there appears to be ample possibility to synthesize oligomers of WALP peptides. However, it is difficult to characterize these covalent assemblies of WALP peptides and purification is not straightforward. This is mainly due to the lack of charge in these transmembrane helices and their hydrophobicity. To be able to proceed with the study of oligomeric WALP peptides the problems with purification have to be solved. For this purpose, several adaptations of the mod-

el peptides are possible. It was shown in chapters 4 and 5 that a lysine tag facilitates MALDI-TOF analysis of dimers and tetramers, which was a major advance. However, exact molecular weights could still not be determined and the feasibility of purification of the assemblies was not improved. Therefore, other or more radical adjustments will be necessary. A newly designed model peptide should provide additional possibilities for purification or have properties that facilitate purification by established methods, such as an increased solubility in aqueous environments. For example, the possibility to perform size exclusion chromatography in buffer solutions or to add His-tags or other tags for affinity purification would increase the potential for purification. Several options to modify the model peptides will be discussed in the following two paragraphs.

It would be most desirable if the 'core sequence' of the model peptides, the WALP (GWW(LA)nLWWA), is preserved in the design of covalent assemblies. In this way, the results with the oligomers can be related to the extensive amount of previous research that has been performed with the monomeric WALP peptides. A first step may be to use shorter WALP peptides in the oligomers, which would be less hydrophobic. In addition, or alternatively, there are some groups which can be applied in the native WALP sequence that are known to facilitate the synthesis and purification of difficult peptides. For example, the so-called NpSSPeoc group ([2-[(2-nitrophenyl)dithio]-1-phenylethoxy]carbonyl) to purify peptides with affinity chromatography in organic solvents may be used.[9] This group is electrophilic and may be converted to the nucleophilic HSPeoc ((2-mercapto-1-phenyletoxy)carbonyl). It is incorporated in the peptide via a modified amino acid building block. The NpSSPeoc or HSPeoc functionalities can be immobilized on several solid supports that are compatible with organic solvents. Upon cleavage of the peptide from the support, the pure peptide is recovered, without the NpSSPeoc or HSPeoc groups. Another option to facilitate purification is the reversible addition of a PEG-tail or other solubilising groups.[10-12] An especially attractive, recently developed, solubilising strategy would be the incorporation of Fmoc-Trp(Boc-Nmbu)-OH amino acids on the positions of the tryptophan flanking residues.[13] Upon cleavage and simultaneous deprotection of the peptide from the resin, the so-called Nmbu group (N-methyl butyric acid) remains on the indole nitrogen of the tryptophan, providing it with a positive charge. This will greatly enhance its solubility and the feasibility of HPLC purification, as anticipated from the fact that the lysine flanked KALP peptides are much easier to purify than a WALP. After purification, the Nmbu can be cleaved, yielding the required model peptide and NMP that can easily be removed. Synthesis of new peptides would be necessary to test if the purification of the covalent WALP assemblies will benefit from the use of the mentioned groups.

In addition to the use of solubilising groups to be able to purify oligomeric WALP peptides, it may be inevitable to adjust the sequence of the WALP analogues themselves. The WALP was designed to mimic the features of natural membrane proteins. The tryptophan residues were chosen because aromatic residues are frequently found at the interface in natural membrane proteins. Much research on the model peptides has focused on the role of the tryptophan residues and since they proved to have such an important role in anchoring the helices in the membrane, it is not desirable to replace them. The alternating Ala-Leu sequence was chosen to create a hydrophobic α-helix with an irregular surface and intermediate hydrophobicity. Moreover, the repeating unit in this sequence allows for the systematic variation of the hydrophobic length of the peptide. To decrease the hydrophobicity of the peptide while maintaining its properties, a first step could be to increase the alanine:leucine ratio, e.g. by making the repeat-sequence (Leu-Ala-Ala) instead of (Leu-Ala). However, this may still not provide for a large enough increase in solubility. A next step would involve applying amino acids that are more hydrophilic than alanine and leucine, such as ser-

ine, threonine, glycine or methionine. All these amino acids are frequently found in single spanning transmembrane proteins. Thus, their use in a model peptide can still yield a membrane protein mimic. However, it has to be considered that the potential differences in helix-helix and helix-lipid interactions possibly alter the behaviour of the 'new' model peptides with respect to the WALP peptides.

Influence of dimerization and helix-helix interface on protein-lipid interactions

In this thesis the first systematic support of the importance of cross sectional diameter and nature of helix-helix interactions in protein-lipid interactions is presented. Studies were performed with two types of covalent dimers of transmembrane model peptides (Figure 1A and 1B). All peptides proved to form highly defined and stable α-helices in di-14:0 PC bilayers as well as micelles, as measured by CD and ATR-FTIR. It was found that dimerization can affect orientation, dynamics and lipid stretching of the peptides in (mis)matching bilayers, depending on the type of connection between the helices. The structural characterization and differences in behaviour of the two types of dimers and their monomeric counterparts will be summarized in the following paragraphs, together with the implications for the knowledge on membrane protein properties and the comparison with previous research.

The α-helical structure of all dimers and their monomeric counterparts was found to be well-defined and stable (Chapters 2, 3 and 4). The stability of monomeric WALP peptides was shown previously,[14, 15] but the stability of the α-helices in the dimers is not self-evident. In the dimers coupled in the middle of the helices (Figure 1A), the forced interfaces could cause restraints or unfavourable interactions that may make it more prone to destabilization. For the dimers with a forced topology (Figure 1B) differences in structural stability may also be expected on basis of the more favourable helix-helix packing for an antiparallel arrangement, shown in previous research.[16, 17] However, no evidence was found for differences in the stability of the secondary structure of the peptides in a membrane, either under destabilizing conditions or during H/D exchange.

The orientation of the dimers with respect to the monomers in a positive mismatch situation was found to depend on the position of the connection between the helices. Both monomeric references (Figure 1A and 1B) showed a tilt of 20º with respect to the membrane normal of di-14:0 PC bilayers. This is in agreement with tilt angles found previously for a WALP 23 in di-14:0 and di-18:1 PC vesicles.[18, 19] Interestingly, all three dimers that were coupled in the middle of the helices (Figure 1A) did not show an altered orientation as compared to monomers (Chapter 3). The forced helix-helix interfaces of these dimers possibly inhibit the ability to achieve a favourable packing while tilting. In contrast, dimers that were coupled at one of the termini (Figure 1B) did show an increase in tilt to 30-34º (Chapter 4). These dimers have an increased flexibility to change the interface with respect to each other, providing better possibilities for favourable helix packing upon tilting. The small but possibly significant difference in tilt of 30º for the antiparallel and 34º for the parallel dimer may also be related to differences in packing. The antiparallel dimer is assumed to have slightly more favourable helix-helix interactions,[16] which may make changing the interface for tilting less favourable. The comparison of the tilts of all dimers and monomers suggests that dimerization may facilitate tilting, depending on the helix-helix interactions. This would imply that both cross sectional diameter and helix-helix interactions can be important in membrane protein properties.

