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Orientation into the lipid bilayer of an asymmetric amphipathic helical peptide located at the *N*-terminus of viral fusion proteins

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The complete amino-acid sequence of viral fusion proteins has been analyzed by the Eisenberg procedure. The region surrounding the cleavage site contains a highly hydrophilic region immediately followed by a membrane-like region. Since the effective cleavage between these two domains seems required to expose the fusogenic domain (located at the N-terminal sequence of the transmembrane like region) which is assumed to interact with the lipid membrane of the host cell, we have focused our analysis on the conformation and mode of insertion of this membrane-like domain in a lipid monolayer. It was inserted as an α-helical structure into a dipalmitoylphosphatidylcholine (DPPC) monolayer and its orientation at the lipid/water interface was determined using a theoretical analysis procedure allowing the assembly of membrane components. For each viral protein sequence these N-terminal helical segments oriented obliquely with respect to the lipid/water interface. This rather unusual orientation is envisaged as a prerequisite to membrane destabilization and fusogenic activity.

Introduction

Many enveloped viruses penetrate into the host cell by fusion of the viral envelope with the host cell plasma membrane or with the endosomal membrane. Envelope proteins of paramyxoviruses, influenza virus and some retroviruses have been shown to mediate membrane fusion processes involved in viral penetration, hemolysis and syncytia formation [1]. Most viral fusogenic proteins contain a short amino terminal hydrophobic segment which has been proposed to interact with lipid membrane during the fusion process. For several viruses [2–5] mutagenesis studies confirmed that this N-terminal hydrophobic sequence is critical for fusion. The interaction of the N-terminal hydrophobic segment with a cell membrane has been documented [6,7] and synthetic peptides have been used to demonstrate their fusogenic effects on liposomes [8,9].

However, a molecular model tentatively depicting the interaction with the host membrane is still lacking. Here, we bring evidence that a peptide sequence located at the N-terminal region of the fusion protein of viruses adopts a quite unusual oblique orientation when inserted into a lipid bilayer. This new class of membrane penetrating segment is expected to destabilize the host membrane and to generate new lipid phases which might be associated with initial events of membrane fusion between virus and host cell. Peptide segments were identified according to the Eisenberg methodology and their orientation in the lipid layer was calculated according to a procedure described below.

Methods

Hydrophobicity analysis

The hydrophobic moment of a protein segment was calculated according to the procedure of Eisenberg et al. [10–13] using the following equation.

$$\mu_H = \left\{ \left(\sum_i^{i+n-1} H_i \sin(\delta_i) \right)^2 + \left(\sum_i^{i+n-1} H_i \cos(\delta_i) \right)^2 \right\}^{1/2} \quad (1)$$

where μ_H is the hydrophobic moment calculated at an angle δ, H_i is the hydrophobicity of residue i and n is the total segment-length used in the calculations. As the hydrophobic moment is a function of the periodicity in the protein structure, this parameter was calculated

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assuming different values for the δ angle at which the side chains emerge from the backbone, when the periodic segment is viewed down its axis. The normalized 'consensus' hydrophobicity scale, developed by Eisenberg [11–13] was used in all calculations. Therefore hydrophobicity values were expressed in arbitrary units, and hydrophobic moments were plotted on an arbitrary scale. A segment of n amino acids was moved through the protein sequence and the mean hydrophobicity and mean hydrophobic moment per segment were calculated. These two parameters were plotted as a function of the midpoint of, the amino acid segment along the sequence.

Conformational analysis

A. Structure and orientation of the isolated molecule. The method classicaly used to study the conformation of peptides is modified to take into account the variation in the dielectric constant and the transfer of atoms from an hydrophobic to an hydrophilic environment which characterize the lipid/water interface [14]. The total conformational energy is calculated as the sum of the contributions resulting from the Van der Waals interactions, from the torsional potential energy, the electrostatic interactions and the transfer energy. To limit the number of possible degrees of structural freedom, bond lengths and bond angles were assumed to be constant. The values used are those commonly used in conformational analysis [15].

