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The interaction of synthetic analogs of the N-terminal fusion sequence of influenza virus with a lipid monolayer. Comparison of fusion-active and fusion-defective analogs

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The amino terminus of subunit-2 of influenza virus hemagglutinin (NHA_2) plays a crucial role in the induction of fusion between viral and endosomal membranes leading to the infection of a cell. Three synthetic analogs with an amino acid sequence corresponding to NHA_2 of variant hemagglutinins were studied in a monolayer set up. Comparison of the interaction of a fusion-active and two fusion-defective analogs with a lipid monolayer revealed a greater surface activity of the fusion-active analog. Pronounced differences were found if the pure peptides were spread at the air/water interface; the fusion-active analog showed a higher collapse pressure and a greater limiting molecular area. Circular dichroism measurements on collected lipid monolayers indicated a high content of α-helical structure for the fusion-active and one of the fusion-defective analogs. A simple relation between α-helical content and fusogenicity does not seem to exist. Instead, the extent of penetration, a defined tertiary structure or orientation of the α-helical peptide may be essential for its membrane perturbing activity.

Introduction

It is generally assumed that proteins play a key role in the regulation of biomembrane fusion events [1,2]. However, only in the case of virus-membrane fusion has the protein responsible for the induction of membrane fusion been identified [3]. Among these viral fusion proteins influenza virus hemagglutinin (HA) has been characterized in great detail [4,5].

After binding to sialic acid-containing receptors on the plasma membrane, influenza virus is taken up into its host cell by receptor mediated endocytosis [6]. The low pH of the endosomal compartment activates influenza HA and results in fusion of viral and endosomal membrane and release of the viral nucleocapsid into the cytoplasm. Both binding and fusion are mediated by influenza HA [7]. The HA spike protein is a trimer of identical HA monomers, each of which consists of two subunits linked to each other by a disulfide bridge [5,8]. Subunit-1 of HA (HA_1) contains the sialic acid receptor-binding domain, and subunit-2 (HA_2) links HA to the viral membrane. In addition, HA_2 possesses an extremely hydrophobic N-terminus (NHA_2). The fusion activity of HA is only revealed at low pH (pH 5–6), and is accompanied by an irreversible conformational change of the trimeric HA spike protein [9–14]. During this irreversible conformational change the highly conserved hydrophobic N-terminus of HA_2 is exposed and subunit contacts within the HA trimer are partially lost. The molecular structure of the ectodomain of HA at neutral pH is known to 3 Å resolution [8] and also the conformational change of HA that occurs upon lowering of the pH, has been extremely well characterized [9–14]. However, the mechanism by which HA induces membrane fusion after its conformational change remains ill-defined.

Abbreviations: BHA, ectodomain of HA obtained by bromelain treatment of intact virus; Chol, cholesterol; DOPC, dioleoyl-PC; DOPE, dioleoyl-PE; HA, influenza hemagglutinin; HA_1, subunit-1 of HA; HA_2, subunit-2 of HA; NHA_2, N-terminus of HA_2; PBS, phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4); PC, phosphatidylcholine; PE, phosphatidylethanolamine; π, surface pressure; $\Delta\pi$, change in π; Spm, sphingomyelin.

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Although its mode of action is still unclear, NHA_2 is thought to be directly involved in the induction of membrane fusion. This suggestion is supported by the fact that NHA_2 is extremely hydrophobic and its amino acid composition highly conserved [3]. At low pH, the ectodomain of HA (released after bromelain treatment of intact virus, BHA) aggregates, and aggregation occurs through NHA_2 [11]. Recently, it has also been demonstrated that binding of BHA to lipid vesicles at low pH is mediated by NHA_2 [15]. Furthermore, almost any change in NHA_2 by site-directed mutagenesis strongly affects the fusion activity of HA [16]. Finally, synthetic peptides with an amino acid sequence identical to NHA_2 were shown to be able to destabilize small unilamellar lipid vesicles, as evidenced by lipid mixing and a concomitant release of vesicular contents [17–19].

Although these data do suggest a direct and active role of NHA_2 in the induction of membrane fusion, they do not allow any conclusions to be drawn on the actual fusion mechanism. Crucial to the understanding of the mechanism of influenza HA-mediated membrane fusion is the movement of NHA_2 after it has been extruded from HA at low pH. However, no direct data on the movement of NHA_2 (in the intact virion) are available and different models for HA-mediated membrane fusion have been proposed. Depending on the model, NHA_2 either moves upwards or downwards penetrating the target membrane (see for example, Ref. 2) or the viral membrane (see for example, Ref. 20) or, alternatively, NHA_2 is extruded sidewards and penetrates no membrane at all [21]. While these models are totally different with respect to the mechanism by which HA is thought to induce membrane fusion, in all three models NHA_2 interacts with lipids from the target and/or viral membrane (even if NHA_2 penetrates neither membrane, see Ref. 21). This interaction is likely to be of crucial importance to the induction of membrane fusion.

