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Membrane destabilizing activity of influenza virus hemagglutinin-based synthetic peptide: implications of critical glycine residue in fusion peptide

Tomoharu Matsumoto*

Division of Molecular Biology and Information I, Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011 Japan

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Abstract

Peptide III is a 20-residue synthetic model peptide based on the fusion peptide of influenza virus A/PR/8/34 strain and takes a secondary structure similar to the original peptide. While conserving the amphiphilic helical nature, 20 peptides to modify the bulkiness of side chains of peptide III were synthesized, and acid-induced membrane destabilization was assessed by aqueous content leakage from large unilamellar vesicles. Substitutions on the hydrophobic side decreased activity but showed less effect on the hydrophilic side, which confirmed the importance of the hydrophobic side for interaction with the membrane. Interestingly, substitution at the 13th Gly residue enhanced the amphiphilic helical nature but severely reduced activity. Correlation between α-helical content at acidic pH and the activity was not recognized, suggesting rather that the importance of this site was due to helix termination by glycine which allows N-terminal and C-terminal halves to behave as different secondary structural units.

Keywords: Amphiphilic alpha-helix; Fusion peptide; Glycine termination; Influenza virus; Membrane destabilization; Side chain bulkiness

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CD, circular dichroism; DPX, *N*,*N*′-*p*-xylenebis(pyridinium bromide); PC, phosphatidylcholine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HPLC, high pressure liquid chromatography; LUV, large unilamellar vesicle

* Fax: +81-774-38-3256; e-mail: matumoto@bms.Kuicr.Kyoto-u.ac.jp

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1. Introduction

After its endocytosis into the host cell, an influenza virus releases a viral genome into the cytoplasm with the fusion of viral envelope and endosome membrane [1,2]. This membrane fusion process is triggered by the reduction of pH in the endosome and accomplished by the viral membrane glycoprotein hemagglutinin. Hemagglutinin exists as a homotrimer of disulfide-bonded peptide chains HA1 and HA2 [3]. HA1 contains the binding site to sialic acid receptors on the host cell membrane. The highly conserved segment at the N-terminus of HA2 consists of approximately 20 amino acid residues. This hydrophobic segment is essential for membrane fusion and called 'fusion peptide'. Although X-ray crystallography of the hemagglutinin ectodomain at neutral pH showed that the fusion peptide was buried in protein [4], it was suggested that a large conformational change in HA2 taking place under mildly acidic conditions might move the fusion peptide to a position near the membrane to interact with it [5,6].

The formation of a tiny fusion pore or local-point fusion architecture through the two adjacent bilayers is believed to be necessary for membrane fusion [2,7]. Models of the membrane fusion process mediated by hemagglutinin and intermediate membrane structures have been proposed [8–13]. Accumulating evidence suggests that the fusion peptide is insufficient for the complete fusion process, and that the whole hemagglutinin protein may be required. Fusion peptides interact only with outer leaflets of the bilayers [8]. It is necessary for a virus particle to bind to the target membrane through HA1 for an efficient fusion event with less leakage [14]. The cleaved ectodomain of hemagglutinin can not induce membrane fusion [15]. The ectodomains artificially anchored to a liposome induce only merging of outer leaflets but not complete fusion [9]. The viral transmembrane domain of HA2 contains the site necessary for the fusion activity [16]. To date, it is supposed that assembly of three or more trimers would be necessary for the pore formation [2,17]. Despite the above evidence, the participation of the fusion peptide in the membrane fusion process is undoubted, especially at the early stages of interaction when the stable phospholipid bilayers destabilize prior to the reorganization.