The three dimers covalently coupled in the middle (Figure 1A) had an increased lipid effect with respect to the monomers (Chapter 3). This result provides the first step towards systematic evidence of the influence of cross sectional diameter on protein-lipid interactions. In the experiments, the amount of helices in the samples was kept constant and it was found that, per

lipid, the dimers had a more pronounced effect on the order of the acyl chains. This may be related to entropy and enthalpy influences in the lipids surrounding the helices. Acyl chain stretching causes a loss of entropy. Since there are more lipids alongside a helix in the monomer sample, stretching to compensate for mismatch would result in a higher entropy cost in case of the monomers. On the other hand, ordered acyl chains next to one another can have more favourable Van der Waals interactions, resulting in a more favourable enthalpy. Thus, one large ring of ordered lipids surrounding a dimer is likely to be more favourable than two smaller rings of ordered lipids surrounding two monomers. In Chapter 1 indications of the influence of protein size on their lipid effects were described. One example was the fact that the β-helical gramicidin, which has a larger diameter, had a larger effect on lipid order than a WALP monomer.[20-22] The current result suggest that this is at least partly caused by the difference in cross sectional diameter. Another observation described in the general introduction, was that WALP peptides do not tend to localize to microdomains with matching hydrophobic thickness[23], while multispan membrane proteins do[24, 25]. This difference may be related to the influence of cross sectional diameter on protein-lipid interactions, shown with the model peptides. Possibly, unfavourable lipid effects, such as stretching of the acyl chains, are an important part of the underlying mechanism of the localization of membrane proteins to micro domains. The dimers are the first step towards natural membrane proteins and their increased lipid effects may result in a larger penalty to reside in mismatching bilayers.

Remarkably, the dimers covalently coupled at the termini (Figure 1B) and the corresponding monomers did not order the lipid acyl chains (Chapter 4). Most striking is the lack of lipid stretching effect of the monomers, because other monomeric WALP peptides with the same hydrophobic sequence do have a significant effect.[22, 26] When considering the sequences of the monomeric WALP analogues from Chapters 3 and 4 (Figure 1A and 1B), the only difference is the presence or absence of a tail, indicating that the stretching effect is sensitive to variations in the peptides. The origin of the lack of ordering by the monomers with a positively charged tag may be related to insertion of the lysine side chain between the lipid headgroups. Such an insertion might generate more space for the acyl chains, counteracting the ordering effect of the Leu-Ala stretch. It is possible that the tails also contribute to the difference in acyl chain ordering between the dimers in Chapters 3 and 4 (Figure 1A and 1B).

Another factor that is likely to contribute to the difference in acyl chain ordering by the two types of dimers is their tilt. Tilting of the peptides and ordering of lipid acyl chains are both responses to a positive hydrophobic mismatch (Chapter 1). The effect of peptides on lipid acyl chains may depend on the ability of the peptide to adapt to mismatch in a different way. The dimers coupled in the middle of the helix (Figure 1A) may not have sufficient flexibility to tilt and accordingly the acyl chains show significant stretching. Conversely, the dimers coupled at the termini (Figure 1B) have an increased tilt and it is likely that this plays a role in the absence of a significant change in the effect on the lipid acyl chains in comparison to the monomers. A similar effect is observed when comparing WALP (Trp flanked) and KALP (Lys flanked) peptides. KALP peptides show larger tilt angles, but smaller lipid effects than WALP peptides.[27] However, it is difficult to definitively establish any correlation, because of the complicating factor that the WALP monomers do not behave similarly.

The dynamics of the dimers coupled in the middle of the helix (Figure 1A) is restrained in comparison to that of the monomers (Chapter 3). A decrease in lateral diffusion and a restriction of rotation was demonstrated by simulations and supported by their ^{2}H-NMR fingerprints. It is likely that the decreased dynamics relates to other properties of membrane proteins, such as lipid effects. Strikingly, no evidence was found for a

difference in dynamics for a parallel or antiparallel topology of the helices. In the simulations it was found that both arrangements had equal lateral diffusion properties.

Future prospects

The experiments with the two types of dimeric model peptides form the basis for many follow-up studies on the effects of oligomerization on protein-lipid interactions. A key objective is confirming and extending the results and knowledge obtained in Chapters 3 and 4. In addition, the study of oligomeric assemblies offers exciting prospectives for the investigation of other aspects of the influence of size on membrane protein behaviour and lipid interactions. The most evident and some ultimate objectives will be discussed below.

The current results on the orientation and dynamics of the WALP dimers and their monomeric counterparts encourage further studies on these topics. The comparison of monomers and dimers (Chapters 3 and 4) provided the first indication of the importance of cross sectional diameter and helix-helix interactions in the properties of the peptides. However, not all results were conclusive and there are many more possibilities to investigate the tilt, rotation and dynamics of oligomeric model peptides and the factors that influence these properties. Some first steps will be described in this paragraph. It was shown in the research described in this thesis that tilt angles could conveniently be determined by ATR-FTIR. However, it is not straightforward to determine rotation angles with this technique. Moreover, the choice of lipids is limited and subtle variations in tilt may not be detected, complicating systematic mismatch studies. Solid state NMR is suitable, but not straightforward method to establish tilt, rotation and dynamics of peptides in several bilayers. The role of dynamics in the calculation of tilt when using NMR methods is becoming increasingly evident.[28, 29] Therefore, care will have to be taken to account for a difference in motional averaging of the mono- and dimers. With PISEMA[30] and GALA[31] methods this may not be achieved sufficiently. Recent results suggest that the orientation and dynamics of monomeric and oligomeric model peptides can best be established by the NMR-based MACADAM strategy.[19] In this approach a dynamic model is used, considering tilt, rotation and two associated order parameters. To specifically monitor one dynamical parameter, the lateral diffusion of the peptides, fluorescence correlation spectroscopy of labelled monomers and oligomers in giant unilamellar vesicles may be performed.[32] By determining the diffusion of oligomers with a varying number of WALP peptides and correlating this to the estimated sizes of the assemblies, the validity of two models[33, 34] that describe the influence of hydrodynamic radius on protein diffusion, may be tested systematically.

