Chapter V

The interplay between membrane interaction and homo-coiling of coiled-coil peptides on the membrane studied by temperature dependent FTIR and CD spectroscopy

ABSTRACT

Knowledge of the structure of the fusogenic membrane tethered peptides E and K is a key element in the understanding of their role in membrane fusion. Special conditions can be found at the interface of the membrane, where the peptides are confined in close proximity to other peptide molecules as well as to the lipid interface. Consequently, different possible structural states were proposed for the peptides when tethered to this interface. Due to the multitude of possible states, the determination of the structure solely on the basis of circular dichroism spectra at a single temperature can be misleading. Also it has not been possible so far to unambiguously distinguish between the membrane bound and the coiled-coil state of these peptides by means of infrared spectroscopy (IR) using IR reflection absorbtion spectroscopy, due to very similar amide I' bands. Here, the molecular basis of this similarity is investigated by means of site-specific ^{13}C labeled FTIR spectroscopy. Structural similarities between the amphipathic membrane interacting helix of K and the amphipathic coiled-coil forming helix of E are shown to cause the similar spectroscopic properties. Furthermore the peptide structure is investigated using temperature dependent CD and IR spectroscopy and it is shown that the different states can be distinguished on the basis of their thermal behavior. These results will prove useful in further investigation of the membrane fusing lipopeptides as they form the basis for an unequivocal determination of the peptide state by easy spectroscopic methods.

INTRODUCTION

Membrane tethered coiled-coil forming lipopeptides have been examined extensively in different studies[1-12] and in the previous chapters due to their ability to induce lipid membrane fusion. The systems apparent simplicity and possible applications[8,9] have raised a particular interest to conceive the mechanism of lipopeptide mediated fusion. The binding state and secondary structure of the peptide molecular recognition units E: $(EIAALEK)_3$-NH_2 and K: $(KIAALKE)_3$-NH_2 when tethered to the membrane are crucial for understanding of this mechanism.

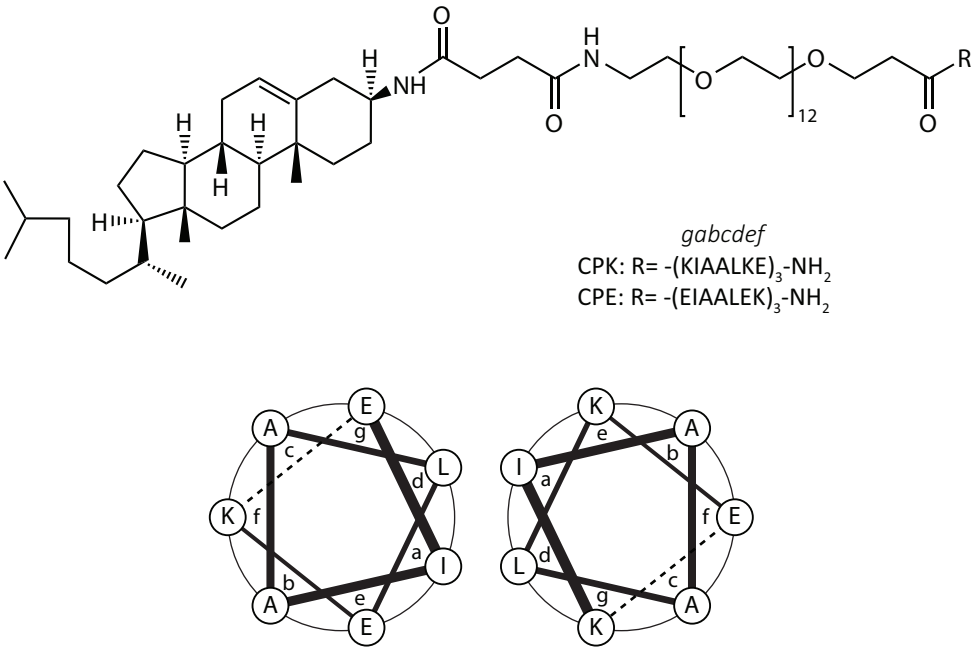
Circular dichroism (CD) spectroscopy is often used in fusion studies to estimate the helical content of the peptides. Generally, the vesicle tethered lipopeptides exhibit increased helical contents compared to their untethered equivalents E and K (*Chapter IV*).[1-8] The increased helical content has often been attributed to the formation of homo-coils EE and KK at the membrane, but is without distinct experimental proof. However, it has been shown in *Chapters III* & *IV* that the helical content of membrane tethered peptide K can be explained by its interactions with the membrane which induces the formation of a monomeric amphipathic A helix.[13] This shows the ambiguity of the CD method in the case of complex systems and demonstrates the necessity of further analytical methods to study them.

Typical CD spectra of α-helices exhibit two minima around 208 and 222nm (cf. *Chapter II* & *IV*). Due to the low absolute ellipticity of other secondary structure elements at this position, the mean residual ellipticity at 222 nm ($[\theta]_{222nm}$) is a convenient measure for helical content.[14,15] However by using $[\theta]_{222nm}$ only, it is not possible to distinguish whether the origin of this helicity is due to coiled-coil complex formation or folding into a single α helix. A second criterion which is commonly used in the characterization of coiled-coil complexes by CD is the ratio $[\theta]_{222nm}$ / $[\theta]_{208nm}$.[16,17] However $[\theta]_{208nm}$ is often perturbed in experiments with vesicles, due to light scattering in this wavelength region. Also, the ratio $[\theta]_{222nm}/[\theta]_{208nm}$ might lead to inconclusive results in systems with coexisting unordered peptide chains. However, the monitoring of peptide melting curves by means of CD spectroscopy was proven to allow for distinctive conclusions on the molecularity of peptide unfolding processes (*Chapter II*) and therefore poses a promising approach to distinguish coiled-coil formation from peptide membrane interaction.

An additional convenient method for the study of peptide and protein structures is Fourier transform infra-red (FTIR) spectroscopy. The position of the amide I band which mainly originates from the carbonyl stretching vibration of the peptide bond is known to strongly depend on its secondary structure.[18] In D_2O this band is usually referred to as amide I'. Coiled-coils have been shown to

exhibit characteristic amide I' bands with two main components, which are not observed for single helices.[19-23] This pattern is caused by the different accessibility of the amide carbonyls for the solvent. Coiled-coils are amphipathic helices, due to the arrangement of hydrophobic (h) and polar (*p*) amino acids in the so called heptad repeat: *hpphppp*. The positions in this pattern are denoted *abcdefg* (Chart 1). The carbonyls on the hydrophilic face, especially in the *b*, *c*, and *f* position of the heptad repeat can form additional hydrogen bonds with the solvent and absorb ~20 cm^{-1} lower than the amides on the hydrophobic face.[22,23] Surprisingly, a similar pattern was also found in amide I' bands of peptide K interacting with lipid monolayers using infrared reflection absorption spectroscopy (IRRAS, *Chapter III*). As an interaction of the peptide as the homomeric coiled-coil complex with the membrane seems unlikely, it was hypothesized that the pattern arises from a different accessibility of the carbonyls to water, caused by the shallow insertion of the single amphipathic A helix. This hypothesis allowed fitting of the angle dependency of the IRRA spectra, yielding the model of the helix insertion parallel to the membrane interface. Although a coherent bigger picture resulted from this approach, the origin of the two band pattern in the lipid bound form of K remains questionable, also due to sparse reports of comparable effects in literature.[24-27] Therefore, further investigations towards the IR spectroscopic properties of the membrane incorporated

Chart 1. Chemical structures of CPK and CPE lipopeptides used in this study and helical wheel profection of E/K coiled-coil complex.



state of K together with an unequivocal band assignment are necessary to substantiate the helix insertion model. The latter can be done by site specific labeling of the amide with ^{13}C, which is a convenient way to gain structural information for peptides at the residue level.[22,28-34]

In this chapter a combination of CD melting curves and temperature dependent IR spectra is applied to the coiled-coil forming peptides E, K and their lipid tethered equivalents. The lipid tethers in this study are made by cholesterol connected via a polyethylene glycol (PEG_{12}) spacer to the peptide moieties (lipopeptides CPE, CPK; Chart 1). It is shown that a detailed analysis by means of singular value decomposition (SVD)[35-37] of these temperature dependencies can shed light on the intrinsic temperature dependencies of the amide I' two band pattern and reveals structural properties of the peptides. Additional evidence is presented to support the model of the homo-coiling of peptide E on the membrane surface. Furthermore, the origin of the low absorbing amide I' component is determined by studying ^{13}C labeled peptide K.

