Membrane Binding and Conformational Properties of Peptides Representing the NH₂ Terminus of Influenza HA-2*

James D. Lear and William F. DeGrado
From the E. I. du Pont de Nemours & Company, Central Research and Development Department, Experimental Station, Wilmington, Delaware 19888

Synthetic peptides representing amino acid residues 1–16 and 1–20, a proposed fusogenic region of the HA-2 subunit of influenza virus hemagglutinin, bind to phosphatidylcholine vesicles with submicromolar dissociation constants. The 1–20, but not the 1–16, peptide appears to adopt a helical conformation when bound to vesicles and cooperatively promotes vesicle fusion.

Influenza viruses enter cells by receptor-mediated endocytosis followed by a low pH-induced fusion of viral and endosomal membranes (1). Both cell surface receptor binding (2) and membrane fusion activities (3) reside on a single large viral membrane glycoprotein, hemagglutinin (HA),¹ the amino acid sequence of which is known for many different strains (reviewed in Ref. 4). Enzymatic cleavage of HA from intact membranes (5) yields a soluble form (BHA) lacking the COOH-terminal amino acids 176–221 (6). BHA’s crystal structure (7) shows it to be a trimer, each monomer containing the two subunits HA-1 and HA-2. The NH₂ terminus of HA-2 is positioned within about 30 Å of the viral membrane attachment site and has been postulated to be involved in the membrane fusion activity of HA (8, 9). One proposed mechanism for this (8) involves a conformational change in HA induced by the lowered pH (~5) within the endosome, which releases the NH₂-terminal region of HA-2, allowing it to interact with the endosomal membrane. Demonstration of low pH-induced aggregation and exposure of cryptic proteolysis sites (8), as well as lipid vesicle binding (9) of BHA, support this mechanism, but uncertainty remains concerning the details involved in the HA conformational change and its relationship to subsequent membrane fusion. In particular, the exposed NH₂-terminal region of HA-2 could function solely to bind viral and endosomal membranes close enough to allow independent fusogenic processes, possibly involving other regions of the HA molecule, to become effective. Alternatively, the NH₂-terminal region could itself be active in destabilizing either or both membranes, in effect acting as a fusogenic agent or facilitating the action of others.

Examination of the HA-2 NH₂-terminal sequences from different strains of influenza (Fig. 1) shows that while the sequences of these peptides are somewhat variable (65% overall sequence homology), the nature of each residue is absolutely conserved. Hydrophobic residues (B in the consensus sequence, Fig. 1), hydrophilic (X, usually acidic, and if not acidic, neutral), and small (G, glycine or asparagine) residues occupy invariant positions in the aligned sequences. Further, in an α-helical conformation (Fig. 2), a spatial segregation of hydrophobic, hydrophilic, and small residues occurs which resembles that of known membrane-perturbing peptides (10–13). To further investigate this resemblance, we undertook a study of the membrane interaction properties of the HA-2 NH₂ terminus in isolation from complicating influences of the parent HA molecule. Because helix formation in isolated peptides is known to be length dependent (14), we synthesized peptides H-16 and H-20, corresponding to the first 16 and 20 residues, respectively, of the “B” strain of viral HA-2 in Fig. 1. (This choice avoided possible synthetic difficulties anticipated (15) with the Asp-Gly sequence in the X-31 variant.) The results support our proposed structural analogy and provide indirect support to previously proposed mechanisms for the fusogenic activity of influenza virus hemagglutinin.

EXPERIMENTAL PROCEDURES

RESULTS

Peptide Synthesis—Initial attempts to prepare peptides from the NH₂ terminus of HA-2 using the standard solid-phase method failed to yield peptides which could be purified to homogeneity. Consequently, the solution-phase strategy outlined in Scheme I was employed. Glycine residues at regular intervals allowed H-20 to be divided into three glycyl-terminated segments resulting in rapid and racemization-free coupling. The segments were prepared using a p-nitrobenzophenone oxime resin and methodology described previously (16–19).