Another important aim is to further investigate the influence of topology on helix-helix and helix-lipid interactions. The study on the terminally coupled dimers, described in chapter 4, showed that dimers with an antiparallel arrangement may have smaller tilt angles than dimers with a parallel arrangement. The current model system and results should be validated and there are many possibilities to extend the study of the dependence of the properties and lipid interactions of the dimers on their arrangement. First, to confirm if the dimers coupled at the termini have both helices in one bilayer, the vesicles may be investigated using small angle X-ray scattering. Second, the properties of the parallel and antiparallel dimers can be further compared. By reproducing the ATR-FTIR results, it is feasible to establish if the differences in tilt between the parallel and antiparallel dimers are significant. In addition, as mentioned above, the orientation and dynamics of the two dimers may be established by NMR, using the MACADAM method.[19] Moreover, simulations of the parallel and antiparallel dimers would probably yield new insights on dimer tilt, rotation and dynamics and possibly further explain the experimental data.

Finally, by incorporating a fluorophore-quencher pair in the two helices, as a molecular ruler, it might be possible to determine potential differences in interhelical distances.

The lipid effects of the dimers coupled in the middle of the helix (Chapter 3) indicate that diameter may be important in the extent of acyl chain stretching. However, the ordering of acyl chains is sensitive to variations in the peptides, as suggested by the lack of effect of the (anti) parallel dimers. This issue may be resolved by comparing one, two, three or four WALP peptides, possibly with and without a charged tail, all covalently coupled to a scaffold. A potential consequence of unfavourable lipid effects, such as stretching, may be localization[24, 25, 35, 36] of membrane proteins to micro domains. It would be interesting to establish a potential correlation, using the same system as used for the ordering studies mentioned above. The scaffold may be equipped with a fluorescent label, enabling the visualisation of localization. The systematic study on this topic is an important objective. It was shown previously that WALP peptides did not tend to localize to matching liquid ordered domains[23] and that a WALP27 does not localize to thicker cholesterol rich domains,[23] maybe because the high order of the domain had more influence than mismatch. It would be informative to determine if oligomers of WALP27 would localize to such domains. Related to the ordering effect, also the influence of cross sectional diameter on phospholipid flop and on the thermotropic behaviour of lipid bilayers can be investigated. It would be interesting to establish if phospholipid flop indeed relates to the size of proteins, as is suggested by differences in transbilayer movement of lipids by a subset of membrane proteins.[37] This could be systematically studied by comparing WALP monomers and oligomers with varying membrane spanning segments, following methods described previously.[38] Regarding lipid phase transition, it was described in the general introduction (Chapter 1) that single span WALP peptides[39] show only minor effects as compared to natural membrane proteins.[40-42] This difference may be coupled to size by comparing phase transition temperatures of lipids bilayers with WALP monomers and oligomers incorporated.

The ultimate goal of the study of WALP model peptides is to determine basic principles of membrane protein behaviour and lipid interactions and maybe even establish the ability to predict function on basis of structure. With the oligomeric assemblies, the aim is to reveal all aspects of cross sectional diameter and helix-helix interactions that influence protein-lipid interactions. A key question in the observed influence of dimerization and thus in further studies, is how general the observations are. A systematic approach is important in answering this question. There are multiple properties of the model system that may be varied systematically. One possibility is changing the flanking residues. For example, the influence of tryptophan in the observed differences upon dimerization may be studied by replacing the tryptophan residues with lysine residues. Another parameter that should be studied is the influence of helix-packing. It was hypothesized on basis of the differences in tilting and acyl chain ordering of the two types of dimers (Figure 1A and 1B) that tilting may be a more favoured reaction to positive hydrophobic mismatch than stretching of the lipid acyl chains. However, more systematic experiments on dimeric peptides with different helix-helix packing motifs are required to confirm this. Suitable peptides to test this would for example be dimeric WALP analogues in which the alternating Leucine-Alanine motif is replaced by several motifs of scrambled sequences of these residues. Besides this, many more variations of the sequence are possible, such as including a recognition motif. Important in the systematic approach to study the influence of size on peptides behaviour and lipid interactions is the use of hydrophobic mismatch. The hydrophobic length of the peptides and the hydrophobic thickness of the bilayers can both be varied, to compare the sensitivity of monomeric and oligomeric WALP peptides.