Therefore, we decided to study the interaction of synthetic analogs of NHA_2 with a lipid target. Experiments using photoaffinity labeling have shown that the interaction of BHA with a lipid vesicle at low pH is mediated by NHA_2 [15] and, in addition, that this interaction is probably confined to the outer lipid monolayer [10,22]. Therefore a lipid monolayer spread at the air/water interface seemed an attractive model to study the interaction of analogs of NHA_2 with a lipid target. The characteristics of a peptide mimicking NHA_2 of influenza X31 were compared with those of two peptides mimicking NHA_2 of fusion-defective variants of influenza X31. Earlier experiments [19] had already shown that these 'mutant' peptides had a much restricted capacity to destabilize sonicated lipid vesicles as compared to the 'wild-type' peptide (see also Ref. 23). The aim of our study was to try and explain these differences in 'fusogenicity' (none of these peptides is capable of inducing fusion of biologically more relevant large unilamellar vesicles; see also Discussion), at the same time hoping to gain more insight into the mode of action of NHA_2 during membrane fusion.

Materials and Methods

Materials

Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) were synthesized from egg-yolk PC and purified as described [24,25]. Bovine brain sphingomyelin (Spm) was obtained from Sigma (St. Louis, MO), and cholesterol (Chol) from Baker (Phillipsburg, NY). Phospholipids were pure as judged by high performance thin-layer chromatography. All other reagents and chemicals were of analytical grade.

Synthetic analogs of NHA_2

Peptides with an amino acid sequence corresponding to the sequence of NHA_2 of influenza X31 and of two fusion-defective variants of influenza X31 were synthesized as described before [19]. The sequences of these peptides are shown in Fig. 1. The 19 amino acid peptide ΔG1 was synthesized first, and subsequently a glycine or a glutamic acid were added to this sequence (giving wt and G1E, respectively). Amino acid analysis was performed using standard procedures, and gave the following results (expected number of amino acids is given in brackets); wt peptide: D 2.09 (2), E 2.11 (2), G 5.80 (6), A 2.17 (2), M 0.95 (1), I 2.75 (3), L 1.04 (1), F 1.97 (2), W not determined (1); peptide G1E: D 2.11 (2), E 3.04 (3), G 4.86 (5), A 2.12 (2), M 0.90 (1), I 2.76 (3), L 1.01 (1), F 1.91 (2), W not determined (1); and peptide ΔG1: D 2.37 (2), E 2.01 (2), G 5.07 (5), A 2.30 (2), M 0.89 (1), I 2.80 (3), L 0.97 (1), F 1.88 (2), W not determined (1). Peptides were dissolved in DMSO at a concentration of approx. 1 mg/ml. The peptide concentration was determined by performing a (quantitative) amino acid analysis in the presence of a known quantity of norleucine. Peptides may be considered to be > 90% pure. The membrane destabilizing activity of the three analogs was tested (for experimental procedures see Ref. 19) the results confirmed earlier measurements (see Ref. 19) in that the wt peptide induced lipid mixing of sonicated PC/Chol vesicles at low pH whereas peptides G1E and ΔG1 were found to be ineffective (not shown).

Monolayer measurements

The surface pressure of the monolayer at the air/water interface was measured using the Wilhelmy-plate method and a Cahn 2000 electrobalance (for a concise review, see Ref. 26). Experiments were performed at constant area using a 5 ml teflon trough, 5 cm in diameter. For pressure-area curves, a 17.2 × 33.2

cm teflon trough was used, and the monolayer was compressed at a speed of 98.28 cm^2/min by moving a teflon barrier.

Stock solutions of lipids were made in chloroform/methanol (1:1, v/v). The phospholipid phosphorus concentration was determined according to Böttcher et al. [27]. Final lipid mixtures were prepared at a concentration of 1 mg/ml in chloroform, and contained less than 10% methanol. Lipids were spread at the air/water interface using a small glass capillary. Peptides were injected into the subphase using a Hamilton syringe. In the presence of a lipid monolayer, the peptide-induced increase in surface pressure reached equilibrium within approx. 10 min; these equilibrium values of surface pressure increase were used (Figs. 2 and 3). The pressure-area curves of the peptides were recorded after spreading a known quantity of peptide at the air/water interface. A good reproducibility of spreading was obtained by injecting the peptide solution onto a sandblasted microscope glass slide partially immersed into the subphase (see Ref. 28). The maximally attainable stable surface pressure, or collapse pressure, was also determined by injecting an excess amount of peptide into the subphase and measuring the final surface pressure (at constant area). For CD measurements, the monolayer was deposited onto quartz plates (1.5 cm in diameter) during a single pass through the interface at a rate of 3 mm/min. Throughout this procedure the surface pressure was kept constant. Mixed monolayers were prepared by first spreading the lipid (initial surface pressure π_i of 20 mN/m) and then injecting the peptide into the subphase (final π of 30–32 mN/m). Peptide monolayers were made by spreading the peptide at the air/water interface, and compressing the monolayer until the collapse pressure was reached. CD measurements were performed on eight quartz plates put in series. All monolayer experiments were performed at 37°C under constant stirring, using PBS at pH 7.4 or PBS/citric acid at pH 5.0.