Buckingham's pairwise atom-atom interaction functions have been used to evaluate the London-Van der Waals energy:

$$E^{\mathrm{VdW}} = \sum \left(A_{ij} \exp(-B_{ij} r_{ij}) - C_{ij} r_{ij}^{-6} \right) \tag{2}$$

where $ij = 1, 2, \ldots$ are non-bonded atoms, r_{ij} their distances from each other, and A_{ij}, B_{ij} and C_{ij} are coefficients assigned to atom pairs. The values of these coefficients have been reported by Liquori and coworkers [16,17]. In order to compensate for the decrease of the function E^{VdW} at small r_{ij}, we have imposed an arbitrary cut-off value of:

$$E^{\mathrm{VdW}} = 418.4 \text{ kJ/mol at } r_{ij} < 0.1 \text{ nm}$$

At distances comparable to the molecular size, it becomes less accurate to deal with point dipoles rather than with atomic point-charge distributions. The coulombic interaction energies corresponding to such distributions include all higher-order terms (quadrupoles, octopoles, ...) which are usually neglected. Formally, the energy can be written as follow:

$$E^{\mathrm{cb}} = 139.2 \left(\sum \frac{e_i e_j}{r_{ij} \epsilon_{ij}} \right) \tag{3}$$

where e_i and e_j are expressed in electron charge units and r_{ij} in Å. ϵ_{ij} is the dielectric constant. To simulate the membrane interface, we assumed a dielectric constant equal to 3 above the interface, while a plane was drawn at the atom most deeply immersed in the aqueous phase, with a dielectric constant of 30. Between these two planes, the dielectric constant increases linearly along the z-axis perpendicular to the interface [14]. The values of atomic point charge are similar to those used for polypeptides [15].

The rotation around the C–C or C–O bonds was calculated according to the equation:

$$E^{\mathrm{Tor}} = \frac{U_{ij}}{2} (1 + \cos \Omega_{ij}) \tag{4}$$

where U_{ij} corresponds to the energy barrier in the eclipsed conformation during the rotation of the angle, and Ω_{ij} is the torsional angle. U_{ij} is equal to 11.7 kJ/mol for the C–C bond and 7.5 kJ/mol for the C–O bond.

The values of the transfer energies used ($E^{\mathrm{Tr}}i$) are similar to those determined experimentally by several authors as summarized elsewhere [18].

$$E^{\mathrm{Tr}} = \sum \delta E_k^{\mathrm{Tr}}$$

where δE_k^{Tr} is the transfer energy of the chemical group k from the hydrophobic into the hydrophilic domain.

The molecule is finally oriented at the interface in such a way that the line joining the hydrophobic and hydrophilic centers is perpendicular to the interface [14].

B. Monolayer formation [19,20,21]. Position and orientation at the interface of the peptide was calculated as described in part A. Its position was maintained fixed at the origin of the x, y, z referential during the monolayer assembly. The structure and the orientation of the dipalmitoyphosphatidylcholine molecule at the interface was calculated as described earlier [14]. DPPC was allowed to move along the x axis in steps of 0.05 nm. For each position the DPPC molecule was rotated in steps of 30° around its long axis z' and around the first molecule. l is the number of positions along the x axis, m the number of rotations of the second molecule around the first one and n is the number of rotations of the molecule itself. For each set of values of l, m and n, the intermolecular energy of interaction was calculated as the sum of the London-Van der Waals energy of interactions, the electrostatic interaction and the transfer energy of atoms or groups of atoms from an hydrophobic to an hydrophilic phase. Then, the second molecule was allowed to move in steps of 0.05 nm along the z' axis perpendicular to the interface and the position of the z' axis was varied in steps of 5° with respect to the z axis, such that the

