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Biochimica et Biophysica Acta 1768 (2007) 30–38
Biochimica et Biophysica Acta
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Configuration of influenza hemagglutinin fusion peptide monomers and oligomers in membranes

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Received 11 April 2006; received in revised form 1 August 2006; accepted 2 August 2006
Available online 22 August 2006

Abstract

The 20 N-terminal residues of the HA2 subunit of influenza hemagglutinin (HA), known as the fusion peptide, play a crucial role in membrane fusion. Molecular dynamics simulations with implicit solvation are employed here to study the structure and orientation of the fusion peptide in membranes. As a monomer the α-helical peptide adopts a shallow, slightly tilted orientation along the lipid tail–head group interface. The average angle of the peptide with respect to membrane plane is 12.4 °. We find that the kinked structure proposed on the basis of NMR data is not stable in our model because of the high energy cost related to the membrane insertion of polar groups. Because hemagglutinin-mediated membrane fusion is promoted by low pH, we examined the effect of protonation of the Glu and Asp residues. The configurations of the protonated peptides were slightly deeper in the membrane but at similar angles. Finally, because HA is a trimer, we modeled helical fusion peptide trimers. We find that oligomerization affects the insertion depth of the peptide and its orientation with respect to the membrane: a trimer exhibits equally favorable configurations in which some or all of the helices in the bundle insert obliquely deep into the membrane.

Keywords: Influenza; Hemagglutinin; Fusion peptide; Membrane configuration; Simulation

1. Introduction

Influenza is a virus with a high death toll, causing considerable suffering and imposing a huge economic cost every year. It has caused several pandemics in the 20th century in which millions of people died. Viral infection occurs by endocytosis of the virus and subsequent fusion of the viral envelope membrane and the endosomal membrane [1,2]. The membrane fusion process of influenza is mediated by the homotrimeric protein hemagglutinin (HA) [3–5]. Each HA monomer contains two subunits, HA1 and HA2. The 20 N-terminal residues of HA2 are highly conserved and are referred to as the fusion peptide. The importance of the fusion peptide has been demonstrated by site-directed mutagenesis [6–8].

HA-mediated membrane fusion is promoted by acidic pH. Low pH induces a structural change in HA [9,10], which exposes the fusion peptide and allows it to interact with the target membrane. pH also affects the interaction of the fusion peptide with the membrane [11–15]. The wild type hemagglutinin fusion peptide (HAFP) has been reported to cause vesicle lysis at enhanced rates at low pH [12] and a HAFP analogue shows pH-dependent capability of vesicle lysis [15]. On the other hand, the fusion peptide has also been shown to fuse vesicles at a rate independent of pH [16]. The reason may lie in differences in the studied peptides or in bilayer curvature. The fusion peptide has been hypothesized to act by perturbing the membrane or by promoting negative curvature and tension in the membrane [17–20]. Oligomerization has been reported to enhance the membrane fusion ability of the HAFP [21].

Significant effort has been put into determining the membrane structure of the HAFP. The peptide and its analogues adopt a mainly α-helical conformation upon binding to membranes [16,22–33], although β-structure is present as well in varying proportions depending on the environment or the exact sequence [22,26,27,29,30,32–36]. Most often the oligomerization state of the fusion peptides is not addressed. The fusion peptide has been reported to most likely be a

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doi:10.1016/j.bbamem.2006.08.008

monomer in Refs. [15,25] but in other studies synthetic influenza virus HAFPs and Glu-rich analogues are reported to form oligomers [23,28,29,37,38]. High peptide concentration has been reported to promote self-association and β-structure in HAFPs with solubilizing hydrophilic extension tails [35].

Electron paramagnetic resonance (EPR) studies have suggested that the α-helical peptide adopts an angle of approximately 45° [24] or 25° [25,39] with respect to the membrane plane, although a parallel-to-membrane orientation for the helices is reported for a glutamic acid-rich analogue [26]. Significant α-helical content [28,30] and oblique orientation [32] have been suggested as prerequisites for fusion. Obtaining a high resolution structure has been complicated because of the high aggregation propensity of the peptide. Han et al. managed to solubilize the peptides by attaching a hydrophilic tail (–GCGKKKK) to the fusion peptide sequence, which prevents aggregation [40]. Based on measurements of the extended peptide in micelles, Han et al. deduced the atomic resolution structure of the HAFP to be an inverted V-like helix–break–helix configuration, in which both the N- and the C-termini insert into the membrane [14]. The break between the two helical regions has been later observed also by Hsu et al. [41] and reported to be significant in the fusion ability of the peptide [36,42,43].