Circular dichroism (CD) measurements

Measurements were performed on a Jasco-600 spectropolarimeter, interfaced to a laser 386 computer. The cuvette chamber was flushed with nitrogen gas. For each sample 10 spectra (185–260 nm) were accumulated at room temperature. After subtracting the spectrum of a protein-free sample, the spectrum was smoothed, and subsequently stored with a resolution of 0.1 nm. The scan speed was 20 nm/min, the time constant 0.5 s, the band width 1 nm, and the sensitivity of the photomultiplier was +20 to −20 mdeg.

Results

The initial interaction of NHA_2 with a target membrane during virus-membrane fusion, is likely to be confined to the lipids from the outer lipid monolayer of either the target membrane, the viral membrane, or both (depending on which model for HA-mediated membrane fusion turns out to be correct; see Introduction). The monolayer experiments were initiated with the purpose of studying this interaction, and a monolayer composed of PC, Spm and Chol in a molar ratio of 1:1:2 was used. This composition is close to the lipid composition of the outer monolayer of the erythrocyte membrane [29], and probably also close to that of the outer lipid monolayer of the viral membrane (compare Refs. 30, 31 and 32). In addition, a mixture of PC, PE and Chol in a 2:1:2 molar ratio was used; large unilamellar vesicles of this composition have been shown to be a particularly good fusion target for influenza virus (see Refs. 33 and 34).

Wild type (wt)	GLFGAIAGFIENGWEGMIDG
G1E	ELFGAIAGFIENGWEGMIDG
ΔG1	LFGAIAGFIENGWEGMIDG

Fig. 1. Amino acid composition of the synthetic analogs of NHA_2.

The surface activities of three analogs with an amino acid sequence corresponding to NHA_2 of variant HAs were compared. The amino acid composition of these peptides is shown in Fig. 1. As well as a 20 amino acids long peptide mimicking NHA_2 of wild-type influenza X31 (referred to as the fusion-active wild-type or wt peptide), two fusion-defective peptides were examined. Both the fusion properties of these peptides [19] and the fusion properties of the corresponding HA molecule in the intact virion or expressed in CV-1 cells, are known. The first peptide has a G to E substitution at position 1 (peptide G1E), and is, in contrast to the wild-type peptide, not capable of destabilizing small unilamellar vesicles composed of PC and Chol at any pH [19]. Furthermore, the corresponding HA molecule is not capable of inducing heterokaryon formation or mediating CV-1/erythrocyte fusion [16]. The second fusion-defective peptide lacks the N-terminal glycine (peptide ΔG1) and is again not capable of destabilizing PC/Chol vesicles [19]. Analogously, thermolysin-treated virus having an NHA_2 lacking the glycine is fusion-defective and therefore not infectious [35].

Interaction of synthetic analogs of NHA_2 with a lipid monolayer at the air/water interface

The fusion activity of influenza virus is strongly pH dependent; in the case of influenza X31, a pH optimum of fusion activity is found at pH 5.0. Therefore the interaction of the synthetic analogs with a lipid monolayer was studied at neutral and low pH (Fig. 2). Each of the peptides was able to induce an increase in surface pressure, which must result from (partial) penetration of the peptide into the lipid monolayer and an interaction of the peptide with the headgroup and/or

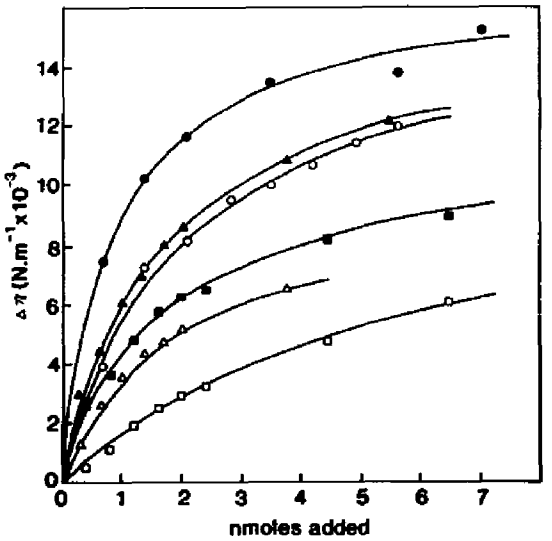

Fig. 2. Surface pressure increase induced in a monolayer of DOPC/DOPE/Chol (2:1:2, molar ratio). Peptides were titrated in at pH 7.4 (open symbols) or pH 5.0 (closed symbols). ○, ●, wt; △, ▲, G1E; □, ■, ΔG1. π_i of 20 mN/m (37°C).