The fusion peptide of influenza virus hemagglutinin contains many hydrophobic residues and small ones such as glycine and alanine. It has been suggested that this peptide binds to the membrane taking an amphiphilic α-helical conformation or 'sided' α-helix with the hydrophobic surface consisting of bulky residues and the hydrophilic surface of relatively small residues [2,18,19]. The correlation of activity and α-helical structure has been discussed by many authors [20–25]. To investigate how the fusion peptide interacts with the lipid bilayers, synthetic peptides with the same amino acid sequence as the viral fusion peptide or idealized ones have been utilized [20,21,26]. Peptide III is one such model peptide which has a 20-residue-long amino acid sequence based on the fusion peptide of influenza virus A/PR/8/34 strain (Fig. 1). Peptides with the native sequence are extremely aggregative and show limited solubility in aqueous media. With the substitution of glutamic acid for some residues in the hydrophilic surface and a little sequence simplification to improve the amphiphilic nature, peptide III shows enhanced solubility and the same secondary conformational character as the original peptide [19,27]. Previous research suggested that an α-helical conformation would be necessary but not sufficient for peptide III to interact with liposomes. Other features such as the distribution of bulkiness of the side chains seem to correlate with the activity [28].

In this work, further research to elucidate such attributes was performed: conserving the amphiphilic nature of the peptide, versions of peptide III modified to change the bulkiness of the side chains were synthesized and their membrane destabilizing activities were compared to that of the parent peptide III. For this purpose, basically an alanine scan substitution was performed, but alanine or glycine was changed to leucine on the hydrophobic side of the supposed α-helix and to glutamine on the hydrophilic side. Results confirmed that substitution on the hydrophobic side was more effective for the interaction with LUVs

a)

A/PR/8/34	GLFGAIAGFIEGGWTGMIDG
peptide III	GLFEAIAEFIEGGWEGLIEG
G13L	GLFEAIAEFIEGLWEGLIEG

b)

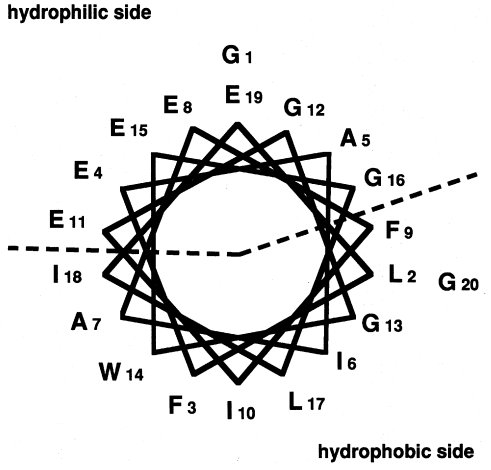

Fig. 1. (a) Amino acid sequences of the peptides and (b) the helical wheel representation of peptide III. The design of peptide III was based on the amino acid sequence of the fusion peptide of influenza virus A/PR/8/34 strain to enhance its amphiphilicity. Substitution of leucine residue for the 13th glycine residue in peptide III produced G13L.

than on the hydrophilic. In addition, it was newly shown that substitution at Gly13 enhanced the α-helical nature of the peptide but severely reduced its activity. This middle site in the peptide may play a key role in the membrane destabilizing activity.

2. Materials and methods

2.1. Peptide nomenclature

All the peptides were named after the residue they substituted for in peptide III, i.e., G13L means this peptide has the amino acid sequence of peptide III, but the 13th residue glycine in peptide III was changed to leucine. Peptide III, G1Q, L2A, F3A, E4A, A5Q, I6A, A7L, E8A, F9A, I10A, E11A, G12Q, G13L, W14A, E15A, G16Q, L17A, I18A, E19A and G20L were prepared.

2.2. Peptide synthesis

Peptides were synthesized by Fmoc solid-phase methods, using a 9050 PepSynthesizer (Millipore-Waters, Milford, MA, USA) and purified with a Waters 600E Multisolvent Delivery HPLC system and a DELTA PAC C18 reversed-phase column (15 μm, 300 Å pore size, 25 × 100 mm). An elution gradient of 10 mM ammonium acetate buffer (pH 7.3) vs. distilled methanol was applied. Eluents were monitored by absorbance at 280 or 220 nm. Purity was confirmed by a single major peak in the analytical HPLC chromatogram with a Waters Puresil C18 column (5 μm, 120 Å pore size, 4.6 × 150 mm). Each purified peptide was freeze-dried and dissolved in HEPES buffer (145 mM KCl, 5 mM HEPES, pH 7.4). Concentration of the peptide stock solution was determined by amino acid analysis. Tryptophan content was verified from absorbance of the peptide solution at 280 nm, assuming the extinction coefficient to be 5690 M^{-1} cm^{-1} at pH 7.4 [29].

