Factors important for fusogenic activity of peptides: molecular modeling study of analogs of fusion peptide of influenza virus hemagglutinin

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Abstract Nine analogs of fusion peptide of influenza virus hemagglutinin whose membrane perturbation activity has been thoroughly tested [Murata et al. (1992) Biochemistry 31, 1986–1992; Murata et al. (1993) Biophys. J. 64, 724–734] were characterized by molecular modeling techniques with the aim of delineating any specific structural and/or hydrophobic properties inherent in peptides with fusogenic activity. It was shown that, regardless of characteristics common to all analogs (peripheral disposition at the water-lipid interface, amphiphilic nature, α-helical structure, etc.), only fusion active peptides reveal a specific 'tilted oblique-oriented' pattern of hydrophobicity on their surfaces and a certain depth of penetration to the non-polar membrane core. The conclusion was reached that these factors are among the most important for the specific destabilization of a bilayer, which is followed by membrane fusion.

Key words: Protein-membrane interaction; Monte Carlo simulation; Molecular hydrophobicity potential; Oblique-oriented peptide; Hydrophobicity

1. Introduction

Membrane fusion activities of conservative fragments of viral membrane proteins, so called 'fusion peptides', consisting of about 20 amino acid residues [1,2], are widely studied because of their ability to perturb the phospholipid bilayer in a way which is strongly dependent on the peptide sequence. The following features of fusogenic sequences have been elucidated: (1) an α-helix formation upon binding to the lipid bilayer [3,4]; (2) a critical pressure of the insertion into the lipid phase determined by the hydrophobicity of an α-helix [5,6]; (3) an asymmetric distribution of hydrophobic and hydrophilic residues on an α-helix resulting in a hydrophobicity gradient along the helical axis; this defines the mode of the association of a peptide with the phospholipid bilayer; only obliquely oriented peptides were shown to induce negative curvature of the bilayer [7,8] giving rise to the formation of non-bilayer lipid phases [9,10]. A wealth of experimental works is available dealing with fusion peptide of influenza virus hemagglutinin (HA). The wild-type peptide was shown to induce fusion of phospholipid vesicles in a pH-dependent manner [3]. Also, fusion and leakage activities of a number of HA analogs (Table 1) were assayed respectively by lipid/internal content mixing methods and the ANTS/DPX technique [11]. It is important that these measurements were done by the same group and in the same laboratory setup [2], thus providing a consistent set of experimental data.

In this paper we characterize nine analogs of the HA fusion peptide by molecular modeling methods with the purpose of elucidating differences between fusion active and defective analogs. Our hypothesis is that although membrane fusion is a complex and fine-tuned process that often demands oligomerization of the polypeptide segments involved [12], the fusogenic activity should be encoded in the amino acid sequence, thus determining the peptides' mode of action at the bilayer-water interface. To 'decode' such specific features inherent in the fusion peptides, we made use of two independent approaches: (1) calculation of spatial hydrophobic properties using the method of molecular hydrophobicity potential (MHP) [13]; (2) exhaustive Monte Carlo (MC) simulations of the peptides with a solvation model which represents a hydrophobic layer surrounded by hydrophilic media, thereby imitating a fully hydrated biological membrane [14].

2. Materials and methods

The peptides under study were subdivided into two groups: with and without fusogenic activity; they are given in Table 1. The average hydrophobicity index ($\langle H \rangle$), maximal hydrophobicity moment ($|\mu_{max}|$, 9-residue window), and indices of α-helix (I_α) and β-sheet (I_β) were calculated, as described in [15,16]. The MHP created by peptide atoms in the surface points of helical segments was calculated and visualized by means of two-dimensional (2D) isopotential maps in coordinates (α,z) (α is the rotation angle around the helix axis, z coincides with the helix axis), as described earlier [17]. α-Helical structures used for the MHP calculations were the lowest-energy states found in the result of MC simulations in membrane-mimetic media (see below).