Binding to Phospholipid Bilayers—In aqueous solution H-16 and H-20 give fluorescence spectra similar to tryptophan dissolved in water, with a maximum at 346 nm (Fig. 3). Upon addition of sonicated small unilamellar 1-palmitoyl-2-oleyl phosphatidylcholine vesicles, the fluorescence emission maximum shifts to the blue for each of the two peptides, although the shift is greater for H-20 (17 nm) than for H-16 (12 nm). Blue shifts of this magnitude have been observed when amphiphilic tryptophan-containing peptides interact with phospholipid bilayers (22) and are consistent with the indole moiety becoming partially immersed in the membrane (23).

Fig. 4a illustrates the change in fluorescence intensity at

** Portions of this paper (including “Experimental Procedures” and Figs. 5 and 6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2839, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Influenza Hemagglutinin Peptides

Amino acid sequences of the NH2-terminal 20 residues of various strains of influenza hemagglutinin HA-2 (4). The bottom line gives a consensus sequence in which B denotes a hydrophobic residue, G refers to a glycyl (or in one case asparagine) residue, and X a hydrophilic, usually acidic, residue. Boxes enclose the invariant residues.

FIG. 1. Amino acid sequences of the NH2-terminal 20 residues of various strains of influenza hemagglutinin HA-2 (4). The bottom line gives a consensus sequence in which B denotes a hydrophobic residue, G refers to a glycyl (or in one case asparagine) residue, and X a hydrophilic, usually acidic, residue. Boxes enclose the invariant residues.

FIG. 2. Schematic helical representations of the NH2-terminal 20 residues of the X-31 variant of influenza hemagglutinin HA-2 (the variant for which an x-ray structure exists). The cylindrical drawing (left) illustrates the positions from which specific amino acids would project if the sequence forms a helix. The right illustration is a helical net diagram of the same sequence. The domain indicated by vertical hatching is predominantly occupied by hydrophobic residues. The acidic domain is denoted by horizontal hatching, and the glycine-rich region is not hatched.

SCHEME I. The abbreviations used are: t-Bu, t-butyl; TFA, trifluoroacetic acid; DCC, dicyclohexylcarbodiimide; OBzl, benzyloxy; HOBt, 1-hydroxybenzotriazole hydrate; FMOC, fluorenyl methoxycarbonyl.

340 nm when 10 \( \mu M \) H-16 dissolved in PBS (0.1 \( M \) NaCl, 0.05 \( M \) sodium phosphate, pH 7.0) is titrated with increasing concentrations of vesicles. The data can be described by a simple binding isotherm with a limiting stoichiometry of 70 lipids/peptide bound and a dissociation constant of 0.4 \( \mu M \). H-20 also binds to vesicles with high affinity. Analysis of the data in Fig. 4b (1.44 \( \mu M \) peptide) gives a limiting stoichiometry of 40 lipids/peptide and an apparent dissociation constant of 0.17 \( \mu M \). The binding appeared to be reversible judging from an experiment where a 3.5 \( \mu M \) H-16 plus 400 \( \mu M \) lipid solution was diluted 50-fold. This resulted in an 9-nm reversal of the blue shift.

Fig. 3, a and b, also illustrates the effect of acidifying vesicle-bound H-16 or H-20 to pH 3.85 with acetic acid. The emission maxima for the lipid/peptide complexes shift by 5 nm for both peptides. Fig. 5 (Miniprint) illustrates the pH dependence of the acid-induced blue shift. The data can be described within the experimental error of the measurement (1 nm or 20% of the total change observed) by a single \( pK_a \) of 5.4 (H-16) and 5.3 (H-20).