RESULTS AND DISCUSSION

CD OF FREE PEPTIDES AND VESICLE TETHERED LIPOPEPTIDES

Melting curves of peptides, monitored by CD spectroscopy have been shown to contain relevant information about the molecularity of the peptide unfolding (*Chapter II*). The CD unfolding curves of untethered peptides E and K in the absence and presence of vesicles and tethered to the vesicle interface were measured (Figure 1). In these and all further experiments the lipid composition was DOPE : DOPE : Cholesterol (2 : 1 : 1), and the buffer was phosphate buffered saline (PBS, pH 7.4). It is shown in *Chapter VI* that the free peptides E and K exhibit shifts of the melting curves towards lower temperatures with decreasing peptide concentration, showing that the observed process is the unfolding of a homomeric coiled-coil complex.[38,39]

In a mixture with vesicles the $[\theta]_{222nm}$ of K was significantly lower over the complete temperature regime, i.e. the helical content was increased (Figure 1A). This is caused by the interaction of the peptide with the lipid bilayer which is accompanied with its folding into an amphipathic helix. Compared to the experiment without vesicles, the shape of the curve appears almost linear with a shallower, uniform slope. In peptide folding studies similar linear increases of folded coiled-coils are often considered as an intrinsic temperature induced change of the optical properties of the helix that does not relate to significant structural change.[40] Another possibility is that it is caused by non-cooperative changes such as end fraying of helices.[41,42]

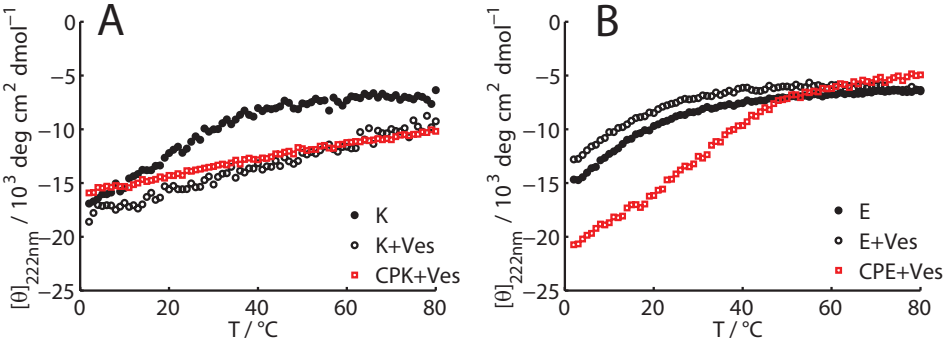


Figure 1. Temperature dependency of ellipticity at 222 nm for (A) K in buffer, K mixed with vesicles, and CPK tethered to vesicles, and for (B) E in buffer, E mixed with vesicles, and CPE tethered to vesicles. Experimental conditions: total peptide concentrations [E] = [K] = 40 μM; [CPE] = [CPK] = 20 μM; [lipid] : [peptide] = [lipid] : [lipopeptide] = 50 : 1. All measurements in PBS.

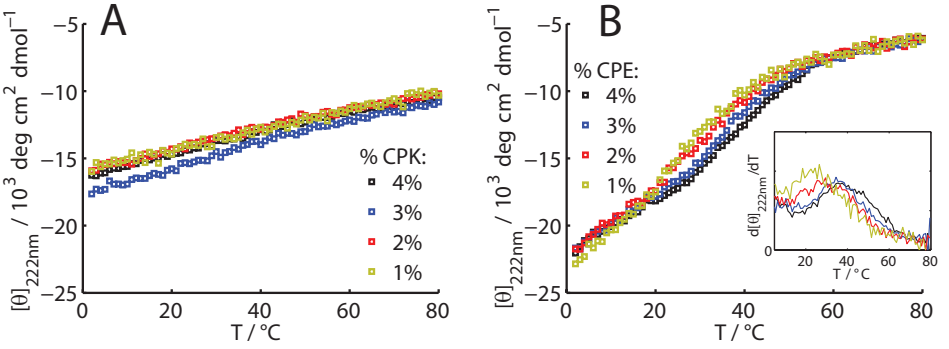


Figure 2. Temperature dependecy of ellipticity at 222nm for (A) CPK and (B) CPE tethered to lipid vesicles at varying molar fractions (total lipid concentration in all experiments 1 mM, in PBS).

In contrast, E in a mixture with vesicles shows a very similar $[\theta]_{222nm}$ curve to the lipid free solution (Figure 1B). The slight shift to higher values is attributed to an artifact caused by light scattering by the vesicles. Thus, the untethered E shows no interactions with the vesicles that induce the formation of a helix.

The membrane tethered CPK also shows decreased $[\theta]_{222nm}$ over the measured temperature range and closely resembles K mixed with vesicles indicating similarities in the structures and binding states of the peptides (Figure 1A). Based on $[\theta]_{222nm}$ at 25 °C the helicity was found to be 45% and 48% for K and CPK respectively. Assuming that all peptides are membrane bound this would imply that the peptides are only partially folded as a helix. However, from this data one cannot determine whether the observed helicity originates from a single state, populated by all peptide molecules or multiple states, differently populated.

CPE, in contrast, shows a $[\theta]_{222nm}$ curve that differs from its untethered equivalents (Figure 1B). A lower ellipticity was found that increases up to ~50 °C where the curve approaches those of the untethered peptides. This increase in helical content is generally attributed to the homo-coiling of the peptides (*Chapter IV*).[1-8] To scrutinize this, further experiments were performed by varying the lipopeptide concentration (Figure 2). CPK showed no significant changes in the temperature dependent $[\theta]_{222nm}$ with concentration change. In contrast, the $[\theta]_{222nm}$ curves of CPE shifted slightly to lower temperatures with decreasing concentration. The derivatives do not consist of a single peak indicating that the observed transition is not a simple two state unfolding. Consequently, fitting the data with several cooperative melting models with molecularities in the range $n = 2…5$ as described in *Chapter II* yielded insufficient results (data not shown). The maxima in the first derivative also illustrates the shift of the apparent melting temperature T_m (Figure 2B, inset). Therefore it shows that the unfolding of the peptide on the membrane is of a molecularity bigger than 1, meaning it is a coiled-coil complex.

The folded fraction (α) of peptide can be determined from the ellipticities at fully folded (θ_F) and fully unfolded (θ_u) states. Assuming values for a coiled coil with high helical content[15] of θ_F = 32,000 deg cm^2 dmol^{-1}, and θ_u = -5,000 deg cm^2 dmol^{-1} the value of $[\theta]_{222nm}$ at 25 °C of CPE 1 mol% suggests that more than 37% of the CPE molecules are folded as homo coils in a standard vesicle fusion experiment.

The melting of the untethered peptides E and K in buffer proved, that they are able to form homo coils at relatively low folding constants K_F compared to the E/K hetero coil (cf. *Chapter II* & *VI*). However, when tethered to the interface of the vesicles the local peptide concentration is drastically increased, leading to a dramatically increased T_m of CPE compared to the untethered E, despite the fact that the overall concentration is lower (Figure 1B). Using the experimental conditions of the melting curves in Figure 1B this effect can be illustrated: the mean molecular density in a 40 µM solution of untethered E is ~24000 molecules / µm^3, which is slightly less than the number of peptides that are confined to the lipid bilayer interface (~33000 molecules / µm^2) at 2% lipopeptide concentration and an average area of 60 Å^2 per lipid. Considering that one lipid leaflet including its direct aqueous periphery is only ~5 nm thick, it is clear that this corresponds to an immense agglomeration of molecules and thus to a high local concentration. This explains the substantial amount of homo coiling of CPE on the vesicles.