2.3. Preparation of liposomes

Egg PC was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and used without further purification. The fluorescent probes, ANTS and DPX, were purchased from Molecular Probes, Inc. (Eugene, OR, USA). LUVs were prepared by the freeze-thaw and extrusion method [30]. Briefly, egg PC in chloroform was dried under a stream of nitrogen to produce a thin film on the wall of the tube. The remaining solvent was removed under high vacuum. The lipid film was hydrated in the aqueous contents of LUVs consisting of 12.5 mM ANTS, 45 mM DPX, 75 mM NaCl and 5 mM HEPES (pH 7.4) for leakage experiments or in the HEPES buffer for other measurements. The lipid dispersion was frozen in liquid nitrogen for 1 min and thawed in a lukewarm water bath at 50°C for 15 min. This freeze-thaw procedure was repeated five times. The resultant multilamellar vesicles were successively

extruded through 0.4 μm, 0.2 μm, and then 0.1 μm polycarbonate membrane filters (Nucrepore Corp., Pleasanton, CA, USA) under nitrogen pressure. The 0.1 mm extrusion step was repeated three times. In the preparation for leakage experiments, the LUVs were separated from uncapsulated materials on a Sephadex G-25 fine column (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Vesicle size was estimated by dynamic light scattering analysis, utilizing a Dyna Pro 801 MSTC (Protein Solutions Inc., Charlottesville, VA, USA) at 20°C. The hydrodynamic radii of the preparations were in the range of 51.4–59.2 nm. Phospholipid concentration was determined by the method of Bartlett [31].

2.4. Assay of leakage

Assay of aqueous content leakage from LUVs was performed by the ANTS/DPX method [32]. ANTS- and DPX-containing LUVs were suspended in citrate buffer (100 mM NaCl, 50 mM sodium citrate, pH 5.0) or HEPES buffer. Peptide stock solution was manually added to a LUV suspension at time zero. At this stage, concentration of the peptide was 12.5 μM and that of the lipid was 0.5 mM (lipid to peptide ratio was 40:1). The increase in ANTS fluorescence intensity at 530 nm with excitation at 384 nm was monitored for 6 min at 27°C, using a FP-550A fluorescence spectrometer (JASCO, Tokyo, Japan), and then Triton X-100 was added to a final concentration of 0.3%. After correction of the dilution by the addition of peptide or Triton X-100 solution, the fluorescence intensity of the LUV suspension before the addition of peptide was taken as 0% leakage, and the intensity after the addition of Triton X-100 was taken as 100% leakage.

2.5. Spectrum measurement

Circular dichroism spectra were taken with a JASCO J-20 spectropolarimeter equipped with a quartz stress-modulator (JASCO) and a Model 5209 lock-in amplifier having a built-in analog-to-digital converter (EG & G, Princeton, NJ, USA). A quartz cell with a 2 mm pathlength was used. The instrument was calibrated with *d*-10-camphorsulfonic acid [33]. Digitalized data were stored in a PC-9801muinn computer (NEC, Tokyo, Japan) and each data set was fitted to an appropriate curve by the non-linear least square method. CD spectra were recorded at 26°C. All CD samples contained 145 mM KCl, 5 mM HEPES and 12.5 μM peptide with or without LUVs, and the lipid to peptide ratio was 40:1. The CD data were expressed as the mean residue ellipticity.

Fluorescence emission spectra of tryptophyl residue in peptides (12.5 μM) with or without LUVs (0.5 mM lipid) excited at 280 nm in HEPES or citrate buffer were measured at 28°C. The spectrum of the LUV suspension or buffer solution was subtracted, respectively. Spectra in the range of 290–430 nm were recorded.