The heterogeneous three-layer membrane model used in this study is based on the combined use of atomic solvation parameters (ASP) for gas-cyclohexane and gas-water transfer, which mimic the hydrophobic core of a bilayer and hydrated head groups of lipids, respectively. The all-atom potential energy function and corresponding bulk solvent ASPs have been described elsewhere [14,18,19]. The bilayer thickness was 30 Å. Full-atom starting models of the peptides with an *N*-methyl group on the C-terminus were built in α-helical conformation and had different orientations relative to the membrane: transmembrane (TM), external, internal, and partly immersed in the bilayer. To change the orientation of the peptides with respect to the bilayer during MC simulation, fragments of 12 dummy residues were attached to their N-termini. The conformational space of the peptides was explored in non-restrained MC simulations in torsion angle space using the modified FANTOM program [20]. The ω dihedral angles were fixed (except those in the dummy residues), a spherical cutoff of 30 Å for non-bond interactions, and distance-dependent dielectric permeability $\varepsilon = 4 \times r$ were used. Before the MC simulation, all starting structures were subjected to 100 cycles of conjugate gradient minimization in the presence of heterogeneous solvent. The simulation length was 5000 MC cycles with the adaptive temperature schedule protocol [20]. At each step, one randomly selected dihedral was

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sampled, and the structure was minimized via 70 conjugate gradient iterations. Other details of the simulations can be found in [14,18,19]. Analysis of the results was done using auxiliary programs written especially for this.

3. Results and discussion

The choice of peptides studied in this work (Table 1) was motivated by their high degree of homology, as well as by the fact that all these peptides were tested in the same laboratory setup, in similar conditions. In addition, the spatial structure in DPC micelles of one of them, namely E5, was recently solved by NMR spectroscopy [21] and, therefore, theoretical predictions (at least for E5) could be checked against experimental data. In order to determine whether the specific features inherent in fusion peptides could be delineated based solely on the analysis of their sequences, in the next section we employ a number of commonly used parameters, like hydrophobicity and helicity indices, hydrophobic moment, etc.

3.1. Analysis of amino acid sequences with hydropathy methods

Amino acid sequences of the peptides, along with their average hydrophobicities ($\langle H\rangle$), maximal hydrophobic moments ($|\mu_{max}|$), and indices of α-helix (I_α) and β-sheet (I_β), are given in Table 1. Analysis of these data leads to the following conclusions. (1) All the peptides demonstrate a pronounced amphiphilic character (high values of $|\mu_{max}|$) and strong preference to adopt an α-helical conformation ($I_\alpha > 2.4$; $I_\beta < 2.2$). (2) Depending on the values of $\langle H\rangle$ and $|\mu_{max}|$, the peptides are assigned to the class of peripheral α-helices which usually associate with a bilayer surface [15]. (3) Analysis of I_α, calculated for 9-residue windows moving from the N- to the C-terminus of the sequences, reveals that all fusion peptides, as well as E5P, have a similar dependence on $I_\alpha(n)$ (n = number of the central residue in the window), while this is not the case for E5CC, E5NN, and E8 (data not shown). In addition, all the peptides demonstrate higher values of I_α in the N-terminal part. This means that under certain conditions the α-helical structure in the N-terminal half (residues 1–11) has a higher probability of occurring compared with that near the C-terminus (residues 14–18).

For peptide E5, the inferences made in the result of the analysis of its hydrophobicity properties (Table 1) agree well with the NMR data on its spatial structure in DPC micelles [21]. Therefore, the methods based on hydropathy scales for amino acid residues might be employed for a rough characterization of peptides, e.g. for discrimination between a TM or peripheral disposition, while these techniques do not delineate any specific features inherent in the fusion peptides but not in the others presented in Table 1. We propose that the reason for this lies in the too approximate description of hydrophobic characteristics of residues. A detailed assessment could be achieved if the heterogeneous nature of the side chains, their conformation and microenvironment are taken into consideration. To do this, we used the MHP approach [13], which has been successfully applied to a number of membrane-bound segments [17,22].