IR SPECTROSCOPY OF PEPTIDES IN SOLUTIONS

FTIR spectroscopy is a common technique to estimate the secondary structure of peptides and proteins, because the position of the amide I' band is strongly

influenced by its secondary structure and hydrogen bond formation. Also, the melting processes that were followed by CD spectroscopy are expected to manifest in this band.[33,37,43]

First, the IR spectra of E, K and EK in PBS prepared from D_2O (d-PBS) were measured at 5 °C. At a total peptide concentration of 1 mM these peptides are expected to predominantly exist in the coiled-coil state at low temperatures. Accordingly, the amide I' bands of the peptides are all dominated by two main components at ~1630 cm^{-1} and ~1649 cm^{-1}, which is a typical pattern for coiled-coils (Figure 3, Table 1). The origin of the additional small component at 1668 cm^{-1} is most probably residual trifluoroacetic acid (TFA) from the peptide purification. Additionally at 1564 cm^{-1} the carbonyl stretching mode of the glutamic acid side chains can be found.

The positions of the underlying components in the amide I' band were determined from the maxima in the smoothed second derivative and the shape of the bands could be adequately fitted with three Gaussians in these positions (Figure 3, Table 1). Unless otherwise stated all band fitting procedures yielded well separated peaks according to the 95% confidence intervals (Table 1 and Table 2). The lower relative absorbance ratios of the two main components at 1630 cm^{-1} and 1649 cm^{-1} (A_1 / A_2, Table 1) of E and K compared to E/K might arise from contributions of unordered structures. These are expected to overlap as a broad band centered at ~1645 cm^{-1} (see below) and cannot be resolved in this manner. The addition of more than three bands will also result in an adequate description of the band shape and underlying hidden contributions cannot be excluded. For instance Pähler et. al. used five strongly overlapping bands to describe the amide I' band of an E/K variant tethered to a supported lipid bilayer.[12] However these bands appear overfitted and the authors state no rationale for this model. Since the simpler pattern with two main bands reported here can be explained with a consistent physical model, this interpretation is used in the following.

The two main components of the amide I' band originate from the heterogeneous environment of the helix in the coiled coil.[19-23,26,44] The frequency of the amide I' band is known to shift down by ~20 cm^{-1} per hydrogen bond to the amide carbonyl. The carbonyls on the hydrophobic face of the coiled-coil form exclusively intra-molecular hydrogen bonds in the common i to i + 4 manner. On the other side, carbonyls on the hydrophilic face are available for one additional hydrogen bond from the solvent, which lowers their frequency.

To test this model, a variant of K was synthesized with the alanine residues situated in the b and c position of the heptad repeat containing ^{13}C labeled carbonyls (^{13}CK). The isotope effect is known to specifically lower the amide I' position of the

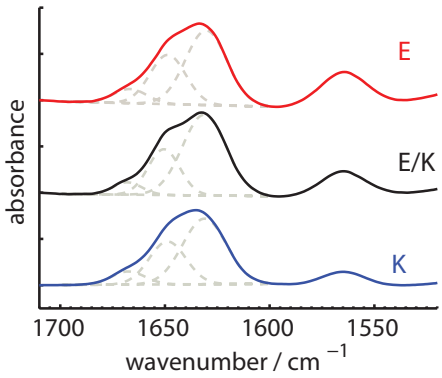


Figure 3. FTIR spectra of E, EK and, K at 5°C (solid lines) and fits of the amide I' bands with gaussians (broken lines) [peptide]= 1 mM, in d-PBS.

labeled residue by 35 - 40 cm^{-1}.[22,28-34] The IR spectra of ^{13}CK and E/^{13}CK at 5 °C showed strongly reduced intensities at 1630 cm^{-1} compared to their unlabeled equivalents K and E/K (Figure 4). Additionally a band at ~1592 cm^{-1} arised, the amide I' band of the ^{13}C labeled carbonyls. The amide I' bands of ^{13}CK and E/^{13}CK could be fitted with 4 Gaussian bands. The carbonyl band of glutamic acid was additionally included in the fit at 1564 cm^{-1}, to enhance the band shape modeling at low frequencies. The fit of E/^{13}CK yielded $A_1 / A_2 = 1.24$, which is smaller than the value for E/K of 2.45 showing that the absorbance at 1630 cm^{-1} is significantly reduced (Table 1, Figure 4B). It is

Table 1. Peak positions and absorbance ratio of peptides determined from band fitting.. Values in parenthesis are 95% confidence intervall with respect to the last two digits.

	$\tilde{\nu}_1$ (cm^{-1})	$\tilde{\nu}_2$ (cm^{-1})	$\tilde{\nu}_3$ (cm^{-1})	$\tilde{\nu}_{13C}$ (cm^{-1})	A_1/A_2[a]
E	1630.3 (4)	1648.6 (3)	1666.7 (11)	-	1.75
K	1631.1 (5)	1648.8 (4)	1667.6 (9)	-	1.82
E/K	1631.2 (2)	1650.5 (1)	1668.0 (5)	-	2.45
^{13}CK	1628.0[b]	1645.0 [b]	1666.4 (5)	1593.3 (1)	1.05
E/^{13}CK	1631.0 (11)	1649.5 (6)	1669.5 (7)	1592.6 (3)	1.24

[a] Absorbance ratio: $Abs(\tilde{\nu}_1)/ Abs(\tilde{\nu}_2)$, determined from area of fitted Gaussians.
[b]Value fixed during band fitting procedure.

Table 2. Peak positions and absorbance ratio of vesicle tethered lipopeptides and peptides mixed with vesicles determined from band fitting. Values in parenthesis are 95% confidence intervall with respect to the last two digits.

	$\tilde{\nu}_1$ (cm^{-1})	$\tilde{\nu}_2$ (cm^{-1})	$\tilde{\nu}_3$ (cm^{-1})	$\tilde{\nu}_{13C}$ (cm^{-1})	A_1/A_2[a]
E	1630.3 (4)	1648.6 (3)	1666.7 (11)	-	1.75
K	1631.1 (5)	1648.8 (4)	1667.6 (9)	-	1.82
E/K	1631.2 (2)	1650.5 (1)	1668.0 (5)	-	2.45
^{13}CK	1628.0[b]	1645.0 [b]	1666.4 (5)	1593.3 (1)	1.05
E/^{13}CK	1631.0 (11)	1649.5 (6)	1669.5 (7)	1592.6 (3)	1.24

[a] Absorbance ratio: $Abs(\tilde{\nu}_1)/ Abs(\tilde{\nu}_2)$, determined from area of fitted Gaussians.
[b]Value fixed during band fitting procedure.

expected that some absorbance remains at 1630 cm^{-1} because the amide I' bands of amino acids in the *f* position is expected at this wavenumber.[22] The amide I' band of the *e* and *g* positions have not been studied in detail yet. However for ^{13}CK no specific, narrow band for the remaining ^{12}C carbonyls on the hydrophilic face at ~1630 cm^{-1} could be resolved. Instead, band fitting yielded a relatively broad band at ~1628 cm^{-1}, which might be the result of an overlap of the expected hydrogen bound ^{12}C carbonyl and contributions from coexisting random structures. Nevertheless the reduction of the water exposed helix band in this position is obvious (Figure 4A).

The reduced absorbance shows that the 1630 cm^{-1} component consists mainly of contributions from the alanine residues, which are situated on the hydrophilic face of the amphipathic helix in the *b* and *c* positions of the heptad repeat. This is further strengthened by the position of the ^{13}C amide I' at ~1592 cm^{-1} the isotope shift is ~38 cm^{-1} which is in range of the typical values of 35-40 cm^{-1} reported for different model peptides independent of their secondary structure and hydrogen bonding.[22,28-34] Manas et. al. reported the position of ^{13}C-Ala in the *b* and *f* position of a GCN4-p1' leucine zipper with 1587 cm^{-1} whereas a ^{13}C-Leu in the d-position, i.e. the hydrophobic face of the same peptide absorbed at 1607 cm^{-1}.[22] Thus, the position found in the present study reflects the expectation for a water accessible helical ^{13}C amide I' band.