3. Results

3.1. LUV leakage measurement

To change the bulkiness of the side chains conserving the amphiphilic nature of peptide III, 20 peptides were synthesized and purified. E4A was very aggregative and difficult to dissolve in aqueous media like the viral fusion peptide. This impediment to its handling as a solution was the reason for introducing the model peptides [19,20], so E4A was omitted from measurements below. However, based on the preliminary measurements of the crude samples and its highly aggregative nature, the activity of E4A would be the same as or higher than peptide III.

Leakage of aqueous contents from liposomes was monitored for 6 min after the addition of each peptide. Typical time courses are shown in Fig. 2. The aqueous fluorescent probe ANTS and its quencher DPX were released from LUVs and the fluorescence intensity of ANTS was raised with reduction of the concentration of DPX. Each peptide induced more leakage at acidic pH than at neutral pH. At neutral, the leakage was less than 10% for each peptide. At acidic pH, the fluorescence signal of peptide III increased to 45% in 1 min and then gradually increased to as high as 80% after 6 min. Other peptides showed

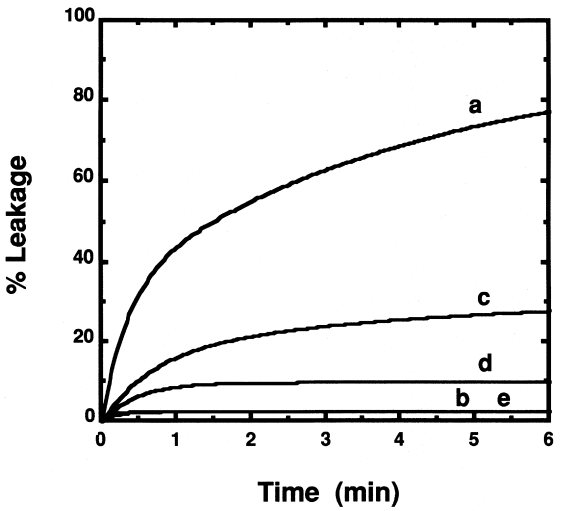

Fig. 2. Typical time courses of leakage of LUVs containing ANTS/DPX induced by peptides. Peptide stock solution was added to LUV suspension. Final concentration of peptides was 12.5 μM, and liposome concentration was 0.5 mM lipid (L/P ratio was 40:1). (a) Peptide III, pH 5.0, (b) peptide III, pH 7.4, (c) G13L, pH 5.0, (d) G13L, pH 7.4, (e) buffer blank, pH 5.0.

the same biphasic patterns, but leakage levels reached varied from 21 to 97%.

Fig. 3 compares the leakage activity of each peptide in the acidic condition. In almost all cases of amino acid substitution on the hydrophilic side, the degree of leakage observed was the same as or higher than that of peptide III. By contrast, substitution on the hydrophobic side resulted in an extreme decrease in most cases. Exceptionally for the latter, the activity of A7L increased over that of peptide III. This would be due to the enhancement of the hydrophobicity in the hydrophobic surface with this substitution.

3.2. CD and fluoresence spectra measurements

Paradoxically enough, the addition of hydrophobicity to the hydrophobic side comparatively decreased the activity of G13L. To detect the difference in the secondary structure, CD spectra of peptides at various pH values in the presence or absence of LUVs were measured. Results of peptide III and G13L are shown in Fig. 4. Other peptides showed spectra similar to peptide III. CD spectra of peptide III at acidic pH suggested the presence of some ordered secondary structures, but this was not totally clear. The amount of ordered structures seemed to increase with acidification or addition of LUVs.

On the other hand, G13L clearly showed α-helical structure without LUVs at low pH. To verify the interaction of G13L and liposomes, fluoresence spectra of tryptophan in G13L were measured. At neutral pH and without LUVs, the peak of the spectrum was the same as that of tryptophan in the aqueous phase (Fig. 5d). The addition of LUVs resulted in a blue shift of the peak, implying the transfer of tryptophan to a more hydrophobic environment [34] and peptide binding to the liposome (Fig. 5c). Acidity increased the blue shift with (Fig. 5a) or without (Fig. 5b) LUVs and the intensity of fluoresence with LUVs (Fig. 5a). As probed by the hydrophobicity of the environment of tryptophan, G13L should interact with liposomes in acid conditions, but some part might be in self-association.