3.2. Spatial hydrophobic/hydrophilic properties of peptides

The formalism of MHP utilizes a set of atomic physicochemical parameters evaluated from octanol-water partition coefficients (log P) of numerous chemical compounds [13]. It permits detailed assessment of the hydrophobic and/or hydrophilic characteristics of various parts of the molecules. Previously, the MHP-based approach was applied to analyze the hydrophobic properties of membrane-bound peptides and to

Table 1
Hydrophobic characteristics of peptides and parameters of their interaction with a membrane: results of sequence analysis and MC simulations with the heterogeneous solvation model

Peptide [2,29]	Sequence[a]	Hydrophobic properties					Results of MC simulations		
		$\langle H\rangle$[b]	$\lvert\mu_{max}\rvert$[c]	I_α[d]	I_β[e]	ϕ[f] (deg)	α-helical fragments	$\langle z\rangle$[g] (Å)	$\langle\theta\rangle$[h] (deg)
Fusion active peptides									
HA	GLFGAIAGFI**E**GGWTGMI**D**G	0.60	0.34	2.42	2.15	70.7	2–19,2–18,2–17	1.46 ± 0.12	15.5 ± 1.2
E5	GLF**E**AIA**E**FI**E**GGW**E**GLI**E**G	0.47	0.54	3.47	1.34	80.6	2–19,2–18,2–17	1.67 ± 0.45	12.8 ± 3.0
E5L	GLL**E**ALA**E**LL**E**GGW**E**GLL**E**G	0.41	0.48	3.43	1.39	74.1	2–19,2–18	1.36 ± 0.36	10.3 ± 2.3
D4	GLFGAIA**D**FI**E**GGW**E**GLI**E**G	0.53	0.50	3.22	1.23	70.0	2–18,2–17	1.26 ± 0.28	18.0 ± 2.2
K5	GLF**K**AIA**K**FI**K**GGW**K**GLI**K**G	0.27	0.73	3.35	1.64	69.8	2–19,2–17	1.34 ± 0.14	16.2 ± 0.8
Fusion defective analogs									
E8	GLL**EE**LL**E**LL**EE**LW**EE**LL**E**G	0.26	0.60	3.90	0.23	90.9	2–19,2–18	2.37 ± 0.04	10.6 ± 0.4
E5CC	GW**E**GLI**E**GI**E**GW**E**GLI**E**G	0.40	0.44	2.91	1.74	92.5	2–18,2–17	2.99 ± 0.17	7.9 ± 1.2
E5NN	GLF**E**AIA**E**FI**E**AIA**E**FI**E**G	0.52	0.63	4.81	0.20	92.2	2–17	0.86 ± 0.07	13.2 ± 0.5
E5P	GLF**E**AIA**E**FIPGGW**E**GLI**E**G	0.51	0.48	3.37	1.30	–	2–9	2.04 ± 0.20	24.5 ± 0.8
							11–17/11–18	5.09 ± 0.19	−2.6 ± 0.6

[a]Charged residues are in boldface, glycines are underlined.
[b]$\langle H\rangle$, average hydrophobicity index.
[c]$|\mu_{max}|$, maximal hydrophobicity moment (window size 11 residues).
[d]I_α, index for α-helix.
[e]I_β, index for β-sheet.
[f]ϕ, tilt angle for the hydrophobicity pattern on the corresponding 2D MHP map (values of Z in Å, values of α in degrees). The angle is calculated between the axis X and the straight line $Z = a\times\alpha + b$ (a and b are coefficients) which minimizes the sum $|Z_i - a\times\alpha_i - b|$ for the set of points (Z_i,α_i) with MHP > 0.12 on the surface of the lowest-energy conformer found by MC simulation (ϕ values are given for the scale of the MHP map in Fig. 1). Data for E5P are not given because its lowest-energy conformer contains two helical segments. The values of coefficient a for peptides HA, E5, E5L, D4, K5, E8, E5CC, E5NN are: 0.22, 0.47, 0.27, 0.21, 0.21, −5.12, −1.80, −1.38 Å/deg, respectively.
[g]$\langle z\rangle$, average distance (with standard deviation) of the center of mass of the α-helical segment from the membrane surface; z is positive inside and negative outside the membrane.
[h]$\langle\theta\rangle$, average angle (with standard deviation) between the helix axis and the bilayer plane. θ is negative when the C-terminus is inside the membrane.