IR OF LIPOPEPTIDES

Next, the spectra of the lipopeptides CPE and CPK tethered to lipid vesicles were measured (Figure 5A). Just as for the untethered E and K, the amide I' bands were dominated by 2 main components. Under consideration of the strong absorbance of the lipid C=O stretching band at ~1740 cm^{-1} the amide I' bands were fitted by Gaussians yielding A_1/A_2 values of 1.52 and 1.03 for CPE and CPK respectively (Table 2). For CPE this result is in line with the assumption of homo coiling of the peptide on the vesicle interface. For CPK this band shape is similar to amide I' bands found in IRRA spectra from lipid monolayers containing the lipopeptide LPK (*Chapter III*).[13] Although the peptide moieties in these molecules are thought to interact as monomers with the lipid bilayers, they show the typical amide I' band shape of multimeric coiled-coils. However K is thought to insert as an amphipathic A helix into one leaflet of the lipid bilayer with its helical axis parallel to the membrane interface (*Chapter III* & *IV*). This would also result in a shielding of the hydrophobic face from water. The water density in DOPC bilayers is known to drop significantly below the lipid phosphate group.[45] This can explain the appearance of two amide I' components that can be attributed to solvent accessible and inaccessible carbonyls.

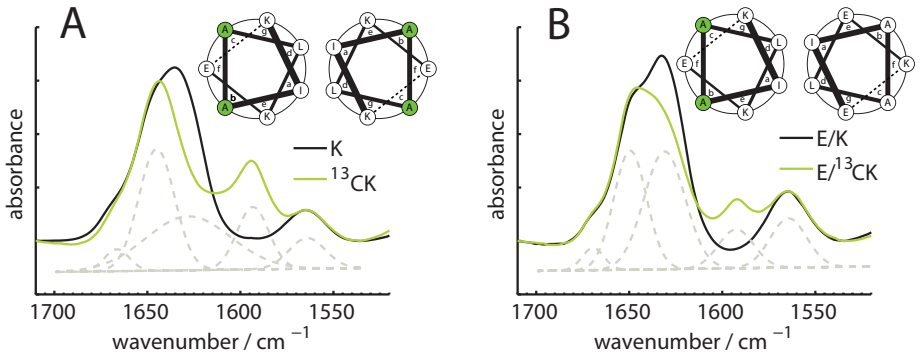


Figure 4. Normalized IR spectra (continuous lines) of (A) K, ^{13}CK, (B) E/K, and E/^{13}CK at 5°C and Gaussian fits of ^{13}C peptide spectra (broken lines). Spectra were normalized with respect to the C=O stretching band of glutamic acid at 1565 cm^{-1}. Fitted curves are offset for clarity. [peptide] = 1 mM, in d-PBS. Insets show helical wheel projections of dimeric coiled-coils with the position of the ^{13}C labeled alanine residues highlighted in green.

Although the assignment of water accessible and buried amide I' helical bands in coiled-coils is a well-studied phenomenon in solution,[19-23,31,37,44,46,47] the related effect in a lipid membrane is reported only scarcely by other groups. A variable two band pattern was reported by Bi et al. for S-palmitoylated N-terminal peptide of pulmonary surfactant peptide SP-C interacting with DPPC monolayers.[24] The authors interpreted a change of the relative band intensities with respect to each other as the expelling of helical fragments of the peptides from the monolayer, leading to a variation in hydration of the helix. Mukerjhee et. al. found amide I' bands with solvent accessible and buried components for alanine rich helical peptides, and amphipathic helices in reverse micelles that mimic membrane water interfaces.[26,27]

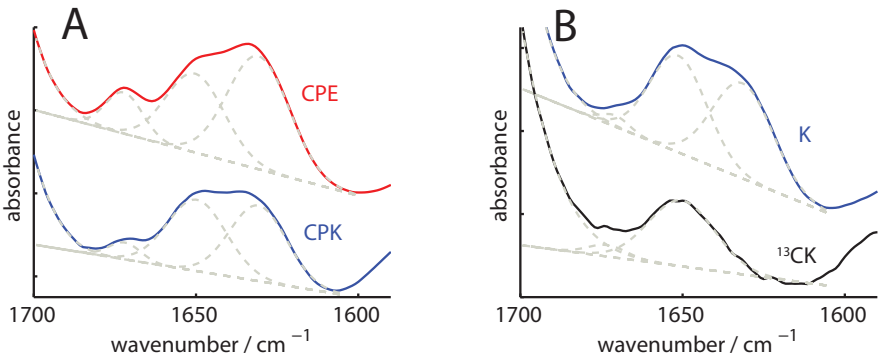


Figure 5. FTIR spectra (continuous lines) of vesicle tethered (A) CPE and CPK; (B) K at 5 °C and ^{13}CK at 10 °C mixed with vesicles at [lipid] : [peptide] = 200 : 1; [peptide] = 100 µM and fits of the amide I' bands with Gaussians (broken lines). All measurements in d-PBS.

To verify the assignment of the two main bands to water exposed and buried helices, the IR spectra of untethered peptides K and ^{13}CK when interacting with vesicles were measured. Due to the need of a relatively high [lipid] : [peptide] ratio (200 : 1) the peptide concentration is relatively low in these experiments and the spectra are markedly affected by residual H_2O below 1600 cm^{-1} and the strong lipid C=O band above 1700 cm^{-1}. While the influence of the lipid C=O could be modeled by fitting additional large Gaussian bands, contributions below 1600 cm^{-1} could hardly be resolved. Despite that, the distinct two band pattern described above can be found in the amide I' band of K in the membrane, with A_1/A_2 similar to CPK of 1.05 (Figure 5B, Table 2). Strikingly, the amide I' band of ^{13}CK in the membrane showed a strongly reduced absorbance at 1630 cm^{-1} and no contribution at this position was necessary to fit the band shape. Remaining bands in this position could not be resolved due to the relatively low peptide signal. The reduced absorbance at 1630 cm^{-1} implies a high structural similarity of the membrane bound and the homo-coiled state of K, in both helices the alanine amide carbonyls are accessible for hydrogen bonds from water. In the monomeric amphipathic A helix that was proposed as the membrane interacting species of K in *Chapter III*, the alanine residues are also expected to be situated on the hydrophilic face, which is in common with their ^{12}C amide I' absorbance at ~1630 cm^{-1}. The remaining peak, mainly centered at 1650 cm^{-1}, is also in common with remaining absorbance of mostly buried ^{12}C amides of the hydrophobic face.