lowest interaction energy state could be obtained for each set of values l, m and n. The energy values together with the coordinates associated to each set of l, m and n, were stored in a hyper matrix and classified according to decreasing values of the interaction energy. The position of the third molecule C is defined as the first energetically favorable orientation stored in the hyper matrix but taking into account the sterical and energetic constraints imposed by the presence of the second molecule. Thus, orientations are disregarded in which overlap of atomic coordinates of two molecules occurs and in which the interaction energy between the two molecules was positive. In order to minimize further the conformational energy, the position of the second and third molecule are then alternatively modified in steps according to the energy classification of the hyper matrix. For the fourth molecule the same process is repeated but now the positions of the three surrounding molecules are modified alternatively in order to find the lowest energy state. In this calculation, the interaction energy between all monomers in the aggregate are considered and minimalized till the lowest energy state of the entire aggregate is reached. We limited this approach to the number of molecules sufficient to surround one central molecule (peptide).

All calculations were performed using an Olivetti CP486, and as software the PC-TAMMO + (Theoretical Analysis of Molecular Membrane Organization), PC-PROT + (Proteins Plus analysis), and the PC-MSA + (Molecular structure analysis) procedures. Graphs were drawn with the PC-MGM + (Molecular Graphics Manipulation) program.

TABLE I

Amino acid sequences of all viruses analysed

Sequences were obtained from 'Swiss-prot protein data bank' and Human retroviruses and AIDS 1988 Edited by Myers, G. Rabson, A.B., Josephs, S.F. Smith, T.F. and Wong-Staal, F. National Cancer Institute. Small letters correspond to the hydrophobic amino acids, capital letters to the hydrophilic amino acids. (...) is the putative cleavage site.

HXB2- BH10- BH8-	
HXB3- PV22- BRU-	KRRvvQREKR..avGi Galf lGfl Gaa GSTm Ga
MAL- ELI- Z6-	KRRvvEREKR..aiGl Gamf lGfl Gaa GSTm Ga
SF2- WMJ1-	KRRvvQREKR..avGiv Gamf lGfl Gaa GSTm Ga
WMJ2- WMJ3- RFENV-	KRRvvQREKR..avGTi Gamf lGfl Gaa GSTm Ga
LAV1A-	KRRvvQREKR..avGi Galf lGfl Gaa GSTmaG
HAT3-	KRRvvQREKR..avGT Galf lGfl Gaa GSTmaG
CDC4-	KRRvvQREKR..avGml Gamf lGfl Gaa GSTm Ga
NDV-	TTSGGRRGRR..fiGaii GSva lGva Taa GiTaaSa
D26-	TTSGGGKQGR..liGaii GGva lGva Taa QiTaaaa
1S-	TTSGGGRQGR..liGaii GGva lGva Taa QiTaaSa
BEA-	TTSGGRRQKR..fiGaii GGva lGva Taa QiTaaaa
A-V-	TTSGGRRQKR..fiGaii GSva lGva Taa QiTaaSa
ITA- MIY-	TTSGGRRQRR..fiGaii GSva lGva Taa QiTaaSa
MEASLES- MEASLESHU2 MEASLESR93	SvaSSRRHKR..faGvvlaGaa lGva Taa QiTa Gial
BOVLV-	SSappTRvRR.. SpvaalT lGlalSv GlT GiNvav
SIV-	TTGGTSRNQR.. GvfvlGf lGflaTaG Sai Gaa
HTLVIV-	TTGGTSRNKR.. GvfvlGf lGflaTaG Sai Gaa
MLVMO-	lfERSNRHKR.. EpvSlTlal lGGl TmG Giaa GiGTGT
MLVAV-	QfERRaKyKR.. GpvSlTlalllGGl TmG Giaa GvGTGT
MLVKI-	RRaRyKK.. EpvSlTlalllGGl TmG Giaa GvGTGT
FSVGA-	HfaKaaRfRR.. EpiSlTvalmlGGl TvG Giaa GvGTGT
FSVST-	HfDKTvRlRR.. EpiSlTvalmlGGl TvG Giaa GvGTGT
MSVFB-	QfKRRaKyKR.. EpvSlTlalllGGl TmG Giaa GvGTGT
MCFFM-	fEKKKTKyKR.. pvSlTlalllGGl TmG Giaa GvGTGTT
AVIRE-	iEyTaGRHKR..avQfi pllv GlGiTGa TlaG GTGlGv
SENDAI-	TQNaGvpQSR..ffGavi GTia lGva TSa QiTa Gial
PF3-	NQESNENTQpRTKR..ffGGvi GTia lGva TSa QiTaavalv
SV5	RNQlipTRRRRR..faGvvi Glaa lGva Taa QvTaavalv
RSV-	TlSKKRKRR..flGfll GvGSaiaS GvavSKvl HlE
VILV-	RRSRRNlQRKKR..GiGlvivlaimaiiaaaGaGlGvaNav
HTLVI-	vpTlGSRSRR..avpvavwlvSalamGaGvaGGiTG
HTLVII-	vpppaTRRRR..avpiavwlvSalaaGTGiaGGvTG