References

1. Killian, J. A., Synthetic peptides as models for intrinsic membrane proteins, *FEBS Lett*, **2003**, 555(1), p. 134-8
2. Nyholm, T. K., Ozdirekcan, S., and Killian, J. A., How protein transmembrane segments sense the lipid environment, *Biochemistry*, **2007**, 46(6), p. 1457-65
3. Holt, A. and Killian, J. A., Orientation and dynamics of transmembrane peptides: the power of simple models, *Eur Biophys J*, **2009**, 39(4), p. 609-621
4. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B., A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes, *Angew Chem Int Ed Engl*, **2002**, 41(14), p. 2596-9
5. Tornoe, C. W., Christensen, C., and Meldal, M., Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides, *J Org Chem*, **2002**, 67(9), p. 3057-64
6. Tuchscherer, G., Scheibler, L., Dumy, P., and Mutter, M., Protein design: on the threshold of functional properties, *Biopolymers*, **1998**, 47(1), p. 63-73
7. Grove, A., Mutter, M., Rivier, J. E., and Montal, M., Template-assembled synthetic proteins designed to adopt a globular, four-helix bundle conformation form ionic channels in lipid bilayers, *J Am Chem Soc*, **1993**, 115, p. 5919-5924
8. Dumy, P., Eggleston, I. M., Esposito, G., Nicula, S., and Mutter, M., Solution structure of regioselectively addressable functionalized templates: an NMR and restrained molecular dynamics investigation, *Biopolymers*, **1996**, 39(3), p. 297-308
9. Sucholeiki, I. and Lansbury, P. T., An Affinity Chromatographic Method for the Purification of Water-Insoluble Peptides, *Journal of Organic Chemistry*, **1993**, 58, p. 1318-1324
10. Becker, C. F., Oblatt-Montal, M., Kochendoerfer, G. G., and Montal, M., Chemical synthesis and single channel properties of tetrameric and pentameric TASPs (template-assembled synthetic proteins) derived from the transmembrane domain of HIV virus protein u (Vpu), *J Biol Chem*, **2004**, 279(17), p. 17483-9
11. Wahlstrom, K., Planstedt, O., and Unden, A., Synthesis and purification of aggregation-prone hydrophobic peptides by the incorporation of an Fmoc dipeptide with the peptide bond protected with a modified 2-hydroxy-4-methoxybenzyl (Hmb) group, *Tet Lett*, **2008**, 49(24), p. 3921-3924
12. Wahlstrom, K., Planstedt, O., and Unden, A., A carbamoyl-protective group for tyrosine that facilitates purification of hydrophobic synthetic peptides, *Tet Lett*, **2008**, 49(23), p. 3779-3781
13. Wahlstrom, K. and Unden, A., A new protecting group for tryptophan in solid-phase peptide synthesis which protects against acid-catalyzed side reactions and facilitates purification by HPLC, *Tet Lett*, **2009**, 50, p. 2976-2978
14. Demmers, J. A., van Duijn, E., Haverkamp, J., Greathouse, D. V., Koeppe, R. E., 2nd, Heck, A. J., and Killian, J. A., Interfacial positioning and stability of transmembrane peptides in lipid bilayers studied by combining hydrogen/deuterium exchange and mass spectrometry, *J Biol Chem*, **2001**, 276(37), p. 34501-8
15. de Planque, M. R., Goormaghtigh, E., Greathouse, D. V., Koeppe, R. E., 2nd, Kruijtzer, J. A., Liskamp, R. M., de Kruijff, B., and Killian, J. A., Sensitivity of single membrane-spanning alpha-helical peptides to hydrophobic mismatch with a lipid bilayer: effects on backbone structure, orientation, and extent of membrane incorporation, *Biochemistry*, **2001**, 40(16), p. 5000-10
16. Sparr, E., Ash, W. L., Nazarov, P. V., Rijkers, D. T. S., Hemminga, M. A., Tieleman, D. P., and Killian, J. A., Self-association of Transmembrane {alpha}-Helices in Model Membranes: IMPORTANCE OF HELIX ORIENTATION AND ROLE OF HYDROPHOBIC MISMATCH, *J Biol Chem*, **2005**, 280(47), p. 39324-39331
17. Yano, Y. and Matsuzaki, K., Measurement of thermodynamic parameters for hydrophobic mismatch 1: self-association of a transmembrane helix, *Biochemistry*, **2006**, 45(10), p. 3370-8
18. Holt, A., Koehorst, R. B., Rutters-Meijneke, T., Gelb, M. H., Rijkers, D. T. S., Hemminga, M. A., and Killian, J. A., Tilt and rotation angles of a transmembrane model peptide as studied by fluorescence spectroscopy, *Biophys J*, **2009**, 97(8), p. 2258-66
19. Holt, A., Rougier, L., Reat, V., Jolibois, F., Saurel, O., Czaplicki, J., Killian, J. A., and Milon, A., Order parameters of a transmembrane helix in a fluid bilayer: case study of a WALP peptide, *Biophys J*, **2010**, 98(9), p. 1864-72
20. Harroun, T. A., Heller, W. T., Weiss, T. M., Yang, L., and Huang, H. W., Experimental evidence for hydrophobic matching and membrane-mediated interactions in lipid bilayers containing gramicidin, *Biophys J*, **1999**, 76(2), p. 937-45
21. Weiss, T. M., van der Wel, P. C., Killian, J. A., Koeppe, R. E., 2nd, and Huang, H. W., Hydrophobic mismatch between helices and lipid bilayers, *Biophys J*, **2003**, 84(1), p. 379-85
22. de Planque, M. R., Greathouse, D. V., Koeppe, R. E., 2nd, Schafer, H., Marsh, D., and Killian, J. A., Influence of lipid/peptide hydrophobic mismatch on the thickness of diacylphosphatidylcholine bilayers. A 2H NMR and ESR study using designed transmembrane alpha-helical peptides and gramicidin A, *Biochemistry*, **1998**, 37(26), p. 9333-45
23. van Duyl, B. Y., Rijkers, D. T. S., de Kruijff, B., and Killian, J. A., Influence of hydrophobic mismatch and palmitoylation on the association of transmembrane alpha-helical peptides with detergent-resistant membranes, *FEBS Lett*, **2002**, 523(1-3), p. 79-84
24. Martens, J. R., O'Connell, K., and Tamkun, M., Targeting of ion channels to membrane microdomains: localization of KV channels to lipid rafts, *Trends Pharmacol Sci*, **2004**, 25(1), p. 16-21
25. Dumas, F., Sperotto, M. M., Lebrun, M. C., Tocanne, J. F., and Mouritsen, O. G., Molecular sorting of lipids by bacteriorhodopsin in dilauroylphosphatidylcholine/distearoylphosphatidylcholine lipid bilayers, *Biophys J*, **1997**, 73(4), p. 1940-53
26. Ozdirekcan, S., Nyholm, T. K., Raja, M., Rijkers, D. T. S., Liskamp, R. M. J., and Killian, J. A., Influence of trifluoroethanol on membrane interfacial anchoring interactions of transmembrane alpha-helical peptides, *Biophys J*, **2008**, 94(4), p. 1315-25
27. de Planque, M. R. and Killian, J. A., Protein-lipid interactions studied with designed transmembrane peptides: role of hydrophobic matching and interfacial anchoring, *Mol Membr Biol*, **2003**, 20(4), p. 271-84
28. Strandberg, E., Esteban-Martin, S., Salgado, J., and Ulrich, A. S., Orientation and dynamics of peptides in membranes calculated from 2H-NMR data, *Biophys J*, **2009**, 96(8), p. 3223-32
29. Ozdirekcan, S., Etchebest, C., Killian, J. A., and Fuchs, P. F. J., On the orientation of a designed transmembrane peptide: toward the right tilt angle?, *J Am Chem Soc*, **2007**, 129(49), p. 15174-81
30. Esteban-Martin, S., Strandberg, E., Fuertes, G., Ulrich, A. S., and Salgado, J., Influence of whole-body dynamics on 15N PISEMA NMR spectra of membrane proteins: a theoretical analysis, *Biophys J*, **2009**, 96(8), p. 3233-41
31. van der Wel, P. C., Strandberg, E., Killian, J. A., and Koeppe, R. E., 2nd, Geometry and intrinsic tilt of a tryptophan-anchored transmembrane alpha-helix determined by (2)H NMR, *Biophys J*, **2002**, 83(3), p. 1479-88
32. Ramadurai, S., Holt, A., Krasnikov, V., van den Bogaart, G., Killian, J. A., and Poolman, B., Lateral diffusion of membrane proteins, *J Am Chem Soc*, **2009**, 131(35), p. 12650-6
33. Saffman, P. G. and Delbruck, M., Brownian motion in biological membranes, *Proc Natl Acad Sci U S A*, **1975**, 72(8), p. 3111-3