4. Discussion

4.1. Activity of peptide III

Peptide III mimics the fusion peptide of the influenza virus A/PR/8/34 strain [19]. Although the N-terminus of the HA2 subunit of influenza virus hemagglutinin is assumed to be an essential segment for the membrane fusion activity [35], we must take care not to equate the free, mimicking peptide with the original peptide connected to the protein. In the case of the protein, other structural factors as well as the fusion peptide may contribute to the fusion efficiency. By binding to the host cell receptor, virus envelope and target membrane are held in close proximity prior to fusion. Formation of a pore and its enlargement might require the participation of several hemagglutinin trimers [2,17]. On the other hand, in the case of free peptides, whether such higher order structure exists is unclear. The activity of the free peptide originate from the improvement of hydrophobicity of the whole peptide resulted from

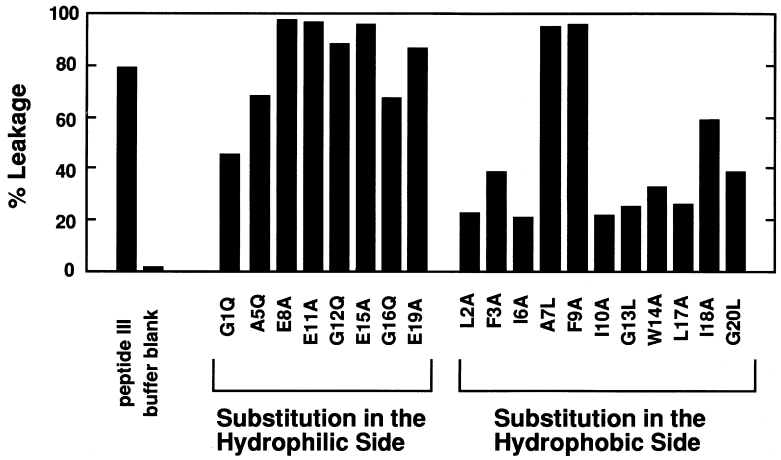

Fig. 3. Comparison of the LUV leakage among the peptides at pH 5.0. Leakage levels at 6 min after the addition of peptides are shown. Each datum is the mean of three measurements.

the protonation of the acidic residues [26,36], so the processes triggered by the peptide or the protein in low pH may be different.

Previous research claimed peptide III showed liposome fusion activity with the same pH dependency as hemagglutinin protein [27,28,36]. However, in these studies fusion was monitored by the lipid mixing of sonicated vesicles or LUVs, not the whole liposome fusion process. Attempts to measure the internal content mixing were made, but due to extensive leakage of contents, reliable data have not been presented. In this study, measurements of lipid mixing and content mixing of LUV were retried at the preliminary stage, but signals were too weak to allow reproduction of the above results. Nonetheless, leakage of contents from LUVs was clearly comparable with the former results. Considering the difference between the free peptide and the protein, failure of the trial to measure fusion events would be rather reasonable. Recently, using micropipette manipulation, lysis of LUV induced by the fusion

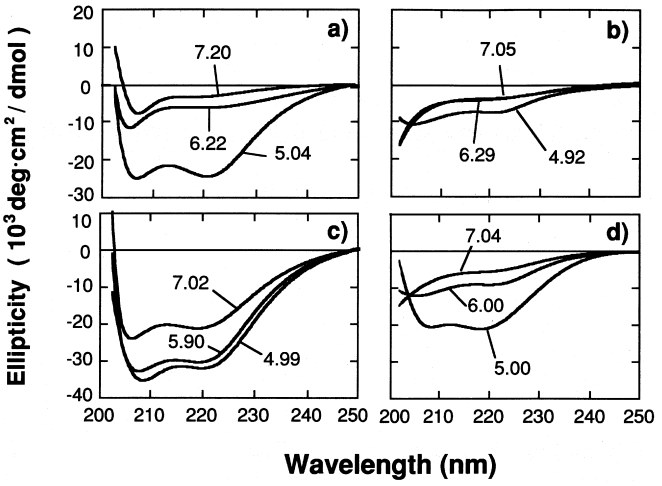

Fig. 4. CD spectra of peptide III (a,b) and G13L (c,d) at indicated pH value in the presence (a,c) or absence (b,d) of LUVs. The peptide concentration was 12.5 μM and, in the presence of LUVs, lipid concentration was 0.5 mM.