Results

The amphiphilicity can be expressed in terms of the mean helical hydrophobic moment and the mean hydrophobicity as proposed by Eisenberg [10]. This procedure allows to classify transmembrane helices, surface-seeking helices and helices from globular proteins.

When the mean hydrophobicity (H_i) and the mean hydrophobicity moment (μ_H) per segment were plotted

Notes to Table I:

HIV:	: human immunodeficiency virus (HXB2,BH10,BH8,HXB3,PV22,BRU,MAL,ELI,SF2, WMJ1,WMJ2,WMJ3,Z6,CDC4)
NDV	: Newcastle disease virus (LS,D26,BEA,A-V,ITA,MIY)
MEASLES	: MEASLES virus (MEASLESHU2,MEASLESR93)
BOVLV	: bovine leukemia virus
SIV	: Simian (macaque) immunodeficiency virus
MLVMO	: Moloney murine leukemia virus
MLVAV	: AKV murine leukemia virus
MLVKI	: Kirsten murine leukemia virus
MSVFB	: FBJ murine osteosarcoma virus
MCFFM	: Mink cell focus-forming murine leukemia virus
FSVGA	: Feline leukemia virus (Strain Garner-Arnstein)
FSVST	: Feline leukemia virus (Strain Snyder-Theilen)
AVIRE	: Avian reticuloendotheliosis virus
SENDAI	: Sendai virus (PF3,SV5)
HTLVI	: human T-cell leukemia virus
HTLVII	: human T-cell leukemia virus (HTLV-IZ)
VILV	: Visna lentivirus (strain 1514)