Theoretical approaches have also been used to study the configuration adopted by the HAFP and analogous peptides in a membrane environment [44–49]. Efremov et al. reported that the peptide adopts a tilted orientation with respect to the membrane plane based on Monte Carlo simulations [44]. A Poisson–Boltzmann approach gave an orientation parallel to the membrane [45]. Spassov et al. employed a generalized Born implicit description of the membrane and predicted a shallow tilted insertion angle [46]. Simulations of HAFP with explicit description of the solvent and the membrane [47–49] predict that the V-shaped NMR structure is stable in the membrane, at least for 5 ns–18 ns.

Both the experimental and theoretical work done on the membrane conformation and orientation of HAFP show a variety of results. Thus, the issue is far from settled. Furthermore, although there is considerable evidence that the fusion peptide may interact with the membrane in oligomeric form [23,28,29,37,38], the influence of oligomerization on the membrane configuration of the HAFP has not been assessed. Here we conduct a simulation study of monomeric and oligomeric fusion peptides using an implicit environment description (IMM1) [50,51] with full atomistic detail in the fusion peptide. The method provides a way to compare the effective energy of a large number of structures. We find that HAFP monomers adopt a slightly tilted orientation along the lipid head group–tail interface but trimers adopt orientations in which some or all of the peptides insert their N-termini deep into the non-polar region and adopt an oblique insertion angle.

2. Computational methods

The simulated peptide is the 20 N-terminal residues of the hemagglutinin protein of influenza strain X31: GLFGAIAG-FI**E**NGW**E**GMI**D**G. Both protonation states of the acidic residues (underlined) are considered. The mutants ΔG1, G1L, G1E, G4V, and G4E were studied in addition to the wild type peptide. The CHARMM 19 force field that treats non-polar hydrogen atoms implicitly is employed to describe the atomic interactions in the peptide [52,53]. The environment is described by EFF1.1 in water and IMM1 in the membrane [50,51]. In the EFF1.1 and IMM1 models an implicit solvation energy term is added to the CHARMM force field [50,51]. This describes the self energy of the molecule in its environment. In addition, the ionic side chains are neutralized and the dielectric constant is distance dependent to account for the screening effects of the surrounding solvent. The membrane environment is described by a hydrophobic slab centered at the plane $z=0$ Å. The thickness T of the slab depends on the type of modeled lipids and typically ranges between 20 Å and 30 Å. A value of $T=26$ Å has been used in this work. The transition between the water and membrane environments is continuous [50,51]. The solvation parameters and the extent of dielectric screening vary between the two environments.

The starting structures for the simulations are either ideal-helices or the V-shaped structures proposed by Han et al. (Protein Data Bank structures 1IBN and 1IBO) [14]. The structures are placed at the orientation reported in Ref. [14]. Furthermore, initial orientations in which the 'V' is rotated 45° on the plane of the 'V' around the center of mass of the peptide, or the plane of the 'V' is tilted 30° with respect to the membrane plane, are sampled. By the plane of the 'V' we mean here the plane defined by the first and second principal axes of rotation of the V-shaped structure. In addition to the insertion depths proposed in Ref. [14], the V-shaped initial configurations are set so that the center of mass initially resides either at $z=0.0$ Å (at the center of membrane), $z=13.0$ Å (at the polar–non-polar-interface) or $z=19.5$ Å (in the polar region). Three different protonation states are tried: Charged N- and C-termini and acidic residues, uncharged N-terminus and acidic residues but charged C-terminus, and uncharged N- and C-termini and acidic residues. A total of 216 simulations were run starting from the NMR structures. The initially α-helical monomer configurations have initial insertion angles between 0 and 90° with respect to the membrane plane at 5° intervals. The helices are inserted into the membrane so that the center of mass resides either at $z=0.0$ Å, $z=13.0$ Å, or $z=19.5$ Å. A total of 228 simulations were run for the wild type peptide from the α-helical initial structure taking into account the different protonation states.

In the initial configuration of the trimer the axes of three identical parallel α-helices span an equilateral triangle with 9 Å sides. Both termini and the acidic residues are neutralized and the helices are rotated around their individual axes such that the three acidic residues are facing the inner core of the bundle and are thus less exposed to the hydrophobic membrane region. 4 different axial rotation angle combinations were sampled. In analogy to the monomer, the bundle of three helices is inserted into the membrane in angles between 0 and 90° at 15° intervals. Two insertion depths are tried: center of mass at the membrane center ($z=0.0$ Å) and at the interface ($z=13.0$ Å).

Two initial orientations are sampled: one corresponding to the equilateral triangle formed by the cross section of the bundle being inserted tip-first into the membrane and the other corresponding to insertion side-first. A total of 112 simulations were run for the trimers.