the glycerol backbone region of the lipid monolayer (see for example, Ref. 36). The interaction of the peptides with the lipid monolayer is strongly pH dependent, probably due to the presence of acidic amino acids (see Fig. 1). Nonetheless, each of the analogs also induced a significant increase in surface pressure at neutral pH.

The fusion-active wt peptide induced a greater increase in surface pressure than the fusion-defective peptides. This difference is particularly obvious if the wt peptide is compared to peptide ΔG1. The higher activity of the wt peptide is observed at low pH and neutral pH, both in the PC/PE/Chol system (Fig. 2) and in the PC/Spm/Chol system (Fig. 3). In the PC/Spm/Chol system, lower values of surface pressure increase were measured. With respect to the comparison of the wt peptide with the fusion-defective peptides, it should be noted that the latter peptides are less active, but still capable of inducing a significant increase in surface pressure of the lipid monolayer.

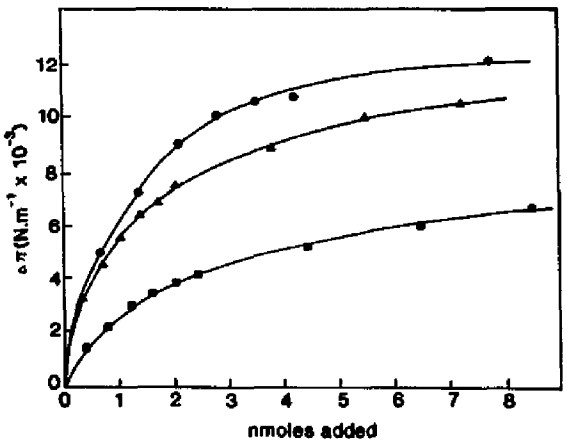

Fig. 3. Surface pressure increase induced in a monolayer of DOPC/Spm/Chol (1:1:2, molar ratio). Peptides were titrated in at pH 5.0. ●, wt; ▲, G1E; ■, ΔG1. π_i of 20 mN/m (37°C).

The activity of the peptides was also examined at higher initial surface pressures of the monolayer. All three peptides were capable of inducing a surface pressure increase at initial surface pressures in excess of 30 mN/m; the pressure increase was reduced by only 30 to 50% if the initial surface pressure was raised from 20 to 30 mN/m (PC/Spm/Chol system; results not shown).

The main difference found between the fusion-active wt peptide and the fusion-defective peptides, G1E and ΔG1 is the fact that the former peptide induces a greater increase in surface pressure of the lipid monolayer. This difference in activity could be due to a higher efficiency of interaction of the wt peptide with the lipid monolayer, resulting in the penetration of more amino acid residues into the lipid monolayer. It could also be related to a difference in molecular area of the part of the peptide that has inserted into the lipid monolayer. A difference in molecular area may, in turn, be related to a difference in secondary structure. In order to reveal possible differences in secondary structure of the analogs after interaction with the lipid monolayer, monolayers were collected and CD spectra recorded; the results obtained at low pH are shown in Fig. 4 (solid lines). It should be noted that the methods used to analyze CD spectra (see for example, Ref. 37) rely on a random orientation of a peptide or protein with respect to incident light. In the current experimental system, a preferred orientation of the peptides with respect to the lipid monolayer (or the air/water interface, Fig. 4d) could greatly influence the shape of the CD spectra (see Ref. 38). In fact, a theoretical analysis of the interaction of a number of different viral fusion peptides with a dipalmitoylphosphatidylcholine monolayer suggests an oblique orientation of these peptides with respect to the lipid/water interface (influenza NHA_2 was not tested; see Ref. 39). The CD spectra of the wt peptide and peptide G1E (Figs. 4a,b) show minima at approximately 209 and 221 nm, indicative of α-helical structure. This conclusion is independent of the orientation of the peptides with respect to the lipid monolayer. The CD spectrum of peptide ΔG1 (Fig. 4c) is characterized by a higher crossover value, and a single minimum at about 216 nm. This result can only be properly interpreted if the orientation of the peptide is known. If the peptide is randomly oriented, the CD spectrum indicates a high content of β-sheet structure. On the other hand, the spectrum could also result from a largely α-helical peptide oriented at a specific angle with respect to the monolayer surface. In conclusion, the data show that