34. Gambin, Y., Lopez-Esparza, R., Reffay, M., Sierecki, E., Gov, N. S., Genest, M., Hodges, R. S., and Urbach, W., Lateral mobility of proteins in liquid membranes revisited, *Proc Natl Acad Sci U S A*, **2006**, 103(7), p. 2098-102
35. Fernandes, F., Loura, L. M., Prieto, M., Koehorst, R., Spruijt, R. B., and Hemminga, M. A., Dependence of M13 major coat protein oligomerization and lateral segregation on bilayer composition, *Biophys J*, **2003**, 85(4), p. 2430-41
36. Briggs, J. A., Wilk, T., and Fuller, S. D., Do lipid rafts mediate virus assembly and pseudotyping?, *J Gen Virol*, **2003**, 84(Pt 4), p. 757-68
37. Kol, M. A., van Dalen, A., de Kroon, A. I., and de Kruijff, B., Translocation of phospholipids is facilitated by a subset of membrane-spanning proteins of the bacterial cytoplasmic membrane, *J Biol Chem*, **2003**, 278(27), p. 24586-93
38. Kol, M. A., de Kroon, A. I., Rijkers, D. T. S., Killian, J. A., and de Kruijff, B., Membrane-spanning peptides induce phospholipid flop: a model for phospholipid translocation across the inner membrane of E. coli, *Biochemistry*, **2001**, 40(35), p. 10500-6
39. Morein, S., Killian, J. A., and Sperotto, M. M., Characterization of the thermotropic behavior and lateral organization of lipid-peptide mixtures by a combined experimental and theoretical approach: effects of hydrophobic mismatch and role of flanking residues, *Biophys J*, **2002**, 82(3), p. 1405-17
40. Riegler, J. and Mohwald, H., Elastic Interactions of Photosynthetic Reaction Center Proteins Affecting Phase Transitions and Protein Distributions, *Biophys J*, **1986**, 49(6), p. 1111-1118
41. Piknova, B., Perochon, E., and Tocanne, J. F., Hydrophobic mismatch and long-range protein/lipid interactions in bacteriorhodopsin/phosphatidylcholine vesicles, *Eur J Biochem*, **1993**, 218(2), p. 385-96
42. Sperotto, M. M., Ipsen, J. H., and Mouritsen, O. G., Theory of protein-induced lateral phase separation in lipid membranes, *Cell Biophys*, **1989**, 14(1), p. 79-95

Appendices

List of abbreviations

Chemicals and reagents

DiPEA	*N,N*-Diisopropylethylamine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DTT	dithiothreitol
Fmoc	9-fluorenylmethyloxycarbonyl
HBTU	*O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uronium-hexafluoro-phosphate
HOBt	*N*-hydroxybenzotriazole
MTBE	methyl-*tert*-butyl ether
N_2	nitrogen
NMP	*N*-methylpyrrolidone
PEG	polyethylene glycol
TBTA	tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]amine
tBu	*tert*-butyl
TCEP	tris(2-carboxyethyl)phosphine
TEA	triethylamine
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TIS	triisopropylsilane
TMS	trimethylsilane
TRIS	tris(hydroxymethyl)aminomethane

Detergents and lipids

DDM	n-dodecyl β-D-maltoside
SDS	sodium dodecyl sulfate
DMPC, di-14:0 PC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DOPC, di-18:1 PC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
di-14:1 PC	1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine
di-16:1 PC	1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine
di-20:1 PC	1,2-dieicosenoyl-*sn*-glycero-3-phosphocholine
di-22:1 PC	1,2-dierucoyl-*sn*-glycero-3-phosphocholine
di-14:0 PC-d54	1,2-dimyristoyl(d54)-*sn*-glycero-3-phosphocholine

Techniques

(ATR-)FTIR	(Attenuated Total Reflection -) Fourier Transform Infrared Spectroscopy
CD	Circular Dichroism
CG simulations	Course-grained simulations
ESI-MS	ElectroSpray Ionization Mass Spectrometry
FRET	Förster resonance energy transfer
GALA	Geometric Analysis of Labelled Alanines
GPC	Gel Permeation Chromatography
HPLC	High Performance Liquid Chromatography
IRE	Internal Reflection Element
NMR	Nuclear Magnetic Resonance
MACADAM	Multiple Anisotropic Constraints And Dynamic Analysis of Membrane peptides
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization - Time Of Flight
SDS-PAGE	Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis

List of publications

Elgersma, R.C., Meijneke, T., de Jong, R., Brouwer, A.J., Posthuma, G., Rijksers, D.T.S., Liskamp, R.M.J., Synthesis and structural investigation of N-alkylated beta-peptidosulfonamide-peptide hybrids of the amyloidogenic amylin(20-29) sequence: Implications of supramolecular folding for the design of peptide-based bionanomaterials, Org. Biomol.Chem, 2006, 4(19), 3587

Elgersma, R.C., Meijneke, T., Posthuma, G., Rijkers, D.T.S., Liskamp, R.M.J., Self-assembly of amylin(20-29) amide-bond derivatives into helical ribbons and peptide nanotubes rather than fibrils, Chem.Eur.J, 2006, 12(14), 3714

Scarpelli, F., Drescher, M., Rutters-Meijneke, T., Holt, A., Rijkers, D.T.S., Killian, J.A., Huber, M., Aggregation of transmembrane peptides studied by spin-label EPR, J.Phys.Chem.B, 2009, 113(36), 12257

Holt, A., Koehorst, R.B.M., Rutters-Meijneke, T., Gelb, M.H., Rijkers, D.T.S., Hemminga, M.A., Killian, J.A., Tilt and rotation angles of a transmembrane model peptide as studied by fluorescence spectroscopy, Biophys.J., 2009, 97(8), 2258

Rutters-Meijneke, T., Nyholm, T.K., Staffhorst, R.W.H.M., Rijkers, D.T.S., Killian, J.A., Dimeric assemblies of transmembrane model peptides: Design, synthesis, purification and characterization, manuscript in preparation