The initial configuration is first relaxed at 300 K. In the case of the trimer the backbone atoms of one of the helices are fixed for 500 ps relaxation after which the constraints are released and the system is let to evolve freely for a relaxation period of 500 ps. This ensures that the trimer is properly relaxed and potential hydrogen bonds have time to form and relax between the helices. For the monomer no initial fixing period is necessary and the system is let to evolve freely for 500 ps for relaxation. In both cases the system is minimized for 300 steps by adopted basis Newton–Raphson (ABNR) method [52] after the relaxation to obtain comparable initial configurations. These configurations are then simulated for 500 ps by employing the Nosé–Hoover thermostat with heat bath coupling constant $q=100$ [54,55]. The dynamics trajectory of the last 400 ps of this part of the simulation is used to calculate the average orientation, membrane insertion depth, and effective energy of the peptide. The angle with respect to the membrane plane and insertion depth are averaged over frames obtained every 1 ps.

When examining the energetics of the V-shaped structure in comparison to the interfacial orientation of the peptide, the V-shaped structures were first positioned with respect to the membrane as proposed in Ref. [14] and then minimized for 300 steps. Two different protonation states were examined: the V-shaped structures either had both termini charged and the acidic residues deprotonated or the termini neutralized and the acidic residues protonated. The interfacial orientation of a particular protonation state was picked randomly from the relaxed sample of highly similar configurations and then minimized.

The fusion peptide monomer is studied with both protonated and deprotonated N-terminus and acidic residues. As a trimer the peptides are examined only with uncharged N- and C-terminus and acidic residues. The effective energy values presented do not contain the cost of (de)protonation and omit entropic contributions to the free energy. Therefore the effective energy values are comparable only within a given protonation state.

3. Results

3.1. Hemagglutinin fusion peptide monomers: the micelle-determined V-shaped NMR structures are not stable in the IMM1 model

The most detailed structural information on the HAFP has been obtained by NMR in micelles. These structures were then docked onto bilayers using EPR spin labeling measurements. We first examined the stability of the proposed structures in our membrane model. We placed the NMR structures in various protonation states, orientations and insertion depths into the membrane and relaxed them with molecular dynamics simulations. We found that the peptides relocate rapidly within ca. 100 ps of the initial 500 ps relaxation period to a slightly tilted configuration along the lipid tail–head group interface. The initial minimized V-shaped configurations and the final configuration are presented in Fig. 1. As the initially V-shaped configurations relocate to the interface, the helix straightens and the configurations show no difference to those obtained starting from ideal-helices.

To find out the reason for the swift interfacial relocation of the V-shaped HAFP we performed an energetics comparison between the two configurations. The results are presented in Table 1. The V-shaped structure is disfavored in comparison to the interfacial orientation mainly because of the solvation energy cost due to embedding the polar residues into the membrane. The same trends are seen for both examined protonation states. The deeper membrane insertion of the V-shaped pH 5-structure shows a larger desolvation cost than the less deep insertion of the V-shaped pH 7.4 structure. Van der Waals energy contributions also favor the interfacial orientation, with the more open pH 7.4 structure now disfavored more. Electrostatic interactions favor the more bent pH 5.0 structure while disfavor slightly the pH 7.4 structure in comparison to the interfacial orientation. The favorable electrostatic contribution is not sufficient to stabilize the pH 5.0 structure. We point out that the energy values have not been corrected for the cost of (de)protonation of the termini and the acidic residues and thus are not comparable between different protonation states.

3.2. Range of configurations accessible by monomeric fusion peptides as ideal helices

To determine favorable configurations of the fusion peptide in an unbiased way, a large number of simulations were performed starting with an ideal helix at different positions

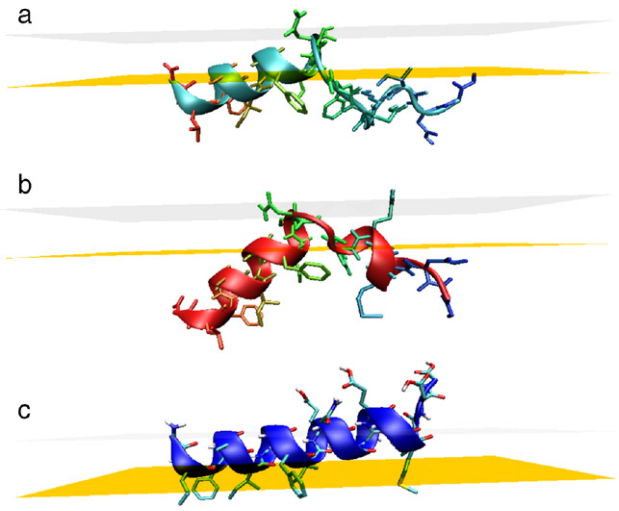

Fig. 1. The initial V-shaped configurations (top: pH 7.4, middle: pH 5.0) and the configuration typically adopted in the relaxation starting from either of the V-shaped structures (bottom). This particular configuration is obtained from the pH 5.0 structure with protonated acidic residues and neutralized termini. The orange plane shows the level of the hydrophilic–hydrophobic interface ($z=13$ Å) and the light gray plane the height at which the phosphate head groups are estimated to reside ($z=16$ Å). This figure and Figs. 2 and 4 were produced with the program VMD [56] after which they were rendered and the planes added with POV-Ray. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Energetics comparison for the V-shaped and the interfacial orientation of the HAFP