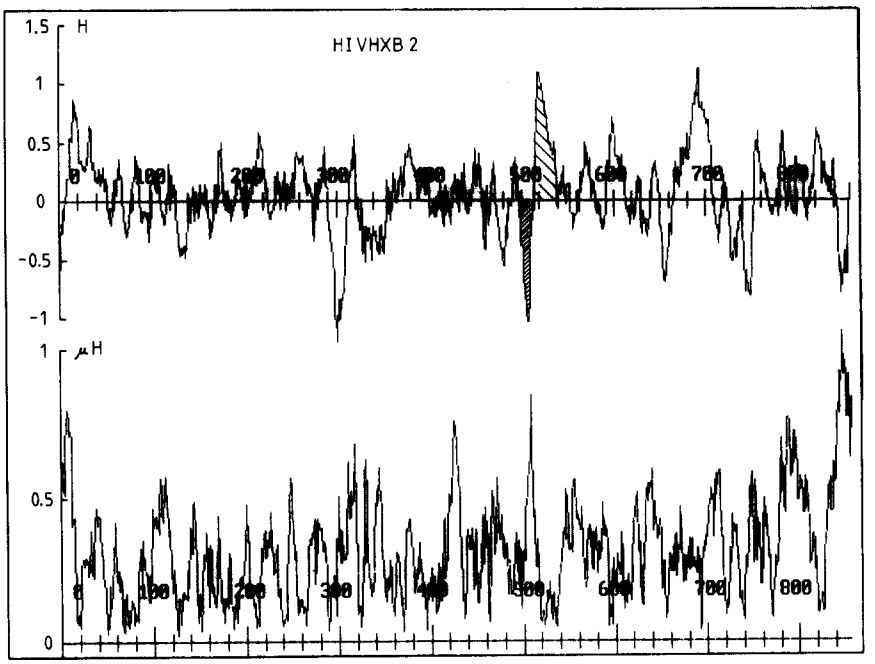
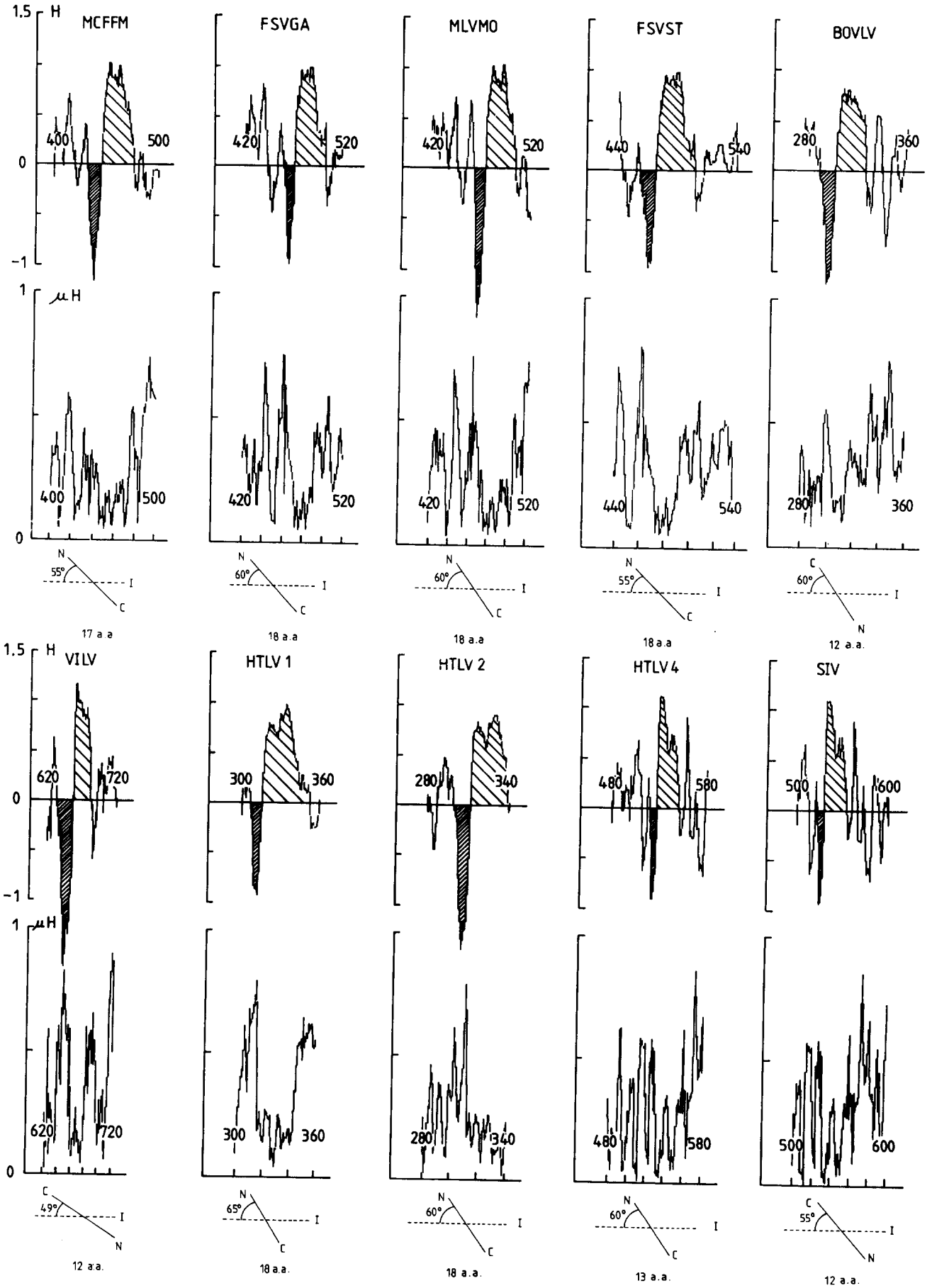