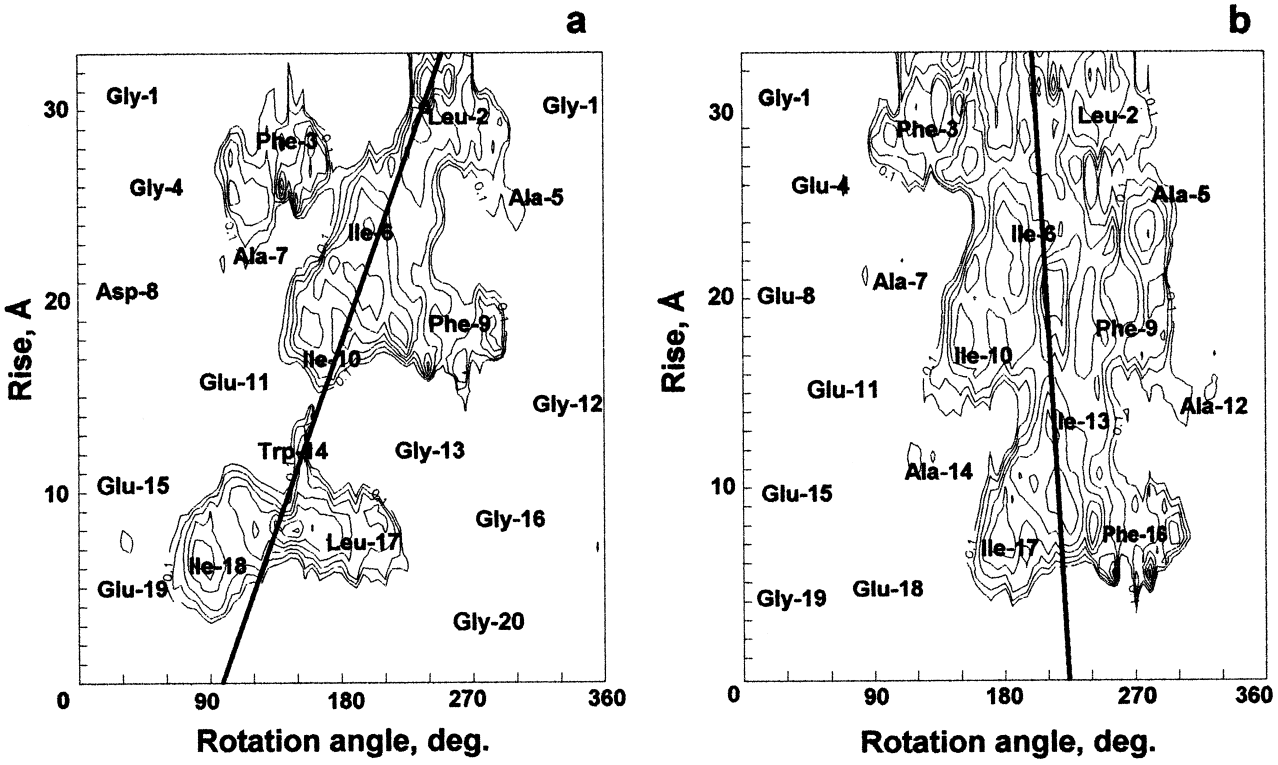

Fig. 1. Two-dimensional isopotential maps of the molecular hydrophobicity potential (MHP) on the surfaces of peptides D4 (a) and E5NN (b). Both peptides have α-helical structures, which correspond to the lowest-energy conformers found in the result of MC simulation (see Fig. 2). The value on the *x*-axis (α) corresponds to the rotation angle about the helix axis; the parameter on *y*-axis (Z) is the distance along the helix axis. Only the hydrophobic areas with MHP >0.100 au are shown. Contour intervals are 0.015. Letters and numbers indicate the positions of residues. Thick straight lines $Z = a \times \alpha + b$ minimize the sum $|Z_i - a \times \alpha_i - b|$ for the set of points (Z_i, α_i) with MHP >0.12.

calculate the location of TM helical hairpins [17,22]. To illustrate the results obtained with the MHP technique for the peptides under study, Fig. 1 displays 2D MHP isopotential contour maps (see Section 2) for the lowest-energy conformers found by MC simulations (see below) of peptides D4 and E5NN, which represent fusion active and defective analogs of HA, respectively. (Similar maps for the other peptides were also analyzed but, for shortness, the data are not shown.) It is seen that the N-terminal parts (residues 1–10) of the peptides are more hydrophobic than the C-terminal ones. The peptides with a 'hydrophobicity gradient' running along the helix axis are related to the group of 'oblique-oriented' peptides which cannot take an orientation parallel to the phospholipid acyl chains, and instead they insert at an angle of 30–60° at a membrane interface [7]. As a result, they facilitate the formation of inverse micelles within the bilayer, thereby favoring membrane destabilization and fusion, formation of TM pores, etc. A number of 'oblique-oriented' peptides are known to possess fusion activity, e.g. β-amyloid peptide, membrane destabilizing segments of prions, apolipoprotein A-II, simian immunodeficiency virus, etc.