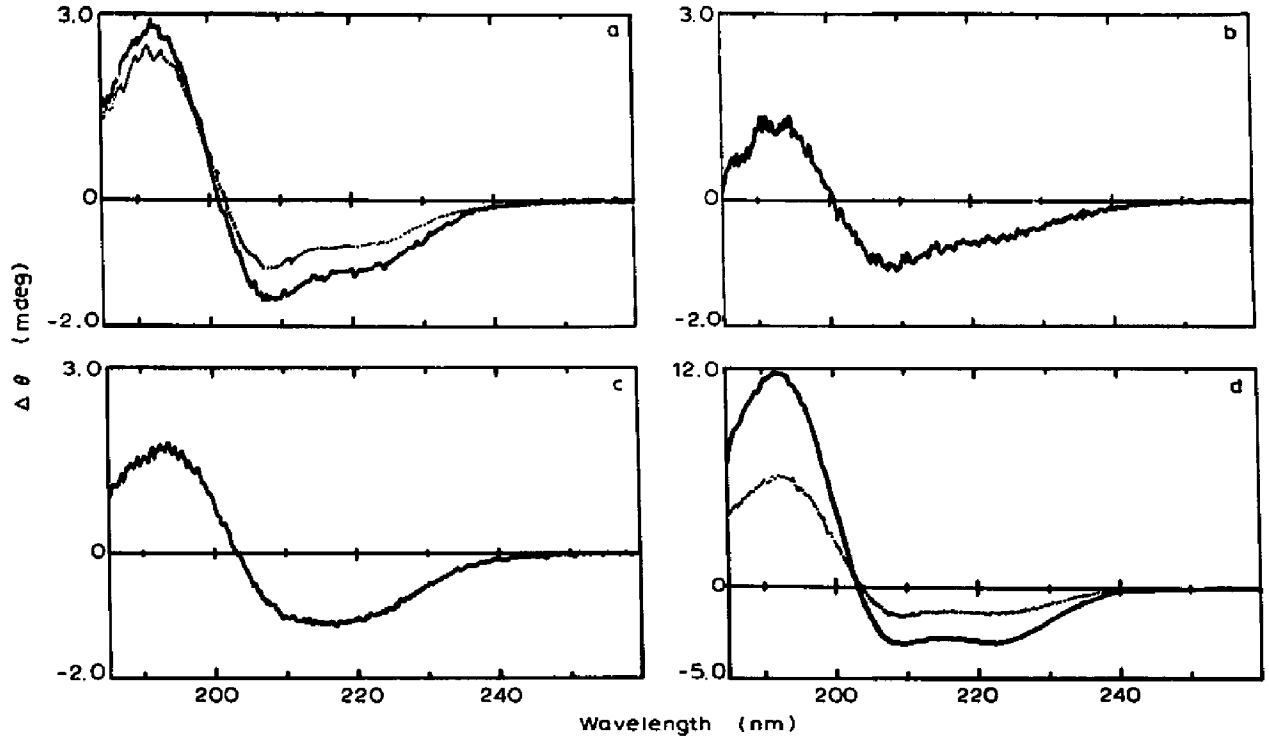

Fig. 4. Circular dichroism spectra of the synthetic analogs. Experiments were performed at 37°C and pH 5.0 (solid lines) or pH 7.4 (dotted lines). In (a–c) a monolayer of DOPC/DOPE/Chol in a 2:1:2 molar ratio was used. (a) wt; (b) G1E, and (c) ΔG1. Final surface pressure was 30–32 mN/m. In (d) the wt peptide was spread at the air/water interface in the absence of a lipid monolayer, and compressed until the collapse pressure was reached. For details on the experimental procedures see Materials and Methods.

after interaction with a lipid monolayer at low pH, at least the wt peptide and peptide G1E have a high α-helical content.

The secondary structure of the wt peptide was also examined after interaction with the lipid monolayer at neutral pH, and the CD spectrum (Fig. 4a, dotted line) was once more indicative of a high content of α-helical structure. Finally, a high content of α-helical structure was also observed after spreading of the wt peptide at the air/water interface in the absence of a lipid monolayer, at low and neutral pH (Fig. 4d).