Rutters-Meijneke, T., Doux, J.P.F., Fuchs, P.F.J., Goormaghtigh, E., Killian, J.A., Dimeric assemblies of transmembrane model peptides: The effect of cross sectional diameter on protein-lipid interactions, manuscript in preparation

Rutters-Meijneke, T., Doux, J.P.F., Rijkers, D.T.S., Goormaghtigh, E., Killian, J.A., Parallel and antiparallel dimers of transmembrane model peptides: The effect of helix-helix interactions on protein-lipid interactions, manuscript in preparation

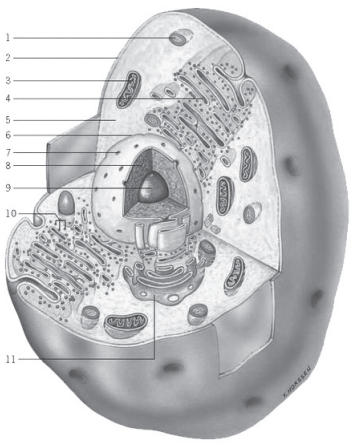
Dutch summary / Samenvatting

Over de grenswachters van onze cellen

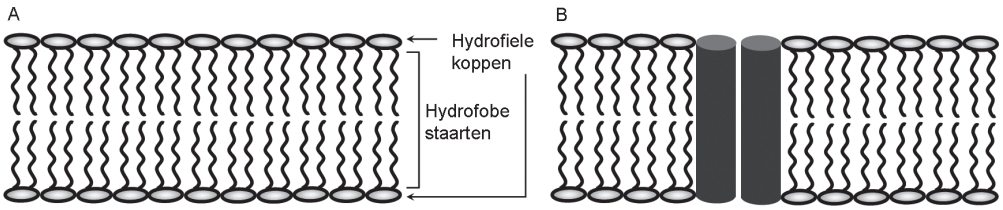
‘Hoe genezen we ziektes?’ Dit is een vraag die we ons al sinds mensenheugenis stellen. De prehistorische medicijnenmannen breidden hun kennis generatie na generatie uit via een ‘trial and error’ methode. Tegenwoordig gebruiken we een meer strategische aanpak die begint met de vraag: ‘Hoe werkt ons lichaam?’ Hoewel we steeds meer te weten komen, zijn er nog altijd meer vragen dan antwoorden. Het begrijpen van ons lichaam op een moleculair niveau is zo complex gebleken dat een onderzoeker slechts kleine stukjes aan één van de vele puzzels van het lichaam kan toevoegen. De puzzel waaraan ik werkte is het begrijpen van zogenaamde membraaneiwitten. Hieronder zal ik meer uitleggen over membraaneiwitten en wat verder ingaan op wat ik onderzocht heb.

Ons lichaam is opgebouwd uit cellen. In Figuur 1 zie je een voorbeeld van zo’n cel. Alle cellen in ons lichaam worden omgeven en beschermd door een wand, het celmembraan. De belangrijkste bestanddelen van dit membraan zijn lipiden en membraaneiwitten. Lipiden hebben een hydrofiele (waterlievende) kop en een hydrofobe (waterafstotende) staart, waardoor ze in de waterige omgeving van ons lichaam zogenaamde bilagen vormen (Figuur 2A). Deze bilagen vormen de grenzen van de cellen. Doordat het voor water oplosbare stoffen veel energie kost om deze grens over te steken, blijft de celinhoud gescheiden van de ‘buitenwereld’. Membraaneiwitten zorgen ervoor dat er toch stoffen door het celmembraan getransporteerd kunnen worden. Ze kunnen gezien worden als de grenswachters van de cellen. Membraaneiwitten bestaan uit één of meerdere segmenten die het membraan overspannen (Figuur 2B), waarmee ze de verbinding vormen tussen binnen en buiten de cel. Dit kan belangrijk zijn bij het voorzien van de cel van voedingsstoffen of in het doorgeven van signalen. Als membraaneiwitten hun functie niet goed uitvoeren kan dit ernstige gevolgen hebben voor de gezondheid van het lichaam. Taaislijmziekte bijvoorbeeld, ontstaat wanneer het zogenaamde CFTR (cystic fibrosis transmembrane regulator) eiwit niet goed in het membraan gaat zitten.

Ondanks de belangrijke rol van membraaneiwitten, is er relatief weinig bekend over hun werking. Dit komt door de grote diversiteit en complexiteit van celmembranen met membraaneiwitten. Een methode om toch meer te weten te komen over de grenswachters van onze cellen



Figuur 1. Schematische weergaven van een cel in ons lichaam. De nummers geven verschillende onderdelen van de cel aan, waarbij nummer 2 naar de celwand wijst



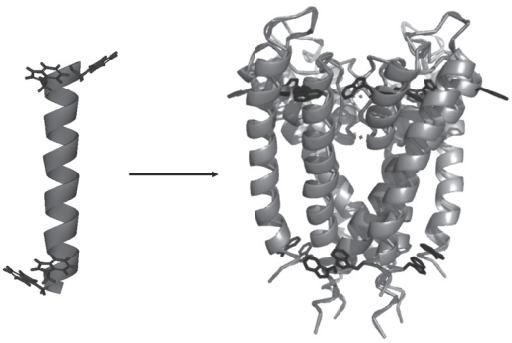
Figuur 2. Schematisch weergave van een bilaag van lipiden met een hydrofiele kop en een hydrofobe staart (A) en een bilaag waardoor een membraaneiwit steekt dat bestaat uit twee segmenten die de bilaag overspannen (B)

is door te versimpelen. Een eerste stap naar een eenvoudiger systeem hebben we gemaakt door te kijken naar de overeenkomsten tussen membraaneiwitten. Zoals in de vorige alinea aangegeven bestaan alle membraaneiwitten uit één of meerdere membraanoverspannende segmenten. Deze segmenten zijn doorgaans 'wokkel-vormig', ze worden helices genoemd. Om het grote complex van helices te kunnen begrijpen, zouden we kunnen beginnen met bestuderen van het gedrag van één helix (Figuur 3). Omdat in de cellen in ons lichaam nog veel meer zit dan lipiden en membraaneiwitten (zie Figuur 1), stoppen we de helices in ons eigen gemaakte celmembranen. Dit zorgt voor een verdere vereenvoudiging van ons systeem.