As follows from Fig. 1, in spite of their common 'oblique-oriented' character, other details of the hydrophobicity distributions for peptides D4 and E5NN are rather different. Thus, the hydrophobic stretch on the surface of D4, created by the residues Leu-2, Phe-3, Ile-6, Phe-9, Ile-10, Trp-14, Leu-17, Ile-18 and spanning the whole helix length, forms an angle (ϕ) of about 20° with the helix axis (Fig. 1a, Table 1). For this type of MHP map we suggest the term 'tilted oblique-oriented pattern'. Like D4, four other fusion peptides studied here reveal the 'tilted oblique-oriented pattern' (2D MHP maps are not shown, although corresponding values of ϕ are given in Table 1). On the other hand, this is not the case for E5NN: its hydrophobic stretch (residues Leu-2, Phe-3, Ala-5, Ile-6, Phe-9, Ile-10, Ile-13, Phe-16, Ile-17) is disposed almost parallel to the helix axis (Fig. 1b, Table 1). Other non-fusion analogs of HA, E8, E5CC, E5NN, and E5P, also do not reveal the tilt of their hydrophobic stretches with the helix axis (Table 1). Therefore we conclude that for α-helical amphiphilic peptides the hydrophobicity gradient along the helix axis itself is not enough to provide fusogenic activity – this also requires the tilt of the hydrophobic stretch. We therefore propose to extend the classification of Brasseur et al. [7] by assigning fusogenic activity primarily to the peptides which have the 'tilted oblique-oriented pattern' or, in other words, exhibit MHP maps similar to that in Fig. 1a.

Along with an assessment of the hydrophobic/hydrophilic properties of peptides, it is interesting to explore their energetically favorable structures and orientations with respect to the bilayer. To check whether there are any differences in behavior of fusion and non-fusion analogs of HA in a medium which mimics a fully hydrated membrane, below we describe for them the results of MC simulations with the heterogeneous implicit solvation model [14].

3.3. Modeling of peptide-membrane interactions

The obtained results show that, whatever the choice of starting orientation with respect to the bilayer (see Section 2), the low-energy states for all studied peptides demonstrate a number of common features. Thus, at the water-membrane

