X-ray diffraction study of feline leukemia virus fusion peptide and lipid polymorphism

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Abstract **The structural effects of the fusion peptide of feline leukemia virus (FeLV) on the lipid polymorphism of *N*-methylated dioleoylphosphatidylethanolamine were studied using a temperature ramp with sequential X-ray diffraction. This peptide, the hydrophobic amino-terminus of p15E, has been proven to be fusogenic and to promote the formation of highly curved, intermediate structures on the lamellar liquid-crystal to inverse hexagonal phase transition pathway. The FeLV peptide produces marked effects on the thermotropic mesomorphic behaviour of MeDOPE, a phospholipid with an intermediate spontaneous radius of curvature. The peptide is shown to reduce the lamellar repeat distance of the membrane prior to the onset of an inverted cubic phase. This suggests that membrane thinning may play a role in peptide-induced membrane fusion and strengthens the link between the fusion pathway and inverted cubic phase formation. The results of this study are interpreted in relation to models of the membrane fusion mechanism.**

Key words: Membrane thinning; Cubic phase; Hexagonal phase

1. Introduction

Biomembrane fusion is a ubiquitous process that plays a crucial role in such fundamental events as spermatozoid-egg fusion and mitosis. Despite this fact, the molecular rearrangements of the lipids and the precise kinetic events involved are still unclear. This is largely because the fusion event is transient and involves only local, isolated patches of lipid. Biomembrane fusion is a protein-regulated event [1,2]. Enveloped virus particles use membrane fusion in order to introduce their infective nuclear material into a host cell. The most widely studied membrane 'fusion protein' is the hemagglutinin of the influenza A virus [2]. This virus, like all animal enveloped viruses, utilises a specialised, extra-membranous glycoprotein 'spike' as a fusion catalyst.

Although the initial triggers of the fusion event show great diversity, the actual macromolecular rearrangements of the membrane are thought to be similar for many fusing systems [3]. The multi-step fusion process involves the merging of two distinct, planar bilayers to form highly curved fusion intermediates [4] and there has been much evidence to support this hypothesis [5–10]. The formation of highly curved lipid mesomorphs also occurs during the lamellar liquid-crystal/inverted cubic (L_α/Q_{II}) phase transition and the lamellar liquid-crystal/inverse hexagonal (L_α/H_{II}) phase transition [11]. The ability of a number of agents to promote fusion appears to be correlated to their ability to lower the L_α/H_{II} transition temperature (T_H) [12–14]. Similarly, some fusion inhibitors raise T_H [15]. Although the Q_{II} and the H_{II} phases, which are kinetically stable, are unlikely to exist at the site of a developing fusion pore, knowledge about the topology of the interface as these phases begin to form may have implications in biological fusion pathways.

The exact structures of the intermediates involved in the L_α/Q_{II} and L_α/H_{II} phase transitions have not been described uniquely [16–19]. Experimental data [20] and theoretical free energy calculations of intermediate lipid structures [21] suggest that the most likely pathway is via the formation of a low energy stalk-like structure [22], then 'hemifusion' intermediates (also known as transmonolayer contacts, TMCs). A recent study showed that intermembrane connections could appear some 22°C below the T_H [23]. The intermembrane connections evolve to form isolated TMCs, then aggregated TMCs, which are a basis for H_{II} phase growth via the formation of quasi-hexagonal phase domains [23]. The integrity of the TMC structure is the committing step in membrane fusion. Ruptured TMCs form interlamellar attachments (ILAs) or fusion pores. Many ILAs in close proximity may nucleate to form Q_{II} phases. There is evidence to suggest that specialised fusion proteins also catalyse the formation of similar lipid intermediates [3,24–26]. Therefore, studying the structural effects of fusion peptides in the proximity of inverted phase boundaries provides instructive insight into the fusion mechanism.