The relatively low A_1 / A_2 ratios of CPK and K interacting with vesicles, compared to CPE or the homo coils of E, K and E/K might be caused by higher amounts of unordered structures (Table 1 and 2). This correlates well with the helicity values found by CD spectroscopy. However, it is difficult to distinguish if unstructured contributions are caused by unfolded domains within individual chains with all peptides being membrane bound or by the existence of different peptide populations, i.e. helical and unstructured. Certain arguments speak for the former. An unstructured form would be expected to be in equilibrium with a homomeric coiled coil and would result in concentration dependent temperature profiles. The absence of this concentration dependence (Figure 2A), therefore might be interpreted as the absence of different populations and supports the model of partially unfolded peptide chains. Short peptides tend to fray at their termini[34] and these ends might reach out of the membrane staying unfolded. In this perspective also the slight positive slope of the temperature dependent $[\theta]_{222nm}$ might indicate an increasing non-cooperative end fraying, leading to a less folded state at higher temperatures.[42]

TEMPERATURE DEPENDENCIES OF PEPTIDES

The temperature dependent unfolding of coiled-coils as measured by CD spectroscopy is also reflected in the temperature dependent amide I' bands. Deconvolution of the amide I' bands of the unfolded peptides E and K at 75 °C yielded broad single bands centered at ~1645 cm^{-1} with the TFA shoulder around ~1668 cm^{-1} (data not shown). Thus, the unfolded band overlaps strongly with the two helical bands. In order to clarify the changes in the amide I' band upon unfolding, difference spectra of the molar absorbance per amino acid residue ($\Delta\varepsilon = \varepsilon_T - \varepsilon_{5°C}$) are interpreted in the following (Figure 6 - Figure 9). These are also sometimes referred to as 'fingerprints' of a conformational change because they can be analyzed in terms of their magnitude, shape, and temperature dependence and classified according to their similarity.[48]

However, the straightforward interpretation of the difference spectra is hampered by the intrinsic temperature dependence of the amide I' band components and artefacts from residual H_2O in the case of low peptide signals (see below). For instance the temperature dependent difference amide I' bands of E/K clearly show no isosbestic point, which shows that more than one process affects the absorbance band (Figure 6A). The overlapping spectral changes can be separated by means of singular value decomposition (SVD) in combination with a global fitting routine.[35-37] For instance a band can show an intensity decrease, due to a reaction or a structural transition, at the same time this band can shift its position, due to changes in the strength of hydrogen bonds with temperature. These two spectral components overlap in the resulting difference spectra, both having their own temperature dependency. Under the assumption that the single spectral components have a temperature dependency of a sigmoidal shape, the different components that overlap in the spectrum can be distinguished. The results of this linear algebra procedure are the separated spectral components $D_1 \ldots D_n$ and their temperature dependencies $F_1 \ldots F_n$ (Figure 6B, D, F) which all together model the original temperature dependent difference spectra. The resulting *D* components can be interpreted to reveal molecular details of the temperature dependent processes. The *F* components yield the midpoints of the transitions $T_{m1} \ldots T_{m2}$ and their widths and describe the physics of the underlying process more precisely than measuring the absorbance at a single frequency.[37]

For the untethered peptides E, K and E/K one transition is assumed to be the unfolding of the coiled-coils, which is modeled in the F_1 component with the thermodynamic parameters ($\Delta H°, \Delta C_p, T°$) known from CD measurements (*Chapter VI*). The corresponding D_1 components of all peptides show a striking similarity with a strong and broad negative component centered at ~1630 cm^{-1} and a positive feature at 1660 cm^{-1} (Figure 6B, D, F). The corresponding F_1 thermal components are all ≥ 0,

which means that the unfolding of the coiled-coils is always accompanied by a vanishing of the band at 1630 cm^{-1}. In K and E/K the D_2 components are very similar with a positive feature at ~1624 cm^{-1} and negative intensity in the region 1640 cm^{-1}-1660 cm^{-1} (Figure 6B, D). Taking into account the negative values of F_2 over the whole temperature regime this corresponds to a blue shift of the water exposed band of the coiled-coil spectrum. The midpoint of this transition (T_{m2}) is below the melting temperature of the peptides which shows that the process influences the spectra less while the coiled coils melt. This blue shift is a known phenomenon for the water exposed amide I' band of coiled-coils. Manas et. al. reported that amide I' bands of water exposed carbonyls show stronger blue shifts at increasing temperature than buried ones, probably due to the weakening of the additional hydrogen bonds.[22]

The D_2 and F_2 components of E mainly overlap with its D_1 and F_1 components, i.e. in this case the SVD global fitting routine could not resolve additional information (Figure 6F). Due to the lower tendency of E to form homo coils (compare *Chapter VI*) there is less influence of the blue shift of the 1630 cm^{-1} band on the difference spectra.

While the water accessible amide I' band vanishes upon melting, the influence of melting on the solvent buried amide I' band is found in the difference spectra of ^{13}CK (Figure 7). The D_1 component indicates a broadening of the band at ~1650 cm^{-1}, resulting from the rising of the band of unstructured peptide chains. The negative feature at 1590 cm^{-1} corresponds to the disappearing of the water exposed ^{13}C amide I' due to melting and is accordingly observed only in difference spectra ^{13}CK and E/^{13}CK (Figure 7, Figure A1). The temperature dependent difference spectra of E/^{13}CK were found to be rather complex due to the multitude of bands. Thus it is not discussed in detail; however these spectra did not contradict the results described before (Figure A1).

Taken together the analysis of the temperature dependent IR difference spectra of E, K, ^{13}CK and E/K revealed that the thermal unfolding of the coiled-coils can be retrieved in these spectra by a specific 'fingerprint' difference spectrum, which is dominated by a strong absorbance decrease at 1630 cm^{-1} and an increase at 1650 cm^{-1}. This overlaps with a more subtle shift of the water accessible helical band of the coiled-coil at 1630 cm^{-1} to higher frequencies, which occurs at temperatures below the cooperative melt of the complex.

Strikingly, the thermal difference spectra of membrane tethered CPE are also dominated by this pattern as can be seen in its D_1 component (Figure 8A-B). The T_{m1} of the corresponding sigmoidal F_1 component is at 38 °C, which lies well in the range of T_m found in the CD unfolding curves (Figure 2). Furthermore, the D_2 component indicates a slight blue shift of the 1630 cm^{-1} band as was found for the untethered coiled-coils. Thus this data strongly suggests that membrane tethered E is in a homomeric coiled-coil state, which unfolds upon heating (Figure 10A).