Surface properties of synthetic analogs of NHA_2 at the air/water interface

Differences in surface properties between the fusion-active and fusion-defective analogs may also be revealed by comparing the compression curves of pure peptide monolayers spread at the air/water interface (Fig. 5). The values found for two important parameters, the collapse pressure and the limiting area per molecule, are summarized in Table I. The shape of the pressure-area curves appeared to be strongly pH dependent (compare Fig. 5a with 5b), and in addition,

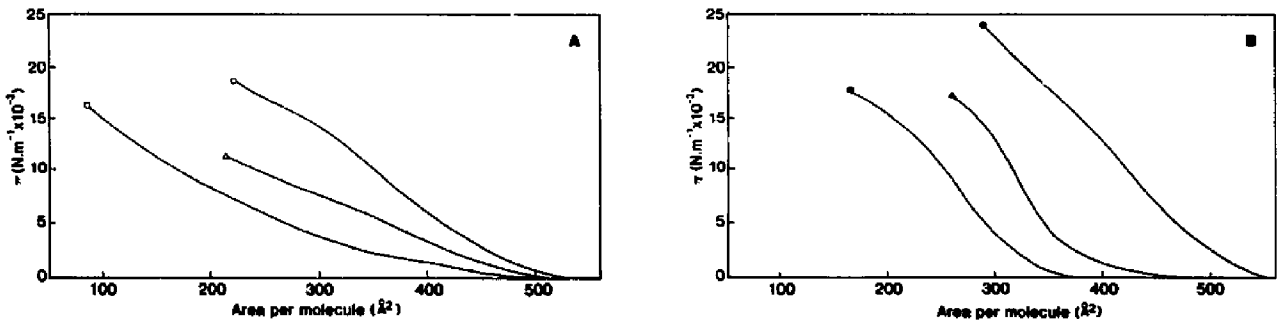

Fig. 5. Pressure-area curves of the synthetic analogs spread at the air/water interface. Peptides were spread at pH 7.4 (a, open symbols) or pH 5.0 (b, closed symbols). ○, ●, wt peptide; △, ▲, G1E; □, ■, ΔG1. The symbols indicate the collapse pressure. Collapse pressures and limiting molecular areas are summarized in Table I.

TABLE I

Characteristics of the synthetic analogs of NHA_2 spread at the air/water interface

Collapse pressures and limiting areas per molecule are deduced from Fig. 6 (S.E.M. ≤ 5%).

Peptide	Collapse pressure (mN/m)		Limiting area per molecule (Å^2)	
	pH 7.4	pH 5.0	pH 7.4	pH 5.0
wt	18.6	24.0	224	290
G1E	11.3	17.3	214	260
ΔG1	16.5	18.0	84	164

significant differences in compression characteristics were observed among the three peptides tested. These differences may, in principle, be caused by differences in molecular conformation, or in the orientation of the molecules with respect to the air/water interface. Alternatively, they may be due to differences in intermolecular interactions, or to a combination of the factors mentioned.

First of all, lowering of the pH resulted in an increase in the slope of the pressure-area curves, and thus in a reduction of the compressibility of the monolayers; the peptide monolayers became more rigid. In addition, the collapse pressures of the wt peptide and peptide G1E increased, as did the limiting areas per molecule for all three analogs. These data suggest that low pH induces a conformational change or a change in the location or orientation of the peptide at the air/water interface. Both the data obtained on pure peptide monolayers and those obtained on peptide–lipid interaction (preceding section) show that the surface activity of the peptides increases upon lowering of the pH.

Secondly, the wt peptide showed the highest collapse pressures and the greatest limiting areas per molecule, both at neutral and acidic pH. Consequently, the wt peptide appears to have the most extended conformation and may be considered most surface active of the three analogs studied. Based on these data, the wt peptide would be expected to interact more extensively with, and induce a more pronounced distortion of a lipid environment.

Discussion

A comparison of fusion-active and fusion-defective analogs of NHA_2

Several studies have focussed on the interaction of synthetic analogs of NHA_2 with pure lipid vesicles [17–19,23,40]. Synthetic analogs of NHA_2 of influenza X31 [19], influenza A/PR/8/34 [18] and influenza B/Lee/40 [17] have been reported to destabilize sonicated lipid vesicles. In some instances lipid mixing was found to be dependent on low pH [18,19]. Furthermore, synthetic peptides mimicking NHA_2 of fusion-defective variants of influenza HA revealed a strongly reduced capacity to destabilize sonicated lipid vesicles [19]. These studies provided support for a direct role of NHA_2 in the induction of membrane fusion but did not answer the question as to which properties of NHA_2 are responsible for its fusogenicity. Instead, it was shown that an analog may efficiently bind to lipid vesicles without destabilizing them (see for example Ref. 19) and although, in general, analogs with a high α-helical content appeared to be more potent, this correlation was not absolute [17,19,40].

With the aim of elucidating those properties of NHA_2 which may be essential for its fusogenicity, we compared the surface properties of fusion-active and fusion-defective analogs of NHA_2 in a monolayer set up. The monolayer data are in close agreement with the results obtained in an earlier study on the interaction of the same synthetic analogs with sonicated lipid vesicles (see Ref. 19).