In this paper, we report the effect of the fusion peptide from feline leukemia virus (FeLV) on the thermotropic lipid phase behaviour of multi-lamellar vesicles (MLVs) composed of *N*-methylated dioleoylphosphatidylethanolamine (MeDOPE). The fusion peptide of FeLV is the hydrophobic N-terminus of p15E, itself part of the viral envelope glycoprotein spike gp85. Sequential small angle X-ray scattering (SAXS) images were recorded from pure lipid dispersions and lipid dispersions containing different amounts of FeLV peptide as the temperature was progressively increased. We interpret the findings in relation to biomembrane fusion.

2. Materials and methods

FeLV fusion peptide was synthesised by Albachem (Edinburgh, UK) to the sequence EPISLTVALMLGGLTVGGIAAGVGTGTK, as used in our previous studies (e.g. [27]). MeDOPE was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. A stock solution of FeLV peptide (1.0 mg/ml) in chloroform:methanol (2:1) was prepared by thorough vortexing and ultrasonication. An ice/water mixture ensured that sonication

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did not heat the solution above room temperature. The appropriate amount of stock solution was dispensed to a pre-weighed amount of MeDOPE in a Pyrex test-tube. The dissolved lipid and peptide were then dried under a stream of oxygen-free nitrogen. The resultant lipid films were placed under vacuum overnight in the presence of phosphorous pentoxide (P_2O_5) to remove trace amounts of solvent. Each film was reconstituted in buffer (20 mM PIPES, 1 mM EDTA, 150 mM sodium chloride and 0.002% sodium azide at pH 7.4) and vortexed thoroughly for 10 min at room temperature. All samples had a low lipid concentration of 100 mM, equivalent to 7.57% (w/v), thereby ensuring an excess water condition. The lipid dispersions were then subjected to five freeze-thaw cycles as previously described [27] to ensure that the lipid was in the fully hydrated L_α phase regardless of the thermal history of the lipid.

X-ray diffraction measurements were performed at station 2.1 of the Synchrotron Radiation Source at Daresbury Laboratory, UK. The X-ray wavelength was 1.54 Å, the specimen to detector distance was 1.5 m. A teflon-lined brass chamber with mica windows and thermocouple was used as a sample holder and a circulating water bath controlled the sample temperature. The temperature increased linearly at a rate of 30 K/h, while data were collected continuously, at a rate of two frames per minute. Geometric and detector response corrections were applied using the XOTOKO program. Angular calibration was achieved using rat-tail collagen as a standard [28] immediately prior to the exposure of each new liposomal sample. The exact location and intensity of Bragg reflection were determined by Gaussian fitting, using PeakFit v4 software (Jandel Scientific, Chicago, IL, USA).

3. Results

3.1. MeDOPE control

This type of experiment may be run as a series of temperature jumps, each one followed by a pause whilst the sample equilibrates to the new temperature before a single frame of data is obtained. Whilst this approach ensures equilibrium across the sample, information is lost during heating. Therefore, the approach chosen for these measurements was a continuous temperature scan, with consecutive frames of x-ray data recording the whole transition process. In this way, it is unlikely that the sample ever reaches equilibrium, but the chance of observing intermediate structures is maximised. This is only possible with bright synchrotron X-ray sources. MeDOPE was chosen for this study because its T_H of approximately 60°C is experimentally convenient, allowing for a long temperature ramp from ambient conditions. MeDOPE is classed as a 'non-bilayer lipid' [29]. Such lipids are thought to play a role in biological membrane fusion. In addition, MeDOPE has an intermediate spontaneous radius of curvature (R_o) value [8] and has been shown to form Q_{II} phases when treated to specific thermal protocols [8,30,31]. MeDOPE has an intermediate R_o value, so this lipid has a tendency to form structures of intermediate curvature [8].