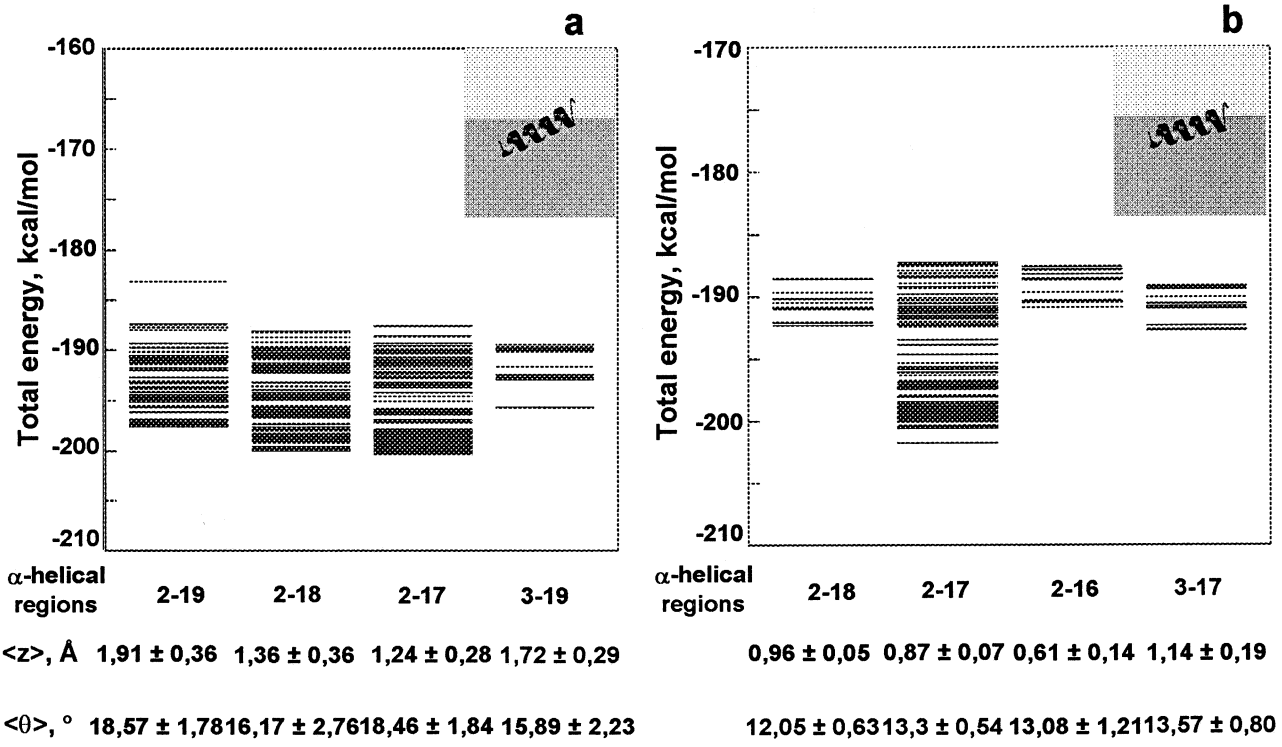

Fig. 2. Low-energy conformers of peptides D4 (a) and E5NN (b) obtained in the result of MC simulation in membrane-mimetic media. Top: Total energy levels for different states grouped according to the length of the α-helical segments. Boundaries of the α-helices are indicated below. On the insert the ribbon diagrams of the corresponding lowest-energy states are shown. Water and non-polar layers of the membrane are shown by light and dark gray hatching, respectively. Bottom: $\langle z \rangle$, average distance of the center of mass of the α-helical segment from the membrane surface; $\langle \theta \rangle$, average angle between the helix axis and the bilayer plane.

interface they are characterized by a high degree of helicity – from entire α-helices to α-helices slightly (1–2 residues) unfolded on the C-terminus (Table 1; Fig. 2). This agrees with the NMR data for peptide E5 in DPC micelles [21]. Typical dispositions of low-energy conformers with respect to the membrane, together with their energies, are shown in Fig. 2 for representatives of fusogenic and non-fusogenic analogs of HA, peptides D4 and E5NN. The simulations correctly reproduce the membrane-induced stabilization of the α-helical conformation – a phenomenon well known from the experiment in [23]. We should outline that all the low-energy conformers are immersed in the non-polar core of the membrane by the N-termini, while the C-termini are more exposed to polar media. This was expected because their N-terminal parts are more hydrophobic. Such orientations of the energetically favorable states correspond well to numerous experimental [24] and theoretical [25] data, which demonstrate preferential insertion of peptides into bilayers with their N-termini. Burial of the N- or C-terminus would require dehydration of NH or C=O groups, respectively, which do not participate in intramolecular hydrogen bonding. However, the dehydration of carbonyl groups is more energetically costly [25]. Therefore, N-terminal insertion is more favorable. In their low-energy states, all the peptides expose most of the polar and non-polar side chains to water and to the membrane, respectively. Finally, water-exposed C-terminal parts of the peptides demonstrate a lower stability of the α-helical conformation – this effect is also well known [26]. To summarize, the known trends of the peptides' behavior at the membrane-water interface are correctly reproduced in the framework of the two-phase solvation model employed here.