First of all, the monolayer experiments clearly show that both the fusion-active wt peptide, and the fusion-defective peptides G1E and ΔG1 (Fig. 1) interact with, and penetrate into, a lipid monolayer spread at the air/water interface. Peptide insertion is not confined to low initial surface pressures of the lipid monolayer, but also occurs at a surface pressure assumed to be relevant for a lipid monolayer being part of a membrane bilayer (about 32 mN/m; see Ref. 41). Analogously, a blue shift in tryptophan fluorescence demonstrated the insertion of all three peptides into the membrane of sonicated lipid vesicles [19]. In both studies the interaction of the peptides with the lipid target is stimulated by lowering the pH, but is not absolutely dependent on low pH. These data may have important implications for the role of low pH during HA-mediated membrane fusion. After the conformational change of HA, low pH does not seem to be an absolute requirement for the interaction of NHA_2 with its lipid target (see later).

Small changes in the amino acid composition of NHA_2 (G1E or ΔG1) completely abolish both the destabilizing activity of its synthetic analog towards PC/Chol vesicles [19] and the fusion activity of the corresponding influenza HA [16,35]. Nevertheless, a comparison of the interaction of the fusion-active and fusion-defective analogs with a lipid monolayer does not reveal absolute differences between these analogs. On the other hand the data do show that the wt peptide is more surface active and induces a greater increase in surface pressure of the lipid monolayer. If for example 3 nmoles of peptide are added at pH 5.0, the surface pressure increase induced by peptide G1E and ΔG1 is lower than that of the wt peptide by on the average 20 and 50%, respectively (see Figs. 2 and 3).

These results are in line with the results obtained on peptide–vesicle interaction showing similar binding characteristics for wt and G1E, and a reduced or more superficial binding of peptide ΔG1 [19]. The relatively minor differences in binding characteristics between the wt peptide and peptide G1E show that although binding is obviously a prerequisite to membrane destabilization it is certainly not a guarantee that destabilization (lipid mixing) will occur.

Despite similar binding characteristics, differences in secondary structure could be responsible for the differences observed in membrane destabilizing activity. After interaction with the lipid monolayer, the wt peptide and peptide G1E have a high α-helical content whereas peptide ΔG1 shows little α-helical structure (or an aberrant orientation of the peptide with respect to the lipid monolayer; see Results). On a qualitative basis, these results are comparable to those obtained after peptide–vesicle interaction indicating a similar α-helical content of wt and G1E and a lower α-helical content of peptide ΔG1 [19]. A correlation between the α-helical content of a peptide and its capacity to destabilize sonicated lipid vesicles has been reported for a number of synthetic analogs of NHA_2 [17,19] and its derivatives [40], as well as for other amphipathic peptides [42]. However, the high α-helical content of both wt peptide and peptide G1E strongly suggests that the formation of an α-helix, as such, is not sufficient for membrane destabilization. The latter is corroborated by the fact that the wt peptide also showed a high content of α-helical structure at neutral pH, a pH at which it is not capable of destabilizing sonicated PC/Chol vesicles [19].

The most noticeable differences in surface properties of the three analogs are revealed when the peptides are spread at the air/water interface. It should be noted that the behavior of a peptide at the air/water interface may not faithfully reflect its behavior upon insertion into a lipid monolayer. Nevertheless, the much higher collapse pressure and greater limiting area per molecule found for the wt peptide upon compression of the peptide monolayer, illustrate its greater surface activity and indicate a more extended conformation of the peptide at the air/water interface.

In summary, the wt peptide distinguishes itself from the fusion-defective peptides by being more surface active towards a lipid monolayer, and, possibly (see Results) by having a slightly higher α-helical content after insertion into the lipid monolayer. The differences mentioned are especially obvious if the wt peptide is compared to peptide ΔG1. However, the differences detected between the wt peptide and peptide G1E seem too small to explain their large difference in fusogenicity; both analogs interact efficiently with a lipid target, and both have a high content of α-helix. The most striking difference between the two peptides is observed when studying the behavior of pure peptide monolayers at the air/water interface; the wt peptide is not only more surface active, it also has a more extended conformation occupying a larger area per molecule. If this would also apply to the wt peptide after insertion into a lipid environment, it may explain the difference in membrane destabilizing activity between the wt peptide and peptide G1E. A greater surface activity and larger molecular area of the wt peptide would be expected to result in a stronger interaction with, and a more pronounced distortion of, the target membrane. Considering the fact that both the wt peptide and peptide G1E have a high content of α-helical structure, a high α-helical content may be a prerequisite to membrane destabilization (as proposed, see Ref. 40) whilst not being a guarantee that membrane destabilization will occur. In addition, the extent to which the α-helical peptide penetrates the target membrane could be an important factor. Alternatively, the orientation with respect to the bilayer normal or the tertiary structure of the peptide may be crucial factors in determining the membrane destabilzing activity of analogs of NHA_2.