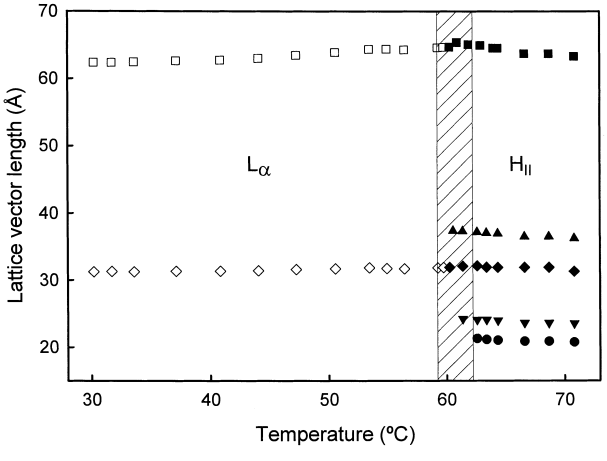

Fig. 1. Lattice basis vector length versus temperature for the L_α and H_{II} phases of MeDOPE, as determined by TRXRD performed at station 2.1 of the Synchrotron Radiation Source at Daresbury Laboratory, UK. Key: □ $d/1$, ⬡ $d/\sqrt{2}$, △ $d/\sqrt{3}$, ◇ $d/2$, ▽ $d/\sqrt{7}$, ○ $d/\sqrt{9}$; L_α phase in hollow symbols, H_{II} phase in black. In the shaded area, the two phases co-exist; the reflections in this region contain contributions from both L_α and H_{II} phases.

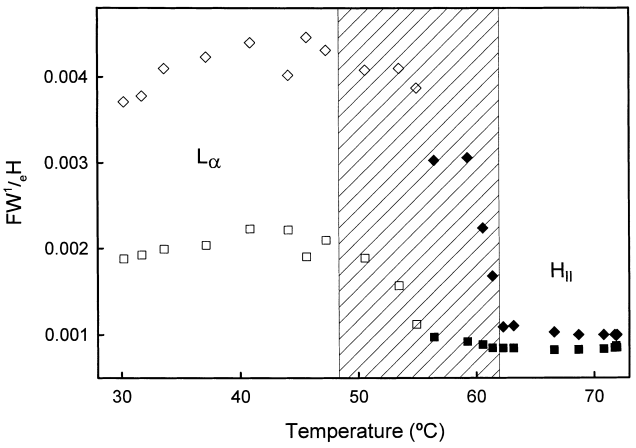

Fig. 2. Variation in sample order as a function of temperature from TRXRD results. Sample order is expressed as the full-width at $1/e$ height ($e = 2.73$) of a Gaussian distribution fitted to each Bragg peak. Symbols as in Fig. 1.

In the X-ray data, two sharp orders of diffraction were observed at 31°C, which indexed onto a lamellar lattice (reciprocal lattice ratio 0:1:2) when measured from the position of the zeroth order. The narrow peak widths and low incoherent scattering inferred that most of the sample mass was in a well-ordered lattice. The lamellar repeat distance (d_L) of MeDOPE at 31°C was 62 Å, which is in agreement with Gruner et al. [8]. Fig. 1 shows the lattice basis vector lengths of the L_α and H_{II} phase as a function of temperature. d_L increases steadily with temperature up to 54°C. H_{II} phase onset is observed at approximately 60°C in agreement with the literature. Although both Q_{II} and H_{II} phases of MeDOPE can co-exist near T_H [31], no Q_{II} phase reflections were observed at our relatively rapid temperature scan rate, as predicted. The exact T_H is difficult to ascertain because the $d/1$ and $d/2$ reflections of both H_{II} and L_α overlie each other with MeDOPE, making differentiation between the two phases difficult. The first reflection unique to H_{II} phase ($d/\sqrt{3}$) was observed at approximately 60°C. The co-existence of L_α and H_{II} phases near the T_H has been reported previously [32]. Above 62°C, some five H_{II} reflections were observed, with the reciprocal ratio of $0:1:\sqrt{3}:\sqrt{4}:\sqrt{7}:\sqrt{9}$.