At the same time, to establish whether there are differences in behavior of fusion and non-fusion analogs of HA in the membrane, a more detailed analysis is required. To this end, we compared the following parameters for these two groups of peptides (Table 1): (1) average angle ($\langle \theta \rangle$) between the helix axis and the bilayer plane; (2) average depth of the peptide penetration into the membrane ($\langle z \rangle$ = average coordinate Z of the center of mass of the helical segment. Averaging was done over the low-energy states). It was found that fusion active and defective analogs of HA have similar values of $\langle \theta \rangle$, between 8° and 20°. It is important that $\langle \theta \rangle$ is close to that ($\sim$20°) observed for E5 by IR spectroscopy [27]. In contrast, the values of $\langle z \rangle$ for the two groups are significantly different (Table 1): for fusion peptides they lie on the interval 1.26–1.67 Å, whereas for the others they are beyond these values. Fig. 3 shows histograms of distributions of the low-energy states of two fusion (E5, E5L) and two non-fusion (E5CC, E5NN) peptides over the $\langle z \rangle$ values. It is seen that for E5NN $\langle z \rangle < 0.86$, while for E5CC $\langle z \rangle > 2.99$ Å. We propose that the membrane insertion depth is important for the peptide fusogenic activity: a certain degree of bilayer destabilization and consequent fusion occur when the center of mass of the helical peptide is buried in the non-polar core – at 1–2 Å from the interface. Probably, the two specific features found for fusogenic HA analogs – 'tilted oblique-oriented pattern' and a certain depth of penetration the into bilayer – are interrelated but this could not be delineated based on the presented data.

Qualitatively, the differences in depth of penetration could also be explained from the analysis of the sequences. For example, compared to E5, peptide E5NN has replacements