	Effective energy kcal/mol	Solvation energy kcal/mol	Electrostatic energy kcal/mol	vdW energy kcal/mol
Charged N- and C-terminus and acidic residues				
V-shaped structure at pH 5.0 (1IBN)	−492.6	−123.7	−316.4	−91.4
V-shaped structure at pH 7.4 (1IBO)	−494.4	−154.1	−297.2	−90.4
Interfacial orientation	−555.5	−182.9	−302.9	−105.4
Uncharged N- and C-terminus and acidic residues				
V-shaped structure at pH 5.0 (1IBN)	−465.9	−103.1	−315.0	−86.5
V-shaped structure at pH 7.4 (1IBO)	−459.8	−111.8	−306.4	−83.2
Interfacial orientation	−513.6	−143.9	−311.8	−92.1

The abbreviations IBN1 and IBO1 refer to the Protein Data Bank names for the structures proposed in Ref. [14]. The energy values have not been corrected for the cost of (de)protonation of the termini and the acidic residues and thus are not comparable between different protonation states.

and orientations (see Computational methods). The initially α-helical peptides adopt the same configuration as the initially V-shaped peptides, see Fig. 2. In addition to the interfacial orientation we also observe a transmembrane configuration when the peptide has a charged N-terminus but uncharged acidic residues, but this orientation has significantly higher energy. Fig. 3 plots the average effective energy and the average or smallest distance of the N-terminus from the membrane center against the average angle of the first 11 residues. The average angle the monomer makes with respect to the membrane is 12.4±4.2 Å where the fluctuation in the angle is estimated by the standard deviation of the averages of individual runs, see Fig. 3. The individual averages of all four protonation states fall well within the standard deviation from one another. Therefore the reported average angle is calculated over all the protonation states presented in Fig. 3 but disregarding the transmembrane configurations. In the final interfacial configurations the α-helical structure is well preserved at the N-terminal part of the peptide while some unfolding and occasionally local changes to α-helicity are observed at the C-terminal region.

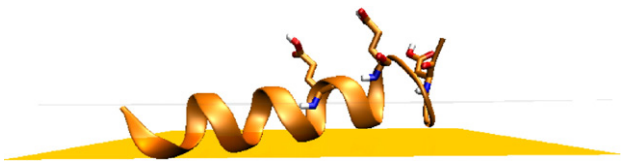

Fig. 2. Interfacial orientation adopted by the hemagglutinin fusion peptide as an initially ideal α-helix monomer. The average angle with respect to the membrane plane is 12.4° and in this particular frame 8.6°. The acidic residues, here uncharged, are presented as stick-models. The orange plane shows the level of the hydrophilic–hydrophobic interface (z=13 Å) and the light gray plane the height at which the phosphate head groups are estimated to reside (z=16 Å). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

On the average, the fusion peptide remains at the membrane interface as is visible in Fig. 3. Regardless of the charge states of the termini and the acidic residues, the distance to the membrane center from the helix corresponds to the interface, ≈14 Å. Neutralizing the acidic residues moves the peptide a little deeper into the membrane, but no major difference is observed in the average insertion. However, neutralizing the N-terminus and the acidic residues, has an effect on the fluctuations of the peptide. If the peptide has a neutral N-terminus, the dots corresponding to the maximum insertion are relocated closer to the membrane center in Fig. 3. The same can be observed if also the acidic residues are neutralized but the major contribution appears to be due to the charge state of the N-terminus, not the acidic residues. The dynamics trajectories show that the peptide becomes more mobile when the terminus is uncharged: the amino terminus takes frequent shallow dips into the membrane but does not stabilize into the center region. We also studied the effect of protonation states of the C-terminus (data not shown). Similar charge-state-related depth fluctuations are observed for the