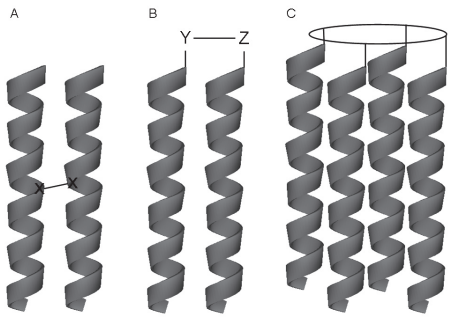
De eigenschappen en het gedrag van membraaneiwitten wordt sterk beïnvloed door de omringende lipiden. Daarom is het van belang om meer te weten te komen over de interacties tussen eiwitten en lipiden. Het is goed mogelijk om deze interacties te bestuderen met de enkele helix in een bilaag. Sterker nog, met deze 'namaak-eiwitten' hebben we in de afgelopen 10 tot 20 jaar al heel veel geleerd over de factoren die belangrijk zijn voor het gedrag van membraaneiwitten. Je kunt je echter afvragen of één enkel segment een goed model is voor eiwitten die uit meerdere segmenten bestaan. Daarom ben ik tijdens mijn promotie een stapje verder gegaan door meerdere helices aan elkaar te plakken. Er ontstaan dan zogeheten oligomeren. In dit proefschrift wordt het maken en bestuderen van deze oligomeren beschreven.

Resultaten

Voordat we onze modellen voor membraaneiwitten kunnen gaan bestuderen, moeten we eerst helices maken. De bouwstenen van de helices zijn zogenaamde aminozuren. Het maken van helices kun je vergelijken met het maken van een kralenketting. Voor het maken van een kralenketting voeg je steeds een kraal toe zoals je voor het maken van een helix steeds een aminozuur toevoegt. De ketting van aminozuren die je aan het eind hebt heet een peptide. Deze peptiden moet je dan vervolgens aan elkaar gaan koppelen. In figuur 4 zie je de ontwerpen van de



Figuur 3. Een enkele α-helix (links) om het gedrag van een membraaneiwit (rechts) te bestuderen



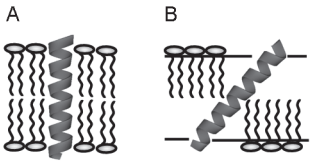
Figuur 4. Drie manieren die we gebruikten om helices aan elkaar te koppelen

oligomeren die we gemaakt hebben. In ontwerp A zijn er twee helices aan elkaar geplakt via één bepaald aminozuur (aangegeven als X) in het midden van het peptide. Dit is een makkelijk te maken ontwerp en een goede eerste stap voor onze studie. Echter, de helices in membraaneiwitten zijn meestal aan het uiteinde gekoppeld. Daarom is ontwerp B gemaakt. Deze is wel iets moeilijker te maken, maar lijkt dus nog net wat meer op een echt membraaneiwit. Ten slotte is ontwerp C bedacht om vier peptiden aan elkaar te kunnen plakken. Met ontwerp A en B hebben we allerlei onderzoek uitgevoerd. Ontwerp C hebben we niet kunnen bestuderen. Dit kwam omdat we deze oligomeer niet konden scheiden van allerlei andere moleculen die ontstonden tijdens het koppelen van de helices. Deze scheiding is nodig om er zeker van te kunnen zijn dat het gedrag dat je bestudeerd echt het gedrag is van de oligomeer en niet van een onzuiverheid.

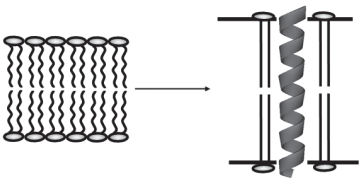
Met onze oligomeren in namaakcellen hebben we nieuwe kennis opgedaan over het functioneren van echte membraaneiwitten. In de volgende alinea's ga ik wat verder in op drie van de aspecten die ik onderzocht heb. Omdat dit de eerste keer is dat deze grotere namaakeiwitten gemaakt zijn, moesten we ten eerst kijken of ze de verwachte vorm hadden. Over de uitkomst van deze studie vertel ik in de eerst volgende alinea. Ten tweede gaf het onderzoek informatie over iets wat een heel aantal membraaneiwitten gemeen hebben: een verandering in de oriëntatie van de helices in het eiwit is vaak essentieel voor hun werking. Dit licht ik in de tweede en derde alinea hieronder toe. Ten derde is, zoals eerder gezegd, de interactie tussen helices en lipiden heel belangrijk voor eiwitten in celmembranen. Hierbij is niet alleen de invloed van de bilaag op de eiwitten van belang, maar ook de invloed van de eiwitten op de bilaag. Hierop zal ik in de vierde alinea verder in gaan. Ten slotte zal ik in de laatste alinea nog wat vertellen over het uiteindelijke doel en nut van al de beschreven studies.

Eerst is dus de 'wokkel vorm' van de oligomeren beter bekeken. Dit is belangrijk, aangezien het mogelijk is dat de helix vorm beïnvloed wordt door het aan elkaar plakken van de peptiden. Het zou bijvoorbeeld ongunstig kunnen zijn voor de helices om zich dicht naast elkaar te bevinden, waardoor ze mogelijk vervormen. Eerst is onderzocht of de enkele en gekoppelde peptiden beiden een helix vorm hadden. De vorm bleek inderdaad hetzelfde te zijn voor de peptiden in de oligomeren als voor de enkele peptiden. Toen is de stabiliteit van de helix vorm voor de enkele en gekoppelde peptiden bestudeerd. Dit werd via twee verschillende methoden gedaan. Ten eerste is geprobeerd om de peptiden te vervormen. Uit het feit dat zelfs onder heftige omstandigheden, waarin vele membraaneiwitten hun helix vorm zouden verliezen, de structuur van de peptiden onveranderd bleef, was af te leiden dat de vorm heel stabiel is. Ten tweede is gekeken of de peptiden reageerden met een speciaal soort water. Een reactie zou betekenen dat de vorm van de peptiden relatief makkelijk te beïnvloeden is. De peptiden reageerden nauwelijks met het speciale water, wat bevestigde dat de bindingen in het peptide sterk waren en daardoor de vorm stabiel.