Fig. 1. Hydrophobicity profiles. μ_H and H_i of HIV (HXB2) protein were plotted as a function of the midpoints of a 7-residues long segment along the protein sequence (X-axis). The left shaded area corresponds to the "cleavage activation domain" and the right shaded area to the hydrophobic N-terminus.

against the amino acid sequence of the envelope fusion proteins (Table I), one main membrane segment directly adjacent to a highly hydrophilic domain was identified as illustrated for HIV (HXB2) and other viruses (Figs. 1 and 2). The high hydrophobicity ($H_i \geq 0.5$) and the low hydrophobic moment ($0 \leq \mu_H \leq 0.4$) calculated for the amino N-terminus segment has been shown to characterize transmembrane structures, as demonstrated for the seven transmembrane helices of bacteriorhodopsin [11] and cytochrome *b* [22]. Close to this transmembrane peptide, one cluster of residues showed a very low hydrophobicity ($H_i = -1.0$) and a high hydrophobic moment ($\mu_H = 0.7$) which might correspond to the domain recognized by the activating host proteinase. Similar domains were identified in Apo E as recognized by the Apo B-E receptor [13]. For all proteins analysed, a 7-residues-long window, calculated according to the procedure described by De Loof et al. [23], was moved through the sequence, since it gave the lowest noise in the μ_H and H_i profile. It should be pointed out that the δ angle, which depends on the secondary structure of the protein segment, was fixed at 100 degrees which is its value in the α-helix. We tried to give a molecular description of the mode of insertion of the N-terminus fusogenic segment in a lipid bilayer. In a tentative model, peptides were allowed to adopt an α-helical conformation consistent with Garnier prediction [24] and with the fact that in the case of fusogenic and signal peptide a hydrophobic medium favors this α-helical secondary structure [8,9,25–27] which seems by far most frequent in membrane penetrating segments of proteins [21].

Relative positions and orientations of the peptides at the lipid/water interface were calculated for increasing peptide length. A maximum deviation between the orientation of the helix axis and the lipid/water interface occurs for a peptide length varying between 13 and 18 residues (Fig. 2). Calculation of insertion of the asymmetric amphipathic peptides into the lipid matrix was carried out as described [20]. The helix is neither oriented parallel to the lipid/water interface, as observed for amphipathic helices [21] nor perpendicular to the interface, as observed for Gramicidin A [19,21] and transmembrane protein segments in general [22] but is oriented obliquely with respect to the lipid/water interface. The asymmetric distribution of the hydrophobic and hydrophilic residues along the helix axis observed in more than 20 viral protein sequences is assumed to

Fig. 2. Part of the hydrophobicity profiles. μ_H and H_i of 10 viruses were plotted as a fonction of the midpoints of a 7-residues-long segment along fusion protein sequence (*x*-axis). The left shaded area corresponds to the 'cleavage activation domain' and the right shaded area corresponds to the hydrophobic N-terminus. Orientation of the peptide at the lipid/water interface is shown at the bottom. Dotted line delineates the interface between the hydrophobic (above) and the hydrophilic (below) medium. The number of amino acids, the angle between the helix axis and the interface and the N and C terminus of the peptide are indicated.