The H_{II} lattice basis vector length of MeDOPE at 60°C was 65 Å. Multiplying this value by the factor $2/\sqrt{3}$ yields d_H, the distance from the centre of one H_{II} cylinder to the next. The calculated d_H was 75 Å, which is comparable with Gruner's value. d_H steadily decreased to 73 Å at 71°C. This occurs because the hydrocarbon chain splay is wider at higher temperatures.

Fig. 2 shows the full-width at $1/e$ ($e = 2.718$) height (FW1/eH) values for the first and second order reflections. This parameter is related to the sample order. The width of the

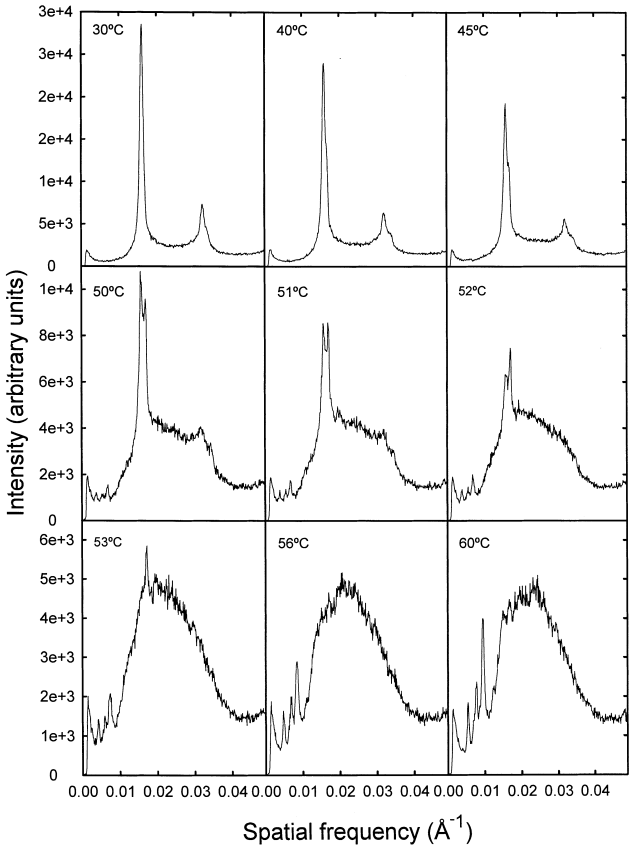

Fig. 3. X-ray diffraction profiles of MLVs composed of MeDOPE plus FeLV fusion peptide (1.0 mol%) at specific temperatures. The diagram clearly shows the emergence of a shoulder on the L_α Bragg reflections and the onset of a Q_{II} phase.

Bragg reflections significantly decreases from those around 50°C, indicating that the lipid system becomes progressively more ordered as the phase boundary approaches. This is because, close to T_H, the recorded reflection is the superposition of a wide L_α Gaussian and a narrow H_{II} Gaussian. As the contribution of the wide L_α Gaussian decreases and the contribution of the narrow H_{II} Gaussian increases, the observed peak appears narrow. Lipid phases may co-exist even without thermal gradients across the sample. The observation that the pure lipid specimen can undergo a L_α/H_{II} phase transition at fast scan rates without entering the Q_{II} phase is in agreement with Hui et al. [7].