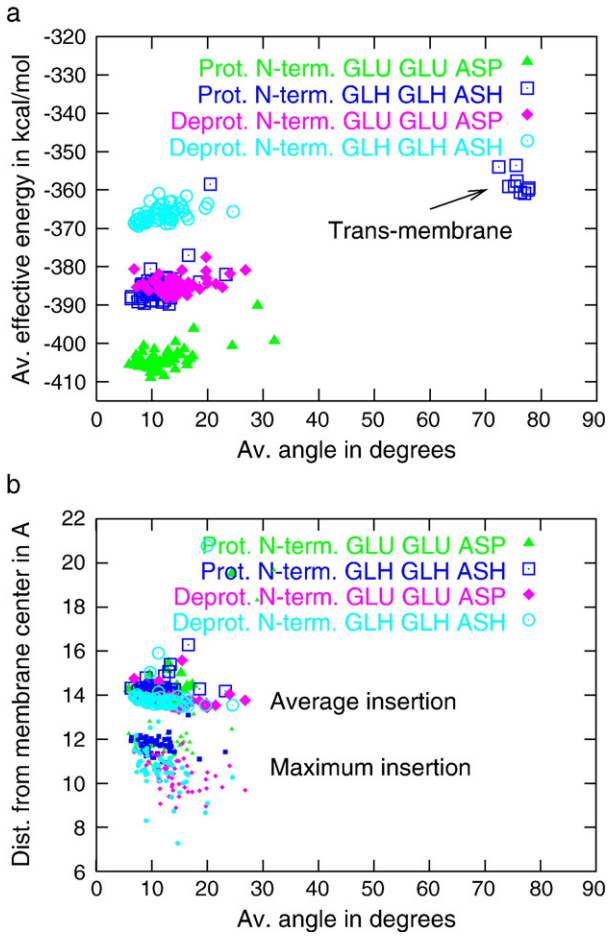

Fig. 3. Effective energy and N-terminal insertion depth as a function of orientation with respect to the membrane plane for the hemagglutinin fusion peptide as a monomer. Four different protonation states are presented. Please note that the energies of the states do not include the cost of (de)protonation and therefore are comparable only within the same protonation state. In the figure legends the GLU and ASP refer to deprotonated glutamic acid and aspartic acid residues in the peptide while GLH and ASH correspond to the protonated, uncharged counterparts.

C-terminus but the C-terminus does not insert into the membrane. In addition, the dynamics runs show that the 20 residue hemagglutinin fusion peptide behaves in a hinge-like fashion; residues around 12–14 allow the helix to partially break into two helical regions connected with a stiff hinge. This region corresponds to the site of the kink in the configuration proposed in Ref. [14] and to the observations of Ref. [41]. Because of this bending, only the 11 N-terminal residues are employed in defining the angle with respect to the membrane.

It has been hyphothesized that fusion impaired HAFP mutants might adopt a different orientation than the wild type peptide [47]. Therefore simulations of five different mutants, ΔG1, G1E, G1L, G4E, and G4V, were performed. Of these, ΔG1, G1E, G1L, and G4V, have been reported to be fusion impaired in experiments [57] although later G1L has been shown to allow viral replication to a small degree [8]. G4E is fusion active but to a lower level than the wild type [11]. With the exception of the G4V mutant all the mutants adopt the interfacial orientation in the simulations. ΔG1 adopts the same insertion depth as the wild type peptide but the removal of the N-terminal glycine causes the peptide to fluctuate much more in its insertion depth and angle. Mutating the N-terminal glycine to glutamic acid, G1E, causes the peptide to adopt a slightly less inserted interfacial orientation than the wild type. This is equally true for the G4E mutant. Replacing the N-terminal glycine by the more hydrophobic leucine moves the peptide slightly deeper into the membrane and induces the N-terminal to take dips into the membrane center. However, the interfacial orientation is maintained. In contrast to the wild type peptide and the other four studied mutants, the G4V mutant shows an oblique orientation with respect to the membrane plane to be equally likely as the interfacial orientation when the N-terminal of the mutant has been deprotonated. Although valine is only slightly more hydrophobic than leucine in the G1L mutant which did not insert, the valine-residue adopts a position in the helix in which it is significantly more exposed to solvent in the interfacial orientation than the N-terminal leucine in the G1L mutant. This explains the emergence of the oblique orientation for this particular mutant.

3.3. Hemagglutinin fusion peptide trimers

Influenza hemagglutinin is a homotrimer. The crystal structures of hemagglutinin do not include the fusion peptide sequence but the trimeric stem region is likely to bring the peptides close to each other, potentially forming fusion peptide oligomers. Next we study whether oligomerization has an effect on the membrane configuration of HAFPs. Because the stem-region of the hemagglutinin protein is a trimer, we set the peptides also as a trimer. Three α-helical peptides are initially bundled and inserted into the membrane at various angles and depths, and simulated for 1.5 ns of which the last 0.5 ns is used for calculating the results, see Computational methods.