1.5
H
MCFFM
400
500
0
-1
1
μH
400
500
0
N
55°
I
C
17 a.a
FSVGA
420
520
N
60°
I
C
18 a.a
MLVMO
420
520
N
60°
I
C
18 a.a
FSVST
440
540
N
55°
I
C
18 a.a
BOVLV
280
360
C
60°
I
N
12 a.a.
1.5
H
VILV
620
720
0
-1
1
μH
620
720
0
C
49°
I
N
12 a.a.
HTLV 1
300
360
N
65°
I
C
18 a.a.
HTLV 2
280
340
N
60°
I
C
18 a.a.
HTLV 4
480
580
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60°
I
C
13 a.a.
SIV
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12 a.a.

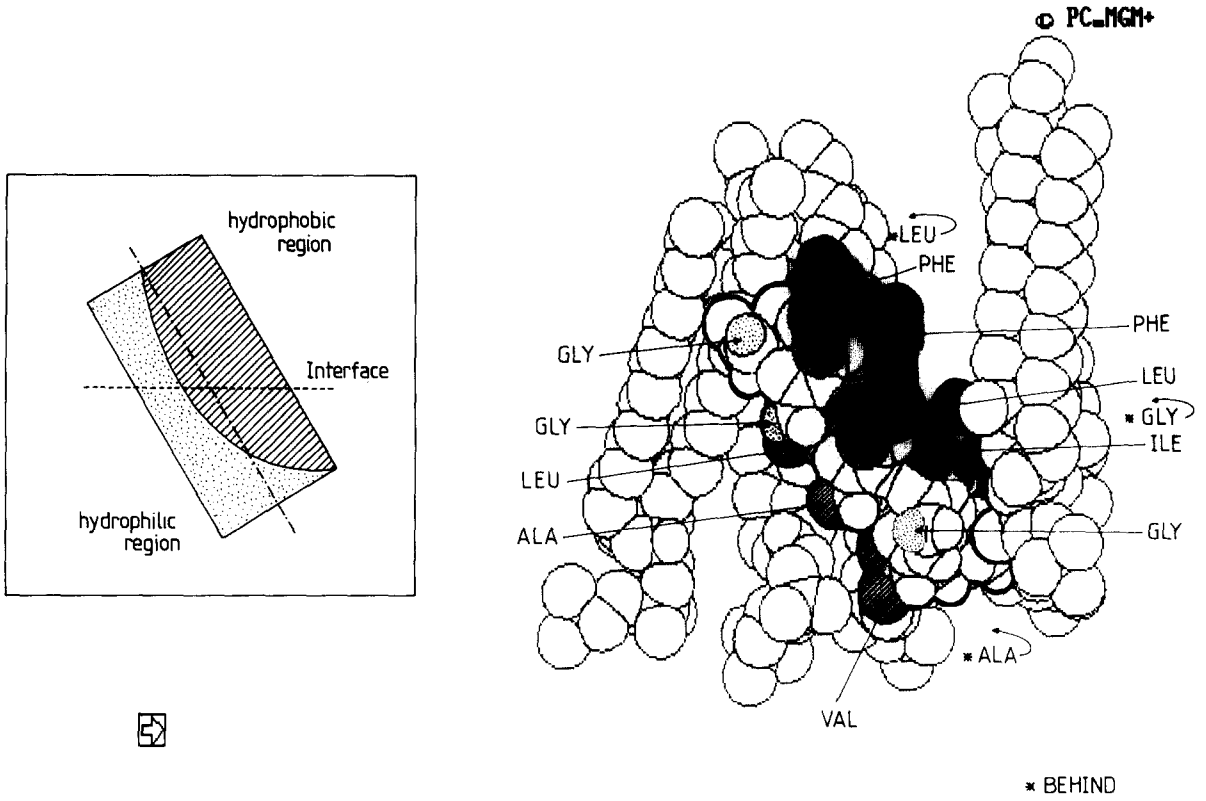

Fig. 3. Right: Space filling representation of the mode of insertion of HXB2 fusion N-terminus peptide in lipid matrix (dipalmitoylphosphatidylcholine). The dotted atoms refer to the hydrophilic amino acids, hatched atoms to the hydrophobic amino acids, and the open atoms to the backbone. The broken line delineates the lipid/water interface. Only four phospholipid molecules are displayed. Left: Schematic representation of the hydrophobic and the hydrophilic amino acid distribution in the asymmetric amphipathic helix oriented at the lipid/water interface. Shaded and dotted areas correspond to the hydrophobic and hydrophilic regions, respectively.

be responsible for the oblique orientation of the peptide into the lipid bilayer and perhaps for the first step in the fusion process.