3.2. MeDOPE and FeLV fusion peptide (1.0 and 0.5 mol%)

d_L for both of these samples was similar to that of the pure lipid. The FW1/*e*H values were slightly less than the lipid control, indicating more order in the L_α phase lattice. In the 1.0 mol% peptide sample, a shoulder was evident on both the diffraction orders of the L_α phase at 35°C (Fig. 3). As the temperature steadily increased, the shoulders on both orders of diffraction grew and resolved into new peaks as the original peak area diminished. These new peaks indexed to a lamellar phase ($d_L = 58$ Å). They overlapped the onset of very low angle Bragg reflections whose spatial frequency indexed onto a ratio of $0:1:\sqrt{2}:\sqrt{3}$. Although the spatial frequencies of only four orders of diffraction were observed (including the zeroth order), both L_α and H_{II} phases can be excluded. The peak coordinates indicate the emergence of a Q_{II} with a large lattice basis vector length of 254 Å at 44°C (Fig. 4). These initial dimensions are approximately 55% greater than previously recorded Q_{II} phases [8,30,32,33]. The lattice basis vector length decreased reciprocally with temperature to 129 Å at 72°C, indicating tighter curvature at higher temperatures. The dimensions at 72°C closely resemble those in previous studies that classed the lipid as possessing Pn3m or Pn3 space group symmetry (unit cell parameter = 125–140 Å) [8]. The size of the Q_{II} peaks increased with temperature. The H_{II} phase was first observed at 71°C, at least some 10°C higher than either the pure lipid sample or the 2.0 mol% peptide sample, both of which showed close agreement with the Q_{II}/H_{II} transition temperature, T_{QH}, measured by Siegel and Banschbach, [31].

As the novel lamellar peaks disappeared at 55°C, there was an increase in sample disorder. The areas under the Q_{II} peaks were much less than the areas under either the L_α phase or H_{II} phase peaks, but the incoherent background did not noticeably increase.

The 0.5 mol% FeLV peptide sample behaved similarly to the 1.0 mol% sample. In the 0.5 mol% peptide sample, the Q_{II} phase emerged at 53°C, nearly 10°C higher than the 1.0 mol% peptide sample. The lattice basis vector length of this cubic phase was 284 Å at 53°C, decreasing to 125 Å at 72°C. Again, shoulders were observed on the first and second diffraction orders of the L_α phase but these were less pronounced than those of the 1.0 mol% peptide sample. As before, the shoulders appeared prior to the appearance of the cubic phase and had the same d_L of 58 Å.

3.3. MeDOPE and FeLV fusion peptide (2.0 mol%)

The highest peptide concentration gave diffraction patterns that were the most similar to those from MLVs composed of pure MeDOPE. Well-ordered diffraction was observed throughout the temperature scan. The d_L, peak areas (including the reduction in amplitude at 60°C), T_H and the FW1/*e*H were all very similar to the lipid control. Only a small amount of Q_{II} phase was observed and then only after the H_{II} phase

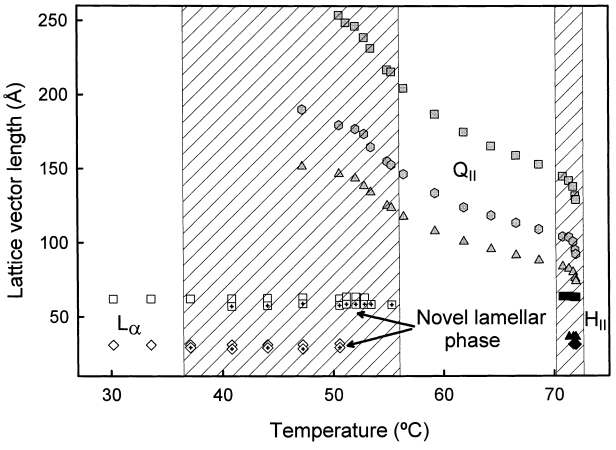

Fig. 4. Lattice basis vector length versus temperature for the L_α and Q_{II} phases of MeDOPE plus FeLV fusion peptide (1.0 mol%) as determined by time-resolved small angle X-ray diffraction. The novel lamellar phase with reduced d_L can be readily observed (crossed symbols). Onset of the H_{II} phase occurs at the end of the temperature scan. The shaded areas represent transition temperature ranges, during which more than one phase is observed simultaneously. Symbols as in Fig. 1; Q_{II} phase symbols in grey.

was established. This may have been accounted for by the existence of discrete membrane patches containing low concentrations of peptide. The peptide in these domains may be able to interact more intimately with the phospholipid in a similar manner to that seen for samples containing the lower peptide concentrations. The Q_{II} phase was observed from 66 to 70°C and had a basis length of 148 Å that did not change significantly during the limited temperature range at which it existed. At this temperature, the Q_{II} basis length was 159 Å for the 1.0 mol% sample and 165 Å for the 0.5 mol% sample.