In the simulations the three peptides typically remain in the trimeric configuration but the bundle shows structural flexibility. Most typically the bundle relocates to an interfacial orientation, but slower than the monomer. A significant number of the trimers do, however, adopt configurations in which either one or several of the peptides are inserted deep into the membrane. A set of low energy configurations for the fusion peptide trimer are presented in Fig. 4. Fig. 5 shows the average effective energies of the simulation runs, the corresponding tilt angles of the helices, and the insertion depths. In Fig. 5 the data points corresponding to trajectories with extremely high average energy in comparison to the lowest energy trajectory are omitted to preserve the clarity of the plots.

The configuration that is lowest in energy in our simulations has one of the peptides inserted and two remaining at the interface. This configuration is presented on the top of Fig. 4. In Fig. 5 the data points corresponding to the low-energy configuration are highlighted in color: in the lowest-energy configuration the helices at the interface are at a more tilted orientation than the monomer, averaging ~30° and ~40° with respect to the membrane plane. The inserted helix has a more oblique, ca. 55° angle to the membrane plane. The other low-energy configurations include configurations in which all three peptides adopt the interfacial orientation (Fig. 4, center) or insert as a bundle (Fig. 4, bottom). In these configurations the helices are also more tilted than as monomers. The configurations presented in Fig. 4 do not show threefold symmetry. In the HA

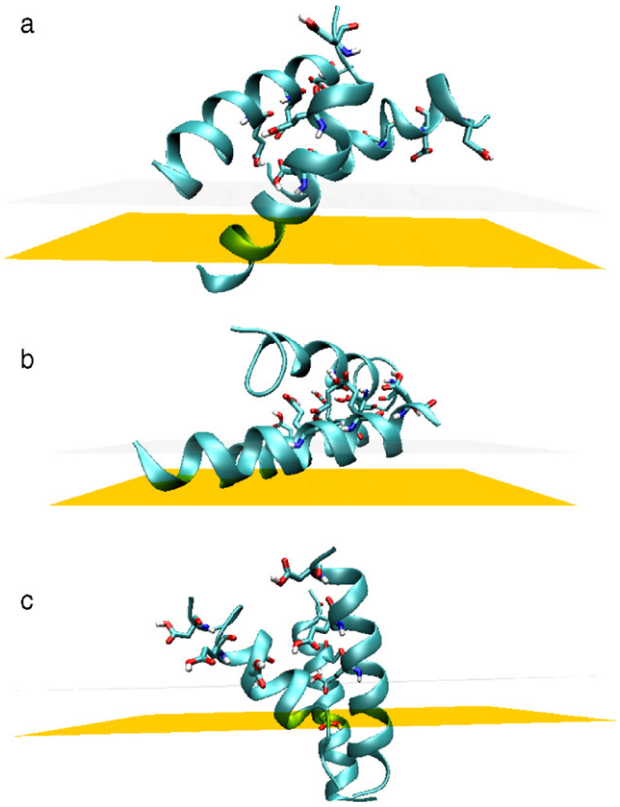

Fig. 4. The lowest energy configurations for the trimer. The figure on the top shows the lowest energy configuration obtained in the simulations. The middle one is the second lowest and represents the interfacial configuration corresponding to a large majority of the low-energy configurations. The third lowest is an inserted bundle represented on the bottom. All structures are within 3.5 kcal/mol of each other, see Fig. 5. The orange plane shows the level of the hydrophilic–hydrophobic interface ($z=13$ Å) and the light gray plane the height at which the phosphate head groups are estimated to reside ($z=16$ Å). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