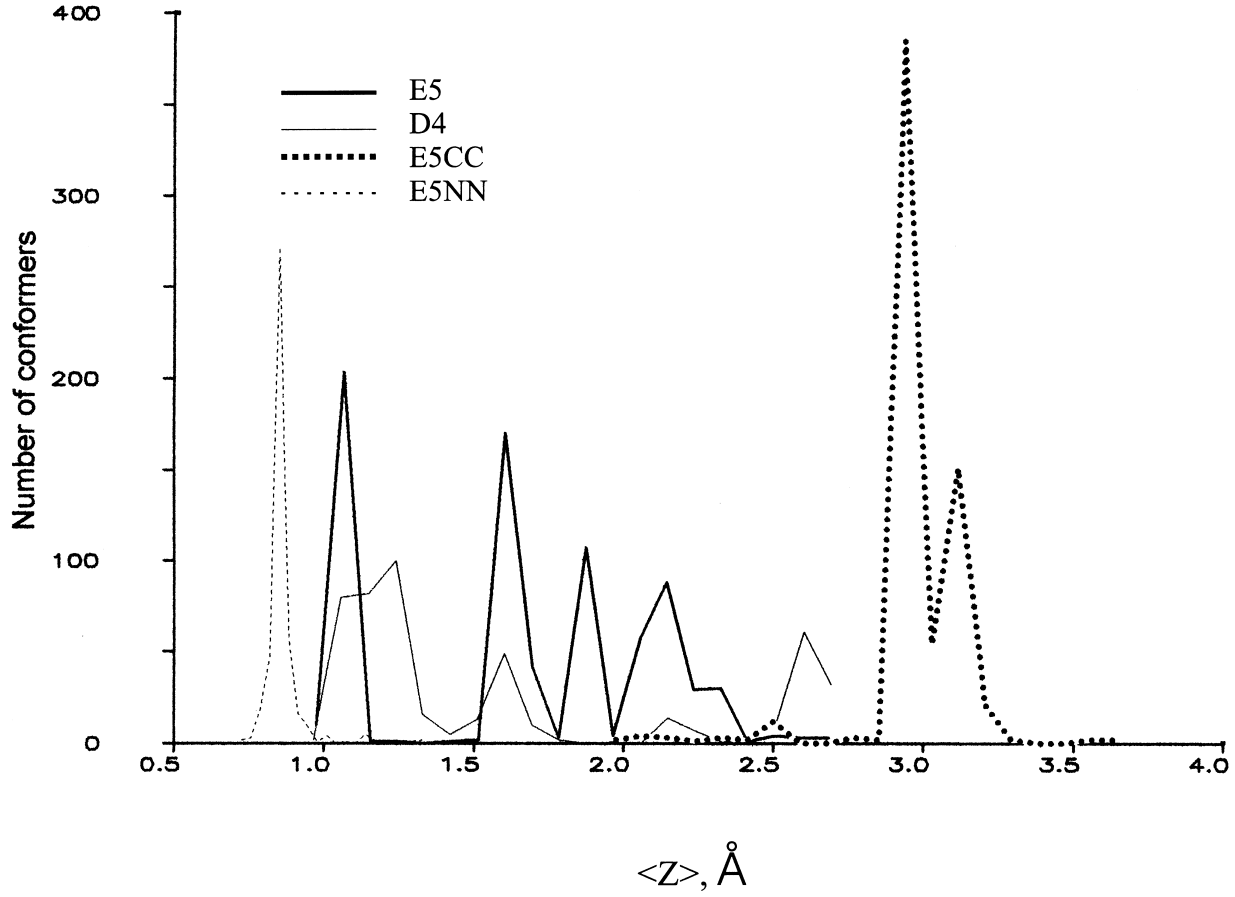

Fig. 3. Histograms of distributions of the low-energy conformers of fusion active and defective peptides obtained via MC simulations, over average distances of centers of mass of the α-helical segments from the membrane surface. Fusion peptides: E5 and D4; non-active peptides: E5CC and E5NN.

of polar Gly-12 and 13, as well as Trp-14 (in membrane-bound proteins tryptophans are preferentially observed on the water-bilayer interface [28]), to hydrophobic Ala, Ile, and Ala, respectively. It is reasonable to expect that this will lead to deeper immersion of E5NN in a bilayer. Similarly, two hydrophobic residues (Ala-5 and Phe-9) in E5 are replaced to glycines in E5CC, thereby inducing larger exposure of E5CC to water. Non-fusion peptide E8 is the most polar one and, therefore, it is most exposed to water. In contrast, the fusion peptide K5, possessing integral hydrophobic features similar to those of E8, is significantly immersed in the bilayer. Probably, this is due to the large size of its Lys side chains – at larger $\langle z \rangle$ their $NH3^+$ groups may still be exposed to the polar phase.

To summarize, the following conclusions were made in the result of this study.

1. Standard methods of sequence analysis based on hydropathy scales for residues might be employed for selection of amphiphilic peptides and a rough assessment of their structural and hydrophobic properties – e.g. for evaluation of the secondary structure, discrimination between TM or peripheral disposition, etc. However, a too approximate characterization of polarity properties of residues with these algorithms does not recognize the fusion active peptides among the other amphiphilic ones.
2. In accordance with previous observations [7], asymmetric distribution of hydrophobicity in peptides is a prerequisite for their ability to destabilize the membrane – all analogs of HA studied belong to the class of 'oblique-oriented' peptides. Their N-terminal parts are more hydrophobic and have larger helix propensities than the C-terminal ones. In addition to that, the fusion active analogs reveal a specific 'tilted oblique-oriented' pattern on their surface: a prominent hydrophobicity stretch running along the helix and tilted to its axis. Therefore, we conclude that not only the hydrophobicity gradient, but its tilt as well are responsible for the fusogenic activity of a given peptide.
3. All peptides studied demonstrate a number of common features at the water-membrane interface: a high degree of helicity; exposure of non-polar and polar groups to the hydrophobic core and water, respectively; N-terminal insertion with similar angles to the bilayer plane. At the same time, fusion active peptides penetrate the membrane into a certain depth.

Based on the results obtained, we propose the following algorithm for recognizing the segments with potential fusion activity in protein sequences: (1) identification of membrane-bound regions, their building in an α-helical conformation; (2) calculation of 2D MHP maps; (3) checking the maps for the presence of the 'tilted oblique-oriented' pattern specific for fusion peptides, selecting the peptides which fit it; (4) MC simulation of found segments in membrane-like media, anal-

ysis of the low-energy states (degree of helicity, orientation with respect to the membrane). Peripheral α-helices with values for $\langle z \rangle$ and $\langle \theta \rangle$ close to those found here for fusion peptides are assigned fusogenic activity.

The systems studied here are too rough to represent the details of the fusion mediated by the influenza virus hemagglutinin: involvement of other protein parts apart from HA, pH dependence, changes in the bilayer, etc. The proposed approach is considered to be a first step toward understanding this process – even regardless of the limited set of peptides, the theoretical analysis permits delineation of several factors which might be important for fusogenic activity, and selection of potential fusion peptides. We are currently working on using the presented approach for studies of other series of peptides with and without fusogenic activity, as well as modeling peptide oligomers in a membrane-like environment which are thought to mediate membrane fusion.

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