4. Discussion

These results clearly show that FeLV fusion peptide has a marked effect on the thermotropic behaviour of MLVs comprised of MeDOPE. This effect was most evident at the two lower peptide concentrations, when the peptide induced both a Q_{II} structure and a novel lamellar structure.

A previous differential scanning calorimetry (DSC) study has shown that, at high concentrations, the FeLV peptide had less effect upon the T_H of DPoPE than was seen at low peptide concentrations [27]. In the same paper, using SAXS, the presence of peptide-induced novel peaks in the H_{II} phase of DPoPE was also less pronounced at high peptide concentrations. Similarly, our X-ray data have shown that the sample containing 2.0 mol% peptide behaved like the pure lipid control. It is possible that, at this high concentration, peptide is simply lying along the membrane surface in sufficiently large numbers to prevent the bilayer from curling into an inverse geometry. Alternatively, peptide aggregation in these higher concentration samples may prevent the peptide from interacting with the phospholipid molecules in the same way. We have already reported [34] that FeLV fusion peptide is able to form amyloid-like cross-β fibrils.

At concentrations of 0.5 and 1.0 mol%, the FeLV peptide reduces the T_H of MeDOPE and increases the amount of isotropic ^{31}P nuclear magnetic resonance [27]. The idea that fusion peptides can induce this isotropic resonance is not new [9,10,33]. However, our data show that the previously reported isotropic resonance [27] is directly related to Q_{II} structure formation. The connection between membrane fusion and the formation of non-bilayer phases is only valid for systems that show these structures during the first heating scan, as was observed here. This is the first observation, to our knowledge, of Q_{II} phase formation by a viral fusion peptide. Previously, this peptide-induced phase has only ever been seen with MeDOPE systems containing influenza A fusion peptide at temperatures above T_H or in samples cooled from T_H [33]. Development of the Q_{II} phase normally takes hours and thus cannot be correlated to the rapid fusion process, but we have shown that FeLV peptide can reduce the time of this transition to minutes. This suggests that the FeLV peptide is an extremely potent trigger of highly curved intermediates.

Previous DSC measurements of MeDOPE-containing MLVs have revealed small shoulders on the low temperature side of the T_H enthalpic peak [13,34], presumably caused by intermediates in the L_α/H_{II} pathway. Later studies using DSC and X-ray diffraction revealed that the L_α/Q_{II} phase transition temperature (T_Q) was approximately 62°C and that the Q_{II} structure undergoes a phase transition to the H_{II} phase at higher temperatures (72–77°C) [31]. Thus, from Fig. 3, it can be readily observed that the FeLV peptide dramatically reduces T_Q by 18°C, when compared to these values. The H_{II} phase that eventually formed had the same lattice parameters as the pure lipid. Importantly, this indicates that the peptide does not affect R_o of this lipid species although it does have a destabilising effect upon the L_α phase. A previous X-ray diffraction study on MeDOPE with the fusion peptide of simian immunodeficiency virus revealed that the H_{II} lattice parameters were slightly less than that of the pure lipid control [32]. It was reasoned that this fusion peptide destabilises bilayers by increasing the strength of R_o. Importantly, the FeLV peptide promotes the facile development of Q_{II} structures rather than H_{II} structures. Precursors involved in the L_α/Q_{II} transition pathway are also thought to be involved in fusion pore formation [35].

An important feature of the SAXS data is the appearance of a lamellar structure with reduced d_L some 25°C below the T_H. This structure occurs at the L_α/Q_{II} transition, co-existing with first the L_α and then the Q_{II} phases. This peptide-induced structure seems to represent a transitory stage in the L_α/Q_{II} phase transition mechanism and possibly in the multi-step fusion event itself. The correlation of the appearance of this novel structure with the greatly reduced T_Q suggests that the two are causally connected. We believe that this effect is a result of the peptide's local action on the membrane rather than due to bulk phase separation into areas of peptide-rich and peptide-poor concentrations. This argument is supported by the fact that no novel peaks were evident at the 2.0 mol% peptide concentration, the concentration where phase separation would be most obvious. The available data do not determine whether the novel structure is thermodynamically metastable.