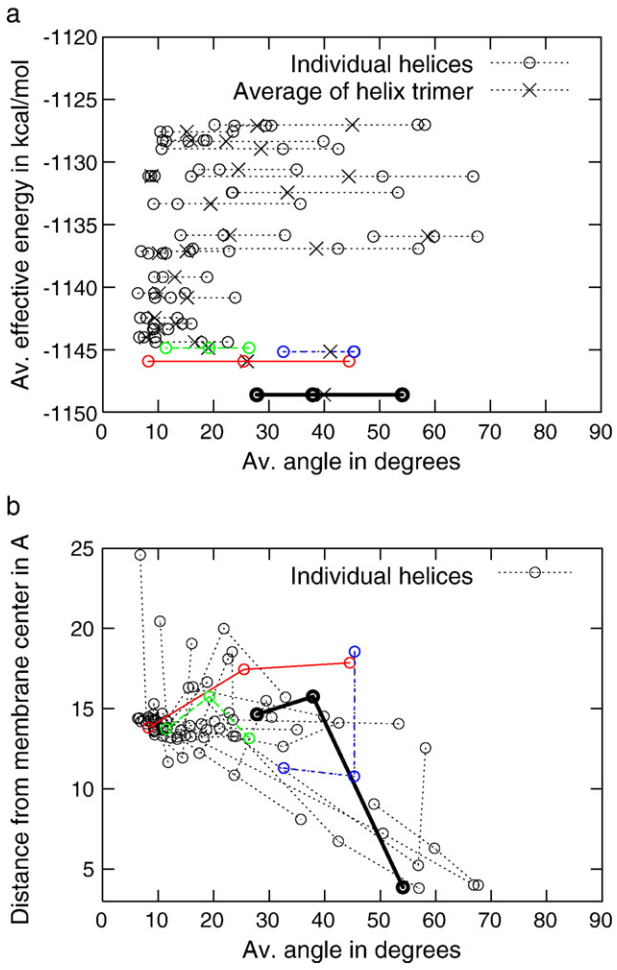

Fig. 5. Effective energy and orientation with respect to membrane for the hemagglutinin fusion peptide as a trimer. The open circles refer to individual helices in the trimer whereas the crosses present the average of the three helices. A dashed line connects the symbols of each trimer bundle. The low-energy configurations are marked with matching color in both figures. The colors black, red and blue correspond to trajectories with the final configuration presented in Fig. 4 from top to bottom. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein the fusion peptide sequence is followed by ca. 20 residues, that do not adopt any ordered structure in the existing crystal structures of the low-pH HA2 [58,59]. In the pre-fusion structure the domain adjacent to the fusion peptide adopts a β-sheet structure [3]. It is likely that the region next to the fusion peptide provides enough flexibility to allow the configurations we observe.

The configurations presented in Fig. 4 reside within an effective energy window of 3.5 kcal/mol. An energy difference this small can easily be induced by changes in the peptide environment, such as differences in membrane composition or interactions with nearby proteins etc. Oligomerization clearly creates stable, low-energy configurations in which some or all of the peptides insert their N-terminus into the membrane and adopt an oblique insertion angle. The wide variety of stable configurations also indicates that the peptide has a large configurational variability and the membrane orientations should be sensitive to environment.

4. Discussion

We studied the membrane configuration of monomeric and trimeric influenza hemagglutinin fusion peptides. As a monomer the α-helical wild type peptide adopts a slightly tilted orientation along the lipid tail–head group interface. For the trimers the inter-helical interactions induce stable configurations in which either one or several of the helices insert much deeper into the membrane and at a steeper angle with respect to the membrane plane than the monomeric peptides. The configurational flexibility of the trimeric peptide is high: a wide variety of stable configurations was observed to be comparable in energy. Therefore small changes in the environment may promote one configuration over the others. Because of the configurational complexity and flexibility of the trimer, we cannot be certain that we have sampled the global energy minimum in our simulations of the trimers. We merely report observations that the peptide exhibits low energy configurations that differ significantly in orientation from that of the monomeric peptide.

Experimental studies of the influenza HAFP configuration in membrane have given a variety of results. Some predict the peptide to adopt an α-helical form and insert into the membrane with N-terminus insertion depth close to the lipid phosphate group [24,25,39], take a parallel-to-membrane orientation [26] or adopt a V-shaped configuration in which both termini are inserted into the hydrophobic membrane [14]. The N-terminal insertion angle with respect to membrane plane has been reported to be 45° [24], 25 or 28° depending on pH [25,39], and 21 or 37° depending on pH [14]. Furthermore, Refs. [14,25,39] use a hydrophobic spin label which may bias the results toward deeper membrane insertion (Sammalkorpi, M. and Lazaridis T., unpublished data). Our results for the monomers clearly favor the lower values and shallow insertion.

The energetics comparison of the NMR-based high-resolution structure of the hemagglutinin fusion peptide [14] and the slightly tilted, essentially straight and less inserted orientation obtained as the monomer equilibrium configuration in this work does not support the V-shaped structures of Ref. [14]. The main reason is the high cost of desolvating the polar residues when they are inserted into the hydrophobic membrane. We do observe the kink reported in Ref. [14] but mainly in the dynamics of the peptide. It may be that the V-shaped configuration with both termini inserted into the hydrophobic core has been brought forth in the experiment of Ref. [14] by the micelle environment. The EPR results were less clear near the C-terminus. Alternatively, the discrepancy may be due to limitations of the simulation method. IMM1 is a highly simplified model of a lipid bilayer. It assumes a perfectly flat, non-deformable membrane and neglects the connectivity between headgroups and acyl chains. In reality, lipid molecules can adapt somewhat to the presence of the peptide. Therefore, implicit membrane results should be regarded with caution and should be corroborated, to the extent possible, with explicit bilayer simulations and further experimentation.