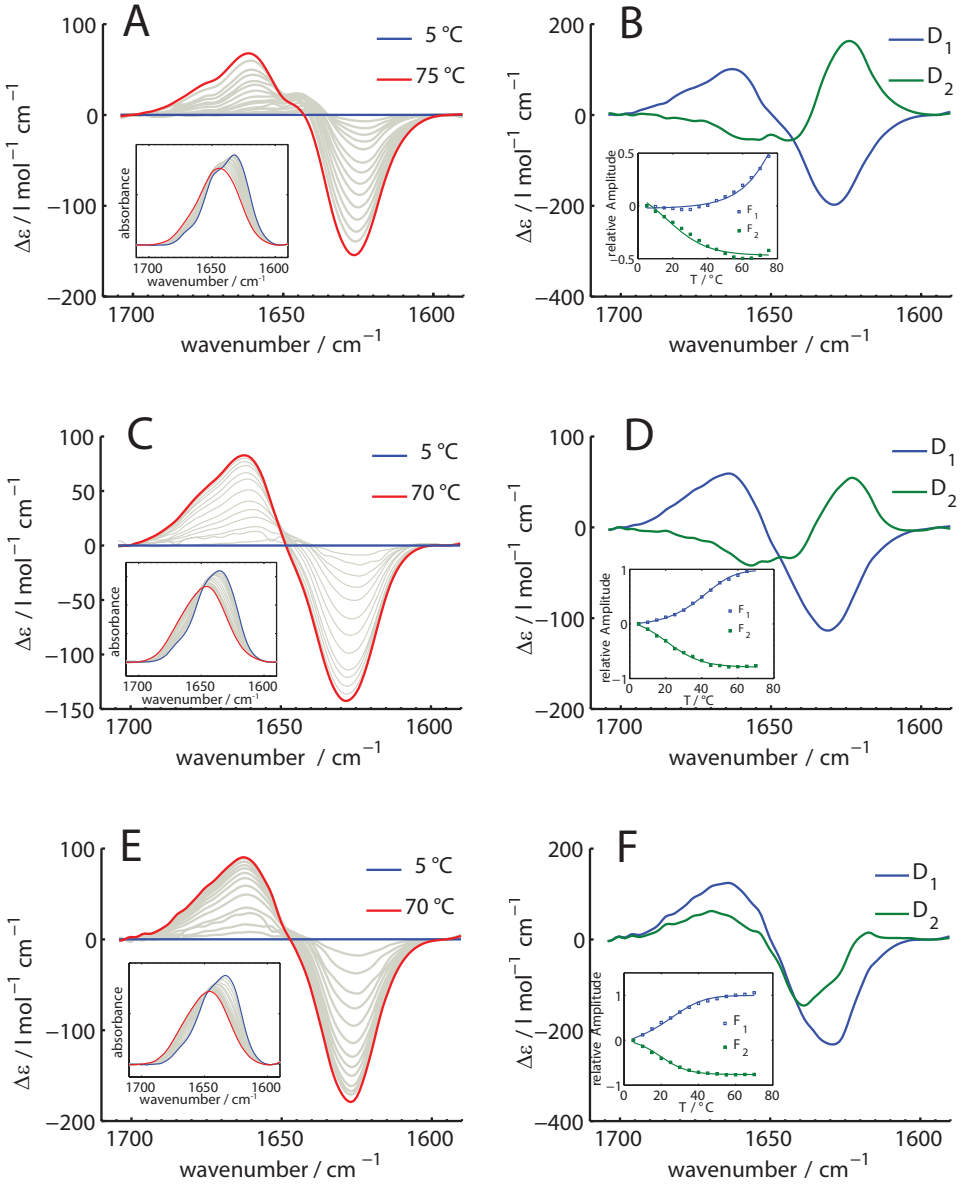


Figure 6. Temperature dependency and singular value decomposition of amide I' bands for (A-B) E/K T = 5 - 75°C, (C-D) K T = 5 - 70°C, and (E-F) E T = 5 - 70°C (A, C, E) Temperature dependent difference spectra in the amide I' region with amide I' bands in the insets in 5 °C steps (B, D, F) Results of singular value decomposition D_1 and D_2 spectral components and F_1, F_2 temperature profiles (squares) and fit results (lines) in the insets; [peptide] = 1 mM, in d-PBS.

Conversely, the difference spectra of the membrane tethered CPK and K mixed with vesicles show both D_1 components with a broad absorbance increase at ~1650 cm^{-1} but only a small decrease at 1623 cm^{-1} (Figure 8C-D, Figure 9). These spectral components

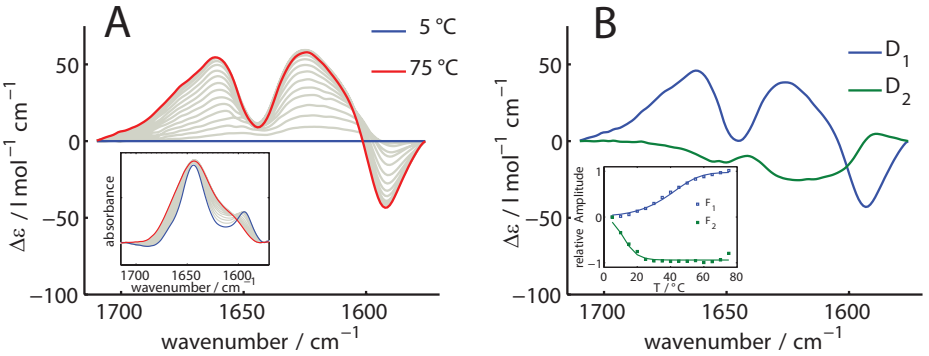


Figure 7. (A) Temperature dependent difference spectra in the amide I' region with amide I' bands in the insets in 5 °C steps of ^{13}CK in d-PBS; (B) Results of singular value decomposition D_1 and D_2 spectral components and F_1, F_2 temperature profiles (squares) and fit results (lines) in the insets, T = 5 - 75°C.

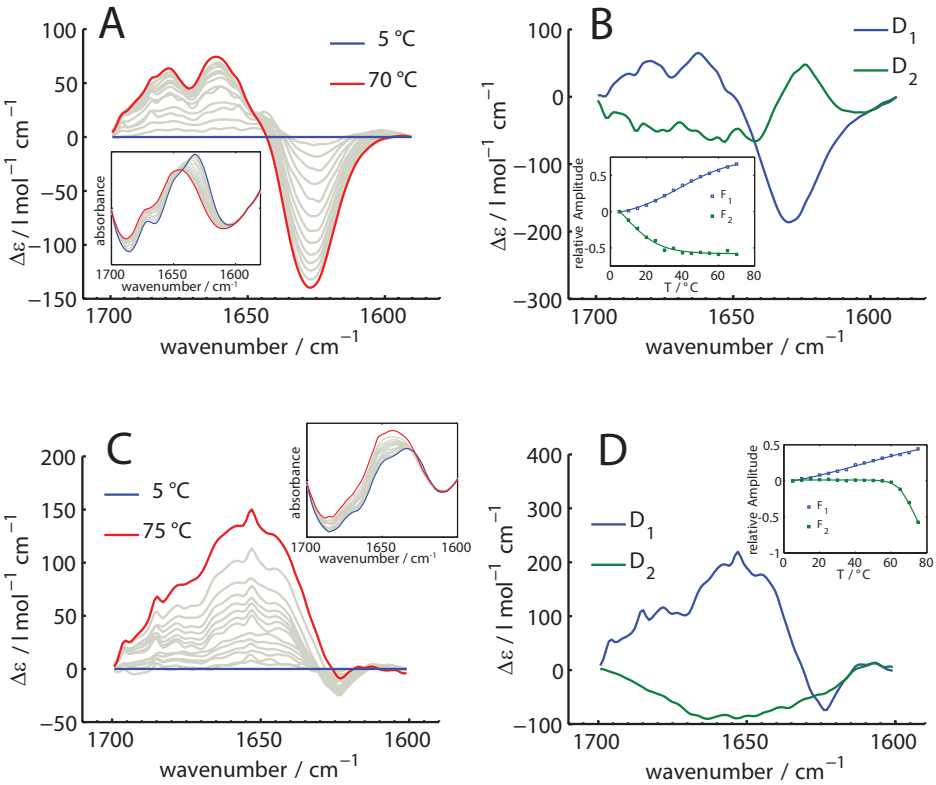


Figure 8. Temperature dependency and singular value decomposition of amide I' bands for lipopeptides tethered to vesicles in d-PBS (A, B) CPE 1 mol% T = 5 - 70°C and (C, D) CPK T = 5 - 75°C; (A, C) Temperature dependent difference spectra in the amide I' region with amide I' bands in the insets in 5 °C steps (B, D) Results of singular value decomposition D_1 and D_2 spectral components and F_1, F_2 temperature profiles (squares) and fit results (lines).

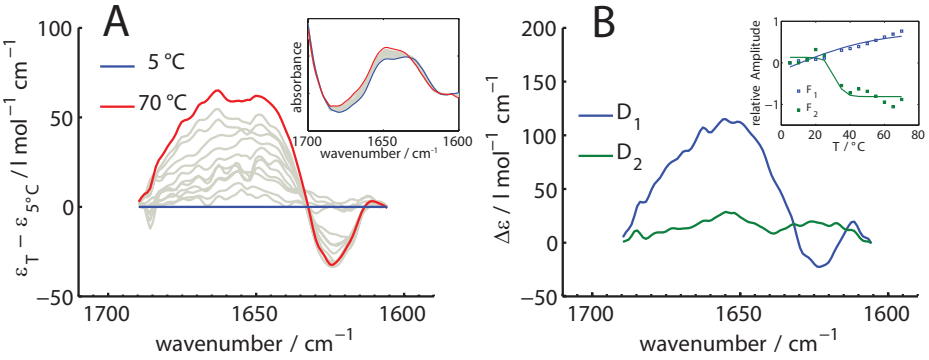


Figure 9. (A) Temperature dependent difference spectra in the amide I' region with amide I' bands in the insets in 5 °C steps of K mixed with vesicles; (B) Results of singular value decomposition D_1 and D_2 spectral components and F_1, F_2 temperature profiles (squares) and fit results (lines) in the insets ([K] = 100 µM; [Lipid]:[K] = 200 : 1, in d-PBS) T = 5 - 70°C.