There are two possible explanations for these lamellar structures of reduced d_L. The thickness of the water layer between adjacent bilayers may decrease or the lipid bilayer may thin.

Interfacial dehydration of the lipid bilayer would decrease the d_L [36]. The close approach of two membranes is hindered, from approximately 20 Å inwards, by an exponentially increasing repulsive force [37,38]. It is necessary to displace tightly bound water molecules from each membrane interface [39]. Surface dehydration, by a fusion peptide, could circumvent repulsive forces and permit close membrane approach. Our SAXS images reveal that the *d*-repeat of the bilayer decreased by some 8 Å in the presence of peptide. This value is similar to that reported by Hui et al. [36] who studied membrane fusion induced by freezing and thawing. They make the point that dehydration does not cause membrane fusion alone but that it increases the opportunity for the event to proceed. The ability of fusion peptides to dehydrate membrane surfaces has already been implicated in modelling studies of a lipid-lined fusion pore [40].

An alternative, or complementary, explanation for the observed reduction in d_L is bilayer compression. X-ray diffraction studies have shown that low concentrations of alamethicin adsorbed onto phospholipid bilayer surfaces can cause bilayer thinning [41]. Wu et al. proposed that alamethicin increases the cross-sectional area of the membrane surface by causing lateral expansion of the lipid headgroups. The cross-sectional areas of the lipid headgroups and hydrocarbon chains must be matched in planar bilayers, so the hydrocarbon chains must increase their cross-sectional area to compen-

sate for the peptide at the surface. As the volume occupied by the chains is constant, there is a resultant decrease in the bilayer thickness. Since monolayers of phosphatidylethanolamine lipids have a natural tendency to curl, at elevated temperatures, the lipid molecules are not able to maintain cross-sectional matching and undergo an inverted phase transition. Like the H_{II} phase, the Q_{II} lattice parameters decrease reciprocally with temperature due to hydrocarbon chain splay. At T_{QH}, the frustration between the free energy of monolayer curvature strain and hydrocarbon chain stretching must be so great that the H_{II} phase forms.

Why does the membrane-thin structure metamorphose to a Q_{II} phase and not H_{II}? At a given temperature, the monolayer curvature free energy and the free energy involved in hydrocarbon chain stretching governs the type of lipid phase present [29]. No lipid assembly can fully satisfy the conflicting demands of a simultaneous low curvature free energy and chain packing free energy, giving rise to frustration. Cubic phases have a lower free energy of curvature than the L_α phase but higher than the H_{II} phase. Conversely, the free energy of the chains is higher than in the L_α phase but lower than the H_{II} phase. This means that the amount of frustration in the cubic phase is smaller than in either the L_α or the H_{II} phases [42], so that the cubic phase represents a compromise between the two. Our data show that the peptide destabilises the L_α phase but stabilises lipid transition intermediates. Compared with the L_α phase, the lipid intermediates have a reduced amount of frustration. The data show that, in the presence of fusion peptide, the inverted structure formed is a Q_{II} structure. The initially large lattice parameter of this phase indicates that the free energy of curvature and the hydrocarbon packing free energy are of an intermediary value.

This work with FeLV fusion peptide supports the proposed link between the mechanism of inverse cubic phase formation and membrane fusion. It also indicates that bilayer thinning may be important in the fusion pathway. This study only deals with simplified peptide lipid interactions. In vivo, the peptide is covalently bound to a larger fusion protein, which is embedded in the viral membrane. During fusion, the primed fusion peptide will interact, at least initially, with only the outer membrane leaflet. In this experiment, the fusion peptide is free to interact with both sides of multi-lamellar bilayers. Nonetheless, these results may well represent many features of the fundamental peptide membrane interactions but without the same control and specificity.

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