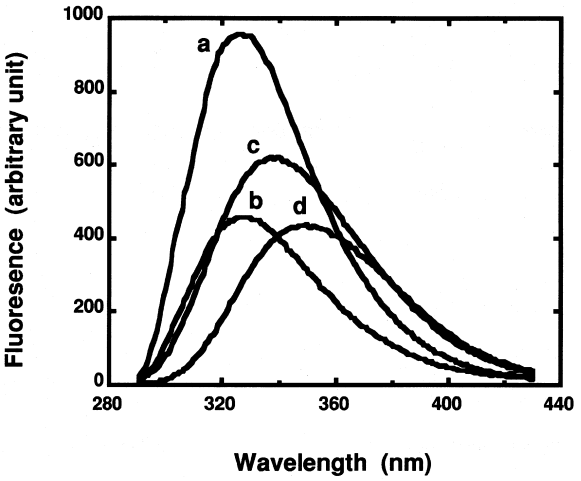

Fig. 5. Tryptophan fluorescence spectra of G13L (12.5 μM) with or without LUVs (0.5 mM lipid) excited at 280 nm. (a) pH 5.0 with LUVs, (b) pH 5.0 without LUVs, (c) pH 7.4 with LUVs, (d) pH 7.4 without LUVs.

peptides was directly observed under the optical microscope [37].

At this point, it seems valid to say that the fusion peptide is one of the segments that interact with membranes to destabilize them at the initial stage of fusion. Limited to the destabilizing activity, the systems of hemagglutinin–cell membrane and of peptide–liposome could show essentially the same nature.

4.2. Substitutions to change bulkiness of side chains

It has been pointed out that the fusion peptide could be a 'sided' amphiphilic α-helix whose hydrophobic side is occupied by bulky hydrophobic residues and hydrophilic surface by smaller residues [2,18]. Many arguments for [20–22] and against [23–25] the correlation of helical structure of the fusion peptide and its activity have been made. The necessity of uneven distribution of the bulkiness of side chains around the supposed axis of the α-helix for the activity of peptide III was also proposed [28].

In this research amino acids were substituted for peptide III to vary the bulkiness of the side chain of one of the residues without destroying the amphiphilicity of the supposed α-helix, and the activities of substituted peptides were assessed by aqueous content leakage from LUVs. In the results, substitutions in the hydrophilic side had little effect or showed slight improvement of the activity (A5Q, E8A, E11A, G12Q, E15A, G16Q, E19A). Substitutions in the hydrophobic side decreased the activity in most cases (L2A, F3A, I6A, I10A, G13L, W14A, L17A, G20L). Exceptionally, A7L did not reduce the extent of leakage from liposomes. Most substitutions in the hydrophobic side, from bulky Leu, Phe, Ile or Trp to small Ala residue, decrease the hydrophobicity of this side. The hydrophobic surface of the amphiphilic peptide is supposed to interface with the membrane, so the reduced hydrophobicity would result in reduced affinity to the membrane and thus its activity. On the contrary, the substitution of A7L adds a hydrophobic bulky side chain to the hydrophobic side so affinity with the lipid bilayer would be improved. The above results can be explained in the scheme of amphiphilicity of peptide III. In addition, F9A and I18A, which substitute a small side chain for a bulky hydrophobic one yet maintain activity as high as peptide III, are substitutions on the border between hydrophobic and hydrophilic sides (Fig. 1b) and thus have less influence.