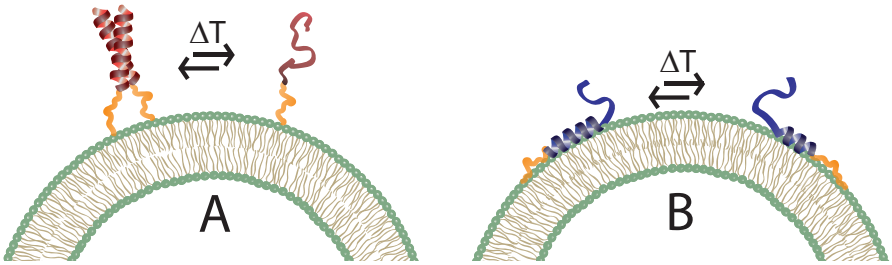


Figure 10. Schematic drawing of temperature dependent changes of peptide states when tethered to membranes. (A) CPE is predominantly folded in an E/E coiled coil which unfolds and dissociates upon heating. (B) CPK is predominantly bound to the membrane in a helical state and stays bound upon heating.

differ substantially from the D_1 component of CPE and therefore indicate a different transition. The increase at 1650 cm^{-1} was also apparent in the D_1 component of ^{13}CK mixed with vesicles (Figure A2). The decrease at 1623 cm^{-1} appears to arise from the temperature dependent blue shift of the water exposed band. It has to be noted that the components found in the D_2 component of these measurements appear to be artefacts arising from variations in the residual H_2O concentration in the sample. Band fitting of the spectra at 75 °C revealed that these amide I' bands still consist of two main components of a similar area at 1635 cm^{-1} and 1652 cm^{-1} (Figure A3). Interestingly, the corresponding F_1 components are relatively shallow and almost linear, indicating that the observed D_1 spectral component does not relate to a cooperative transition, which is in line with the almost linear shapes of the $[\theta]_{222nm}$ curves of CPK and K mixed with vesicles (Figure 1).

This discussed data show that membrane bound K exhibits a fundamental different temperature dependency from an unfolding coiled-coil. Despite the expected blue shift of the water accessible band, the amide I' retains its two main components up to 75 °C. Thus the peptide does not unfold cooperatively and stays bound to the membrane even

at temperatures as high as 75 °C (Figure 10B). However the intermediate helical content, decreasing with temperature found by CD (Figure 1 and Figure 2) and the relatively low absorbance ratio A_1 / A_2 (Table 2) indicate a certain contribution of unordered structure in the peptide chains which might, upon heating, increase in a non-cooperative way.

CONCLUSIONS

The temperature dependent unfolding of the coiled-coil forming peptides E and K in solution and tethered to lipid vesicles could be followed by CD and difference IR spectroscopy and yielded comparable temperature profiles. Membrane tethered CPE showed concentration dependent temperature profiles in CD, typical for an unfolding transition and the IR spectral changes exhibit the characteristics of coiled-coil unfolding. In contrast, CPK on membranes exhibits no transition, remaining in its membrane bound state up to 75 °C. The membrane bound CPK also exhibits a considerable amount of unordered structures, which indicate a partially folded helix in the membrane bound state.

The IR spectra of membrane tethered homo-coiled CPE and membrane incorporated, monomeric CPK show remarkable similarities, despite their different states. This is caused by similar accessibilities of the amide carbonyls for water in the coiled-coil and the amphipathic A helix which are both partially hydrophobically buried. These results contribute further to the model of lipopeptide mediated fusion as they provide additional support for the hypothesis of the asymmetric process. The two recognizing units behave fundamentally differently in the prefusion state. These results show, that the special conditions at the membrane interface have different influence on the state of membrane tethered peptides, depending on their individual properties. This influence is difficult to predict and has to be studied for each peptide individually.

It was proposed earlier (*Chapter III*) that the interaction of K with the membrane induces curvature which helps overcoming the highly curved intermediate states of lipid reorganistion during fusion. Thus, a specific manipulation of the membrane affinity of K appears to be a promising handle to influence the fusion mechanism.

EXPERIMENTAL SECTION

MATERIALS

Fmoc-protected amino acids and Sieber amide resin for peptide synthesis were purchased from Novabiochem, Fmoc-protected (1-^{13}C, 99%) L-alanine was purchased from Cambridge Isotope Laboratories. DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), and cholesterol were purchased from Avanti Polar Lipids. Solvents, buffer salts, D_2O and DCl (deuterium content ≥ 99.9%) were purchased from Sigma-Aldrich. All

water was ultrapure with resistance ≥ 18 MΩ cm^{-1} and TOC ≤ 2 ppm produced from a MilliQ Reference A+ purification system. All experiments were carried out in phosphate buffered saline prepared in water (PBS) or D_2O (d-PBS) of the composition: 150 mM NaCl, 20 mM PO_4^{3-} at pH/pD 7.4.[49]

PEPTIDE SYNTHESIS

The peptides E: Ac-$(EIAALEK)_3$-NH_2, K: Ac-$(KIAALKE)_3$-NH_2, and ^{13}CK with the same sequence as K and a ^{13}C amide labelled alanine residue were synthesized using standard Fmoc-chemistry on a Biotage Syro I and purified by RP-HPLC to yield a purity > 95% based on HPLC. Identity of the peptides was determined by LC-MS. The lipopeptides were synthesized and purified as described elsewhere.[3,6] Peptides were solved in 10 mM HCl and lyophilized three times to remove TFA.[50] Peptide stock solutions in d-PBS were prepared at ~2 mg/ml and diluted accordingly for the measurements. Lipopeptide stock solutions were prepared in a $CHCl_3$: MeOH; 3 : 1 solution and added to the lipids prior to solvent evaporation.

VESICLE PREPARATION

Lipid stock solutions of the composition DOPE : DOPE : Cholesterol (2 : 1 : 1) were prepared in $CHCl_3$: MeOH 3 : 1. For experiments with lipopeptides, lipid stock solutions were mixed with CPK and CPE stock solutions to yield mixtures with the desired molar ratio. Lipid films were created by slow evaporation of the solvents under N_2 stream and kept under vacuum overnight. The films were rehydrated with PBS or d-PBS yielding final lipid concentrations of typically 1 - 2 mM for CD or 20 mM for IR measurements. For measurements with untethered peptides, the lipid films were directly hydrated with solutions of the peptides. Large unilamellar vesicles (LUVs) were formed by sonication at 55 °C for ~15 min. The size of the vesicles was tested by DLS using a Malvern Zetasizer nano-s and was typically found to be ~100 nm.

CIRCULAR DICHROISM SPECTROSCOPY

Thermal unfolding of peptides and lipid tethered peptides were measured as described in *Chapters II* & *IV*. The mean residual ellipticity *[θ]* and the relative helicity *rh* were calculated as described in the aforementioned chapters. The fraction of folded peptide α is calculated, using the ellipticity when all molecules are folded or unfolded (θ_F, θ_u) by:

$$\alpha = \frac{[\theta] - \theta_U}{\theta_F - \theta_U} \quad (1)$$

TRANSMISSION FT–IR SPECTROSCOPY

Transmission FT–IR spectra were measured using a Bio-Rad Excalibur spectrometer equipped with a nitrogen cooled MCT detector. A temperature controlled liquid transmission cell with CaF_2 windows and a fixed nominal path length of 50 mm was used. The precise pathlength (d) was determined by the interference fringe method. Sample spectra in d-PBS and reference spectra of dPBS at 25 °C were measured at a resolution of 2 cm^{-1} with a zero filling factor of 1. Spectra were recorded between 5 and 75 °C in steps of 5 °C. The temperature of the cell was measured and kept constant during measurement at ± 0.2 °C. For each spectrum 128 scans were averaged. Several spectra were averaged and corrected by manual subtraction of a water vapor spectrum. The molar absorptivity per residue (ε) was calculated from the absorbance (A), the peptide concentration (c) and the number of amino acid residues per peptide chain according to Beer-Lambert law:

$$\varepsilon = \frac{A}{ncd}. \tag{2}$$

For band fitting[51] of the amide I' the second derivative of the spectra were smoothed for determination of the position of underlying bands. The positions found were used as input for fitting of the band shape with Gaussian peaks on a linear baseline by means of a trust-region-reflective algorithm.