Discussion

The amino acid sequence of a series of viral envelope proteins was analyzed in terms of hydrophobicity (H_i) and hydrophobicity moment (μ_H) using a 7 amino acid window. For this study the secondary structure was assumed to be fully α-helical, an approximation used in the method originally developed by Eisenberg [10]. A secondary structure prediction [24] was performed for all the viral sequences analyzed here, showing an α-helical structure to be most probable. Even though predictive methods are generally considered to give a correct estimate in only 50–55% of cases, Garnier method has a marked tendency toward an underestimation of helical content in membrane peptide. The choice of an α-helical structure is partly justified by the fact that fusogenic sequences are expected to adopt such a conformation when inserted into a membrane since most intra-membrane segments of proteins investigated to date seem to adopt an α-helical structure in a lipid environment. Synthetic peptides corresponding to the NH_2 terminus of influenza HA-2 were shown by circular dichroism, to increase their helicity when interacting with lipids [8,9]. The same behaviour was observed with a 30 amino acid peptide designed to mimic the properties of viral fusion proteins [28]. The envelope glycoproteins we have analyzed display the same characteristic in their Eisenberg profile (Fig. 2): a 10–15 amino acid segment with high hydrophobicity ($H_i > 0.5$) and low hydrophobicity moment ($\mu_H < 0.5$), immediately preceded by a highly hydrophilic region. Interestingly, this characteristic Eisenberg profile was not observed for Rous Sarcoma Virus which does not induce syncytia formation. This highly hydrophilic domain could allow the accesssibility of this site to a proteinase. As far as we know, cleavage by a proteinase seems a prerequisite to the maturation of most of these envelope glycoproteins. Because we found the same topology in all envelope proteins and since it has been established in several cases that the hydrophobic sequence following the cleavage site is essential for virus-cell fusion, we tried to give a molecular description of the possible mode of insertion of this region in a bilayer membrane. The oblique orientation found for all the fusogenic sequences studied here is a consequence of the asymmetric distribution of the amino acids along the helix axis [29,30].

We have not identified this kind of amino acid distribution in any membrane peptide or protein so far except for signal sequences [25] and we postulate therefore that it could be a feature of fusogenic sequences or more generally sequences which are able to destabilize the membrane lipid bilayer. Our model predicts that as a consequence of the peptide-phospholipid interaction, the parallelism between the phospholipid acyl chains is affected, some of the acyl chains in close contact with the peptide becoming tilted. Such a lipid desorganization could prefigure a more dramatic change in the lipid ordering giving rise to new lipid phases which are thought to be associated with initial events of membrane fusion [31].

Although in recent years it has become obvious that specific membrane proteins play a crucial role in membrane fusion, we know from several studies that lipids are actively involved in this process. It is indeed difficult to imagine that the bilayer structure of membrane lipids at the fusion interface is continuously preserved during this step. Transient lipid organizations have been suggested by a large number of observations made on model membranes to be required for membrane fusion [31]. The role of membrane components and more specifically the role of lipids could explain the varying susceptibility of different cell types towards fusion when using a single viral strain. All the viruses analyzed here do promote syncytia formation at neutral pH when using the appropriate cell line.

In conclusion, the conformational analysis of fusogenic sequences may help to clarify on a structural basis what are the main features necessary for fusion initiation. Of major interest is the possibility to design mutants devoid of fusogenic activity and to test them by the methods of molecular biology. Although the conformational analysis described here is based upon highly simplifying hypotheses, it may be used as a tool to explore the field of protein-lipid bilayer interaction.

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