Voor het gedrag van membraaneiwitten kan de oriëntatie van de helices ten opzichte van de bilaag heel belangrijk zijn. De helix of helices kunnen parallel aan de lipiden zijn (Figuur 5A) of een hoek maken (Figuur 5B). Deze kanteling is belangrijk voor de grenswachters rol van membraaneiwitten, die in de inleiding beschreven werd. Membraaneiwitten zorgen ervoor dat stoffen de cel in en uit kunnen. Dit kan op vele verschillende manieren. Sommige membraaneiwitten staan altijd open en laten bijvoorbeeld kleine stoffen wel door, maar grotere niet. Andere gaan alleen onder bepaalde condities open. Bij het voelen van pijn bijvoorbeeld speelt het doorlaten van de stof kalium door een bepaald membraaneiwit, het kaliumkanaal, een rol. Zo worden pijnprikkels doorgegeven in onze zenuwen. Bij het openen en sluiten van kanalen speelt de oriëntatie van de helices vaak een grote rol. Als alle onderdelen van het membraaneiwit recht staan kan er niks door, maar als er een paar



Figuur 5. Helices die rechtop (A) of gekanteld (B) in de bilaag zitten



Figuur 6. De vervormende werking van een helix op de staarten van lipiden

schuin staan wel. Om stoffen door te laten moeten dus één of meerder onderdelen van het eiwitten opzij kantelen.

Wij hebben een aantal zaken ontdekt over het kantelen van helices in membraaneiwitten. Ten eerste hebben we gezien dat het aan elkaar koppelen van helices invloed kan hebben op hun oriëntatie. Een enkele helix maakt een hoek van 20º ten opzichte van de lipiden, terwijl de helices in ontwerp B (Figuur 4) een hoek maken van 30º. We kunnen hieruit afleiden dat eiwitten die uit meerdere segmenten bestaan, waarschijnlijk makkelijker hun oriëntatie kunnen veranderen. Dit bleek echter wel af te hangen van de interacties tussen de helices. De helices in ontwerp A (Figuur 4) hadden namelijk niet de neiging om extra te kantelen, maar maakten gewoon een hoek van 20º met de lipiden, net als de enkele helices. Het is aannemelijk dat dit komt doordat de helices in deze oligomeer in het midden verbonden waren. Op basis hiervan kunnen we zeggen dat het waarschijnlijker is dat helices in een membraaneiwit van oriëntatie kunnen veranderen als ze vrij kunnen bewegen ten opzichte van elkaar.

De invloed van membraaneiwitten op de celmembraan is van belang voor zowel de eigenschappen van de cellen als voor het gedrag van het membraaneiwit zelf. Je kunt je bijvoorbeeld voorstellen dat als een membraaneiwit een hele ongunstige invloed zou hebben op de bilaag, dit eiwit uitgesloten kan worden van het celmembraan. Een voorbeeld van een ongunstige invloed is het vervormen van de hydrofobe staarten (Figuur 2A) van de lipiden door de membraaneiwitten. De zogenaamde strekking van de staarten van lipiden door een helix is schematisch weergegeven in Figuur 6. Net als bij het in de vorige alinea beschreven kantelen van de peptiden, bleek dat gekoppelde helices een ander effect kunnen hebben dan enkele. De helices uit ontwerp A (Figuur 4) strekten de lipidenstaarten namelijk veel meer dan een enkele helix deed. Wederom bleek het gedrag af te hangen van de interacties tussen de helices, want de gekoppelde helices in ontwerp B (Figuur 4) hadden geen groter effect dan een enkel peptide. Uit de resultaten van deze studies is af te leiden dat het waarschijnlijk is dat membraaneiwitten waarin de segmenten vrij star zijn ten opzichte van elkaar een grotere invloed op bilagen hebben dan eiwitten waarin de segmenten heel flexibel zijn.

Het ultieme doel van de studies beschreven in dit proefschrift is om gedrag van membraaneiwitten te kunnen voorspellen. We willen bijvoorbeeld op basis van de eigenschappen van de helices waaruit het eiwit is opgebouwd kunnen zeggen onder welke omstandigheden de helices zullen kantelen. Dit soort voorspellingen is niet alleen belangrijk voor de wetenschap van het lichaam, maar ook van groot belang voor medicijnontwikkeling. Alle medicijnen die hun werk uitvoeren in een cel moeten langs de grenswachters. Om ervoor te zorgen dat ze over de grens komen moeten we weten hoe het doorlaten van stoffen door membraaneiwitten werkt. Stel dat we bijvoorbeeld een pijnstiller willen maken, dan moet we weten hoe het eerder genoemde kaliumkanaal werkt en dus hoe makkelijk de helices kantelen zodat het kanaal opent. Als we achterhalen waardoor het kanaal opent, zouden we een medicijn kunnen maken dat zorgt dat het dicht blijft. Er ontstaat dan geen pijnprikkel. In de toekomst zal blijken of de kennis die wij opgedaan hebben bij zal dragen aan het ontwerpen van nieuwe medicijnen...

Curriculum Vitae Tania Rutters

Tania Rutters Meijneke was born January 26th 1981 in Utrecht, The Netherlands. After obtaining her Atheneum diploma at the dr. F.H. de Bruijne Lyceum in Utrecht (1999), she started a Chemistry study at Utrecht University. In between her second and third year, she was chair of the Chemistry Student Association 'U.S.S. Proton' for one year (2001-2002). Then, a minor research projects was performed at the section 'physical organic chemistry' (2002), where the application of Zn/Mg/Al hydrotalcites as substitute for heavy metals like Pb, in poly vinyl chloride was explored. An internship was carried out at the Dutch institute 'TNO-MEP' (2003-2004) on the topic of bioethanol. A major research project was performed at the section 'medicinal chemistry' (2004-2005) to investigate the β-sheet formation of 'human islet amyloid polypeptide', a protein important in misfolding diseases. After graduation (2005), she started her PhD study in the section 'Biochemistry of Membranes' at Utrecht University, under supervision of prof. dr. J. Antoinette Killian. Oligomeric model peptides were synthesized and biophysically characterized to obtain a better understanding of membrane proteins, which resulted in this thesis. Tania presented the work orally at the European Peptide Symposium, receiving a travel grant, and with posters at several other international congresses. Nationally, she presented work and participated in several symposia and PhD evenings of the 'Bijvoet' and 'Biomembranes' research institutes and several study groups. Two technicians and one bachelor student helped and were guided on parts of the project. During her PhD study, Tania attended courses on biomolecular mass spectrometry, communicating science to layman and bioinformatics were attended. She also assisted students in several theoretical and practical courses during her PhD studies. In 2010, she started in a job as project manager at the 'biochemical support of production' group of Sanquin Plasmaproducts. The first project was the optimization of the manufacturing of C1 esterase inhibitor from plasma, where the C1 inhibitor is used as a medicine for treatment of hereditary angioedema.

Acknowledgement / Dankwoord

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