An important issue to consider in the experimental studies of HAFP membrane configuration is whether the measured

peptides have been monomers or oligomers: Ref. [25] argues that the spin labels in different helices are not likely to be in tertiary or quaternary contact with each other, which the authors deduce to indicate monomers. On the other hand, it could also indicate that the relatively long residue arms of the used methanethiosulfonate spin label point in opposite directions in an oligomeric configuration. In Ref. [14] the hydrophilic extension tail that prevents aggregation most likely also affects oligomerization. However, the same extended peptide that includes the hydrophilic extension tail, has been reported to aggregate in a pH-dependent manner at higher concentrations [35] which indicates that although the tail introduces additional local charge, some attraction between the peptides remains. The aggregation was reported to promote β-structure but a significant content of α-helices were present in the self-associated samples [35]. The peptide to lipid concentrations were still much lower than what would be expected as the local fusion peptide-to-lipid ratio in influenza hemagglutinin mediated membrane fusion, in which most likely several HA trimers are required to act in a cooperative way [60,61]. There is, however, evidence that the fusion peptide forms oligomers [23,28,29,37,38] and oligomerization of the peptide has also been reported to promote fusion [21]. The orientations we obtain for the trimeric peptide agree with the results of Refs. [14,24,25,39]. The configurational variation observed in the simulations of the trimer can also explain in part the variation in observed insertion depths and angles: Refs. [24,39] report the N-terminal to be at the level of the lipid phosphate group while Ref. [14] presents N-terminal insertion deep to the hydrophobic core of the membrane. Similarly, reported angles range between 25 and 45° [14,24,25]. An oligomerization-dependent configurational change in the membrane is in line with the pH dependence reports of HA fusion activity, since at low pH the acidic residues are more protonated, which acts to stabilize the inter-helical interactions. At neutral pH the charges at the acidic residues repel each other and this is likely to prevent the formation of a helix bundle. A trimeric assembly has been proposed earlier in Ref. [28] and the orientations of the acidic residues observed in our simulations are in line with the proposal of Ref. [28], although we observe flexibility in the interhelix binding. The high configurational variety observed in our simulations may play a role in the bilayer destabilization induced by the HAFP.

The existing theoretical studies treat the HAFP as a monomer and several reports of oblique insertion exist [44,46–49], although parallel-to-membrane orientation has been proposed as well [45]. The slightly tilted interfacial orientation reported in this work as the monomer structure is in agreement with the existing studies of HAFP monomers: Efremov et al. reported based on Monte Carlo simulations in an implicit membrane model that the functional HAFP analogs and mutants inserted into the membrane at an angle between 10.3 ° and 18.0 ° to a depth of approximately 1.4 Å below the membrane surface (measured from the center of mass of the helix and counting in the head group region) [44]. Spassov et al. used a generalized Born solvent model and predicted that the peptide is at interface at an angle of 20–25° but only if the N-terminal helix unwrapped for 3–4 residues—for an intact helix the orientation was practically parallel-to-membrane [46].

Regarding simulations in explicit environment, with the short simulation times there is always the question whether the system has reached equilibrium, especially for Refs. [47,49] in which the V-shaped structures of Ref. [14] were used as initial configurations. Refs. [47,48] predict a backbone insertion depth only slightly below the phosphate groups, in Ref. [48] minimum reported side chain distance being approximately 5 Å from the bilayer center. This is only modestly deeper insertion than the one we obtain. In Ref. [49] the insertion depth for residue 12 is reported in line with Ref. [14] but the angle between the helical segments appears to be larger than in Ref. [14]. In our simulations the use of IMM1 environment description speeds the equilibration of the system in comparison to the explicit environment description: By replacing the lipid and water molecules by a static implicit environment description diffusion processes toward equilibrium become orders of magnitude faster. Thus, it is more certain that the configurations reported in this work correspond to thermodynamic equilibrium.

It is worth noting that the data presented here cannot be used to draw any conclusion on the pH dependence of membrane binding of the fusion peptide. As noted in Computational methods, energy values of peptides with different protonation states cannot be compared. It also is not meaningful to compare the energies of the two NMR structures at the same protonation state, which, in any case, are highly unstable in our model. The proper methodology to determine pH dependent membrane binding free energy is described in a recent publication from our group [62].

We conclude that peptide–peptide interactions may play a significant role in the membrane insertion of the influenza hemagglutinin fusion peptide. This may be related to the observation that oligomerization enhances fusion activity [21]. Therefore, influenza infection may be prevented, or hindered, by methods that aim at destabilizing the trimerization.

Acknowledgements

M.S. would like to thank M. Koivunen for fruitful scientific discussions. Financial support was provided by a NIH SCORE grant (3S06GM008168-25S1). Infrastructure support was provided in part by RCMI grant RR03060 from NIH.

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