SINGULAR VALUE DECOMPOSITION AND GLOBAL FITTING

For analysis of difference spectra from baseline corrected molar absorptivity spectra (ε_T - $\varepsilon_{5°C}$) singular value decomposition (SVD) in combination with global curve fitting was applied.[35-37] The data matrix $\mathrm{A}(\tilde{\nu}, T)$ was created by ordering the difference spectra in such a way that each column corresponds to a temperature. SVD is applied to the data matrix (MatLab function: *svd*) yielding three matrices U, S, and V^T:

$$\mathrm{A}(\tilde{\nu}, T) = USV^T, \tag{3}$$

corresponding to the basis spectra (U), the singular values (S), and the transposed of the temperature development of the basis spectra (V^T). From these matrices components above a rank (r) of 2 were omitted as they mainly contained noise. To describe the data matrix based on overlapping physical transitions it is assumed that these matrices can be described by a matrix D containing the spectral components of the overlapping components and F^T containing their temperature dependencies:

$$USV^T = DF^T. \tag{4}$$

Multiplication with the pseudo inverse of F^T (F^{T+}) yields:

$$D = USH \tag{5}$$

with

$$H = V^T F^{T+}. \tag{6}$$

This means the matrix H contains the coefficients determining how the weighted basis spectra (US) must be mixed to yield the spectral component matrix D and these coefficients can be obtained by globally fitting V^T with physical models for each spectral component. The model used for measurements of lipopeptides or peptides with vesicles consisted of two sigmoid functions:

$$f(T)_n = h_{n1}\left(b_1 + \frac{m_1 - b_1}{1 + \exp\left(\frac{T_{m_1} - T}{\delta_1}\right)}\right) + h_{n2}\left(b_2 + \frac{m_2 - b_2}{1 + \exp\left(\frac{T_{m_2} - T}{\delta_2}\right)}\right). \tag{7}$$

In this equation subscript n refers to the n^{th} row of the matrix V^T; h_{n1} and h_{n2} are the corresponding elements of H, while b, m, T_m, and δ are the minimum value, maximum value, midpoint, and width of the two sigmoidal transitions. For fitting a trust-region-reflective algorithm (MatLab function: *lsqcurvefit*) was used, leaving the parameters b, m, T_m, and δ global.

For melting of the coiled-coil peptides E, K, and E/K one transition was assumed to be the thermal peptide unfolding. The parameters $\Delta H°$ and $T°$, the enthalpy and the temperature where the folding constant $K_F = 1$ and ΔC_p, the change in heat capacity upon folding as determined by CD spectroscopy in *Chapter VI* were used and set constant during global fitting. In the applied model, the first sigmoid function was replaced by the temperature dependent fraction of folded peptide $\alpha(T)$:

$$f(T)_n = h_{n1}(\alpha(T)(m_1 - b_1) + b_1) + h_{n2}\left(b_2 + \frac{m_2 - b_2}{1 + \exp\left(\frac{T_{m_2} - T}{\delta_2}\right)}\right). \tag{8}$$

With the unfolded fraction ($\beta = 1 - \alpha$), $\alpha(T)$ was calculated by using the analytical solutions of:

$$a\beta^2 + \beta - 1 = 0, \tag{9}$$

as described in detail in *Chapter II*.
The F matrix was determined from:

$$F^T = H^{-1}V^T. \tag{10}$$

All calculations were performed using MatLab 2013a equipped with curve fitting toolbox.

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APPENDIX

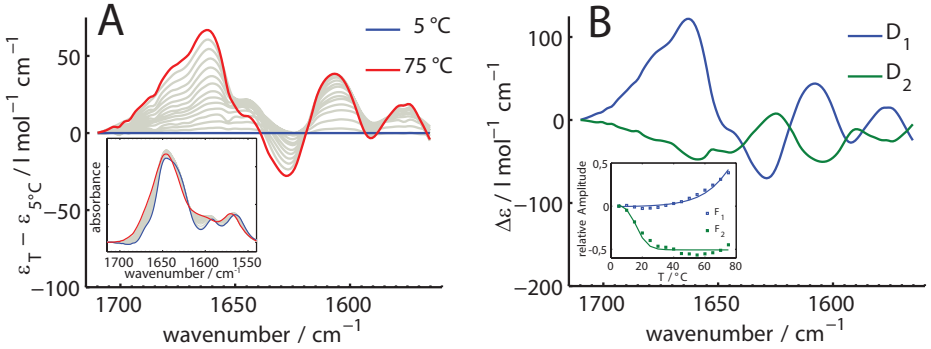

Figure A1. (A) Temperature dependent difference spectra of E/^{13}CK in d-PBS (T = 5 - 75°C) in the amide I' region with amide I' bands in the insets in 5 °C steps; (B) Results of singular value decomposition D_1 and D_2 spectral components and F_1, F_2 temperature profiles (squares) and fit results (lines) in the inset.

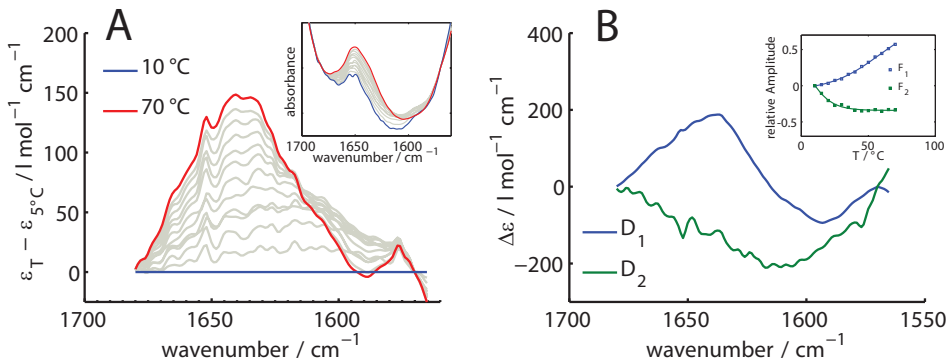

Figure A2. (A) Temperature dependent difference spectra in the amide I' region with amide I' bands in the insets in 5 °C steps; (B) Results of singular value decomposition D_1 and D_2 spectral components and F_1, F_2 temperature profiles (squares) and fit results (lines) in the inset of ^{13}CK mixed with vesicles in d-PBS ([13CK] = 100 µM; [Lipid] : [K] = 200:1) T = 10 - 70°C.

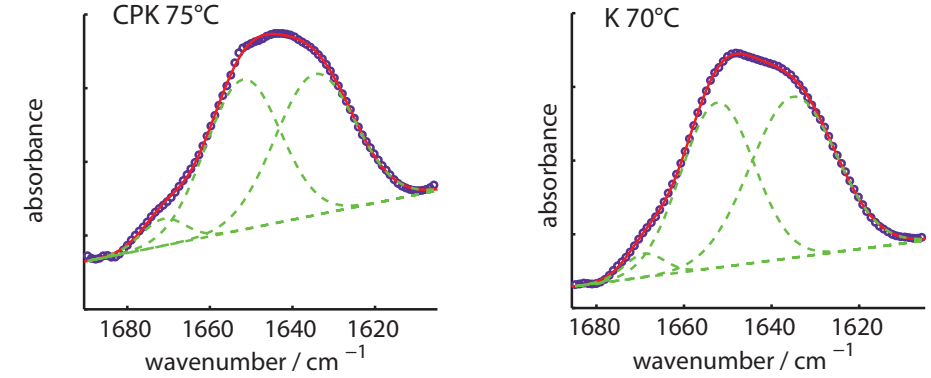

Figure A3. Amide I' bands (circles) of vesicle tethered CPK in d-PBS at 75 °C and K mixed with vesicles in d-PBS at 70 °C; fitted Gaussian bands (broken lines) and resulting band shape (continuous line).