Exceptionally for substitutions in the supposed hydrophilic side, G1Q showed lower activity. On the other hand, G20L should enhance the hydrophobicity in the supposed hydrophobic side but also showed lower activity. The influence of N-terminal residue substitution has been intensively studied [23,24,38–40], and it has been shown that mutation of Gly1 to hydrophilic Glu residue in the fusion peptide reduces the fusion activity of the cell expressing the hemagglutinin [38,40]. In past years, many results were observed in both theoretical [41] and experimental [24,42–44] studies that suggest the fusion peptide is inserted into the membrane with helix axis oblique to the membrane surface, not parallel as simply expected from its amphiphilicity. Recently, utilizing spin labeling to probe the depth of each residue in a membrane, Macosko et al. [45] proposed a model of fusion peptide in which only approximately thirteen residues from the N-terminus were inserted into the lipid bilayer forming an

α-helix whose axis made an oblique angle with the membrane. The positively charged amino group of the N-terminus was thought to project towards the head group region of the lipids, so the N-terminal residue may not take part in the α-helix. Durrer et al. [46] reported that the 1st to 14th residues of the fusion peptide reacted well with hydrophobic photolabeling dye in the membrane but the 15th to 21st residues were weakly dyed. According to these reports the N-terminal residue of peptide III also could be buried in the bilayer in at least one significant stage of the process, so the substitution of the N-terminal Gly residue by hydrophilic Gln residue would be disadvantageous for interaction with the membrane. On the other hand, the C-terminus of peptide III could be thrust into an aqueous phase, so the increase of hydrophobicity around the C-terminal residue might be also disadvantageous for the peptide making this arrangement. The results from G1Q and G20L seem to be consistent with the model in which the N-terminal half of the fusion peptide is inserted into the membrane at an oblique angle.

4.3. Glycine in the middle of peptide

Although excess hydrophobicity was added to the hydrophobic side, the activity of G13L decreased. CD spectra of G13L showed relatively higher α-helical content, and tryptophan fluorescence suggested interaction between the peptide and liposomes. These results support the view that there is no correlation between the activity of the fusion peptide and its α-helical contents [23,24].

Why then was the activity of G13L so decreased? Glycine is known for its potential to terminate the α-helix. The amino acid sequence around G13, in which F9 or I10 is apolar, E11 is polar, G13 is glycine and W14 is apolar, fits a prediction for one of the helix termination motifs, the Shellmann motif [47]. Amino acid substitution of G13L could be understood as an instruction not to terminate the α-helix in the middle of the peptide. Indeed, both peptide III and the peptide of the sequence of natural fusion peptide seem to contain not only the α-helix but also the β-structure or other secondary structural components [19,24]. According to the secondary structure prediction, the N-terminal half of peptide III has a greater propensity for the α-helix than the β-structure and the C-terminal half has higher β-structure potential [28]. Peptides duplicating the residue sequence of N- or C-terminal halves of peptide III showed high or low α-helical content, respectively [28]. The fusion peptide in hemagglutinin crystal structure at neutral pH also showed that not all of this segment was α-helix but rather contained a continuous series of sharp bends [4]. When binding to a membrane, this region is believed to take an amphiphilic α-helical structure [2,18]. The conformational change of the fusion peptide on the membrane may be important for its perturbing activity, and thus a fully elongated helix before the binding to the membrane in acidic conditions would rather be of little use. The results from G13L support this idea.

The relationship between Gly residues in the middle of the fusion peptide and activity on the membrane was suggested not only by the mimicking peptide but also by the intact fusion peptide in proteins. Comparison of amino acid sequences of hemagglutinin fusion peptides of various subtypes of influenza virus A strain, collected from the PIR database [48], is shown in Table 1. Although the fusion peptide is a conserved region, highly conserved residues are concentrated in the N-terminal half. Without exception, the 13th residue is glycine in contrast with the neighboring 12th Gly residue which is allowed to mutate to Gln residue. Gly13 would be the site L-amino acid could not occupy in the Shellman motif [47]. These observations suggest that preserved residues are not only the residues that determine the amphiphilicity or the sided α-helical conformation but also the residues that control the higher order structure [25]. Similarly it was reported that glycines in the middle of the fusion peptide of gp41 in human immunodeficiency virus type 1 are critical for the establishment of infection [49].