Implications for the mechanism of influenza HA-mediated membrane fusion

The interaction of NHA_2 with its lipid target (the endosomal membrane and/or the viral membrane) is thought to play a crucial role in the induction of membrane fusion. The experimental approach we used to study this interaction is a model system approach; synthetic peptides are meant to mimic NHA_2 and the lipid monolayer at the air/water interface is meant to mimic the outer monolayer of its lipid target. It should be appreciated that the conformation of the synthetic analogs and their interaction with a lipid target may not be fully representative of the conformation and lipid interaction of NHA_2 during HA–membrane interaction. For instance, influenza virus fuses efficiently with large unilamellar lipid vesicles without releasing the vesicular contents (see for example, Ref.34) whereas synthetic analogs of NHA_2 have only been reported to induce fusion of small unilamellar vesicles the latter vesicles being intrinsically unstable; destabilization requires high peptide to lipid molar ratios and lipid mixing is accompanied by the release of vesicular contents (therefore referring to these peptides as being fusogenic is questionable; see also Ref. 21). Virus fusion is influenced by variations in the lipid composition of the large unilamellar vesicles [34,43]; in contrast, the interaction of the synthetic analogs with a lipid monolayer was only slightly affected by a change in lipid composition. Not only the amino acid composition but also the length of the synthetic analog strongly affects its membrane destabilizing activity. If, for example, three C-terminal amino acids are added to the wt

peptide used in this study, a much more potent peptide is obtained [19]. Finally, it was recently shown that HA-mediated membrane fusion requires the concerted action of several HA trimers [44]. These data indicate that predictions made based on the behavior of these synthetic analogs should be treated cautiously.

First of all, the monolayer data obtained on the wt peptide predict that after interaction with its lipid target, NHA_2 will form an α-helix. This prediction is in line with the results obtained on BHA–vesicle interaction using photoaffinity labeling. These suggest an amphipathic α-helical NHA_2 interacting with the outer lipid monolayer of the membrane [15,22].

The monolayer data also predict that after extrusion of NHA_2 from the HA spike protein, which normally occurs at low pH, the interaction of NHA_2 with its lipid target is not absolutely dependent on low pH (also see Ref. 23). This prediction is supported by the fact that certain mutants of HA not only undergo the typical conformational change at a pH as high as pH 6.4, but also have fusion activity at this pH (mutations not involving NHA_2; see Ref. 45). On the other hand, in wild-type HA, low pH probably does have a role in addition to the induction of the irreversible conformational change of HA and the exposition of NHA_2 (see Ref. 46). Though low pH may not be absolutely required for the interaction of NHA_2 with its lipid target, it should be noted that the monolayer data predict that this interaction is very likely to be stimulated by low pH. One factor may be the protonation of acidic amino acids (see Fig. 1) resulting in an increase in hydrophobicity of NHA_2. The acidic amino acid residues are situated on the hydrophilic face of the (putative) amphipathic α-helical NHA_2 [21,40] and their protonation should allow for a greater extent of penetration of NHA_2 into a lipid environment. The latter is suggested by model calculations showing that the free energy needed for the transfer of an α-helical NHA_2 from the aqueous phase to a lipid bilayer becomes more negative upon lowering of the pH [47]. Alternatively, charge neutralization may facilitate the cooperative action of several NHA_2's in the induction of membrane fusion.

Finally, a comparison of fusion-active and fusion-defective analogs of NHA_2 in a monolayer set up suggests that a high α-helical content, as such, is not sufficient for fusion activity. The extent of penetration into the lipid target, or a defined orientation or tertiary structure of NHA_2 may be crucial to the membrane fusion activity of influenza HA. In this context it is important to realize that the precise role of NHA_2 in the induction of membrane fusion has not been established yet. It can even not be excluded that NHA_2 may interact specifically with certain (unidentified) components of the target membrane or with protein domains in HA itself. Different models for HA-mediated membrane fusion have been proposed, in which NHA_2 either penetrates the target membrane (see for example, Refs. 2 and 46) or the viral membrane (see for example, Ref. 20) or penetrates no membrane at all. A recent example of the third possibility is a model in which NHA_2 is extruded sidewards, penetrates neither viral or target membrane, but instead forms an hydrophobic bridge allowing a flow of lipids between both membranes [21]. The results obtained in a study on the interaction of isolated HA and intact influenza virions with a lipid monolayer (Burger et al., in preparation) are in good agreement with the latter model; although, as yet, the other models cannot be excluded. In each of the models proposed, NHA_2 interacts with lipids from target and/or viral membrane. In this respect a study on the interaction of synthetic analogs of NHA_2 with a lipid target may prove valuable, irrespective of which model proposed for HA-mediated membrane fusion turns out to be the right one.

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