It is interesting that the reaction curves of leakage measurements showed apparently biphasic patterns. At least two rate-limiting steps exist

Table 1
Amino acid sequences of fusion peptides of influenza virus A strains

Virus strain [hemagglutinin subtype]	Amino acid sequence [a]	PIR entry code
A/Puerto Rico/8/34 [H1]	GLFGAIAGFIEGGWTGMIDG	HMIV
A/Japan/305/57 [H2]	GLFGAIAGFIEGGWQGMVDG	HMIV2
A/equine/Miami/1/63 [H3]	GIFGAIAGFIENGWEGMVDG	HMIVE2
A/duck/Hong Kong/231/77 [H3]	GLFGAIAGFIENGWEGMIDV	JQ1156
A/Aichi/2/68 (X-31) [H3]	GLFGAIAGFIENGWEGMIDG	HMIVHA
A/duck/Czechoslovakia/56 [H4]	GLFGAIAGFIENGWQGLIDG	HMIVF1
A/turkey/Ireland/1378/83 [H5]	GLFGAMAGFIEGGWQGMVDG	HMIVT8
A/turkey/Ontario/7732/66 [H5]	GLFGAKAGFIEGGWQGMVDG	HMIVAT
A/shearwater/Australia/1/72 [H6]	GLFGAIAGFIEGGWTGMIDG	HMIVSA
A/turkey/Oregon/71 [H7]	GLFGAIAGFIENGWEGLIDG	HMIVT7
A/turkey/Ontario/6118/68 [H8]	GLFGAIAGFIEGGWSGMIDG	HMIVTN
A/turkey/Wisconsin/66 [H9]	GLFGAIAGFIEGGWPGLVAG	HMIVTW
A/chick/Germany/N/49 [H10]	GLFGAIAGFIENGWEGMVDG	HMIV49
A/duck/England/56 [H11]	GLFGAIAGFIEGGWPGLING	HMIVDE
A/duck/Alberta/60/76 [H12]	GLFGAIAGFIEGGWPGLVAG	HMIVDA
A/whale/Maine/328 HN/84 [H13]	GLFGAIAGFIEGGWPGLING	HMIVT2
A/duck/Gurjev/263/82 [H14]	GLFGAIAGFIENGWQGLIDG	A46339

[a]Amino acid sequences around the 13th glycine residue fit a prediction for a helix termination motif, in which F9 or I10 is apolar, E11 is polar, G13 is glycine and W14 is apolar.

in the membrane destabilizing process by peptide III. The peptide could be divided into two structural regions, the N-terminal helix and the C-terminal segment. The peptide would be inserted into the membrane from its N-terminal α-helix thrusting the C-terminus into the aqueous phase. What would happen next? It may be elongation of the α-helix on the membrane or the process of association of peptides floating on the same liposome surface to produce non-lipidic domains. But which steps contribute more to the destabilizing activity is not clear, because we couldn't stop the system at any point in the process. Phenomena such as transitions of higher order structures, membrane destabilization, and pore formation are essentially time-dependent and have multiple components. To understand the whole process of interaction between peptide and lipid bilayers and the fusion event, we must know not only what happens but also the timing of its occurrence.

4.4. Conclusions

Direct correlation between α-helical content of peptides at acidic pH and membrane destabilizing activity was not seen. Rather it was suggested that the 13th residue glycine plays a key role in the activity of peptide III and therefore the fusion peptide in hemagglutinin protein. The importance of these glycines may be due to the formation of a helix termination motif by glycine in the middle of the peptide that allows elongation of the N-terminal helix to the C-terminus under acidic conditions or the association with membranes. The peptide also has an amphiphilic nature. Substitutions to change the bulkiness of the hydrophobic side highly influence the activity, so there must be steps in the membrane destabilizing processes to utilize hydrophobic interaction between the hydrophobic side of the peptide and membrane or other peptides.

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