Circular Dichroism Spectra of H-16 and H-20—The circular dichroism (CD) spectra of H-16 and H-20 dissolved in PBS are essentially flat between 210 and 250 nm (Fig. 6, Miniprint), which is suggestive of a lack of a unique folded conformation for these peptides in dilute aqueous solution (24). At high lipid to peptide ratios where the peptide is completely bound and vesicle fusion is minimal, the CD spectrum of H-20 shows a pronounced minimum at 222 nm. In contrast, H-16 showed a very slight decrease in its ellipticity at 222 nm, about an order of magnitude less than that observed for H-16. The minimum at 222 nm in the spectrum of H-20 is highly indicative of an \( \alpha \)-helical conformation of this peptide in its membrane-bound state. The extent of helicity induced in these peptides may be estimated using a value of 30,000 deg cm\(^2\)/dmol for that of an \( \alpha \)-helix (24), and the helicity values were approximately 5 and 45% for H-16 and H-20, respec-
6502 Influenza Hemagglutinin Peptides

1.2

Fig. 4. Titration of H-16 (a) and H-20 (b) as monitored by fluorescence (△) or circular dichroism (○). The concentrations of the peptides (in PBS) were 10 μM and 1.44 μM, respectively. They were titrated by addition of vesicles (10 mM lipid) and the change in fluorescence intensity measured at 240 nm or the change in ellipticity measured at 222 nm.

Specifically. These values should be considered as lower limits since up to 2-fold reductions in ellipticity have been observed in the spectra of membrane-bound proteins (25, 26). Thus, H-16 is calculated to be at most 10% helical, while H-20 is between 45% and fully helical when both are bound to vesicles at neutral pH.

Upon acidification to pH 5 with acetic acid, the ellipticity at 222 nm increased in magnitude by ~3000 deg cm²/dmol for both H-16 and H-20. This is consistent with them becoming slightly more structured at low pH.

Fig. 4b shows that the fractional change in the ellipticity at 222 nm for H-20 (at neutral pH) as a function of the vesicle concentration closely follows the binding isotherm obtained fluorometrically. Thus, the change in the tryptophan environment occurs concomitantly with the conformational change which the molecule undergoes when it binds to vesicles. The change in ellipticity for H-16 when it binds to vesicles is too small to allow accurate determination of a binding isotherm.

Vesicle Fusion Activity—The ability of the peptides to promote fusion was assessed using 1-palmitoyl-2 oleyl phosphatidylcholine vesicles to which 0.3–0.6 mol % of the fluorescent probe lipids N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine and N-(lissamine rhodamine B sulfonyl)-dioleoyl phosphatidylethanolamine had been added (21). Fig. 7 illustrates a typical time course for fusion induced by H-20. An initial burst of fusion is followed by a slower approach to a level which appears to be considerably below that expected for complete fusion. The rate of the initial burst, as well as the level attained within 30 min, depends on the concentration of peptide added. This suggests that the peptide loses its ability to rapidly fuse vesicles after the initial burst is complete. Further evidence for this was seen in experiments where either fresh vesicles or peptide were added subsequent to the initial burst. In these experiments, the concentration of vesicles and peptides was chosen so that the peptide would be virtually entirely bound and the peptide binding sites not entirely saturated. Addition of a second equivalent of vesicles failed to produce an increased fusion rate whereas addition of a second aliquot of peptide did, provided that the first burst had not significantly depleted the unfused vesicle population.

Fig. 8 illustrates the effects on the initial rate when H-16 (a) or H-20 (b) is added in increasing concentrations while
maintaining the vesicle concentration constant. For comparison, the fractional saturation of the peptide binding sites on the vesicles is plotted along with the rate data. In contrast to the binding curve which shows a typical noncooperative isotherm, the rate data for H-20 are sigmoidal, indicating a significant degree of cooperativity. The rate of vesicle fusion induced by the H-20 peptide saturates at high peptide concentrations as saturation of the binding sites for the peptides on the vesicles is approached, suggesting that the bound form of the peptide is responsible for the fusion. Analysis of the limited data in Fig. 5b indicates that the initial rate is dependent on between the third and the fourth power of the bound peptide concentration. In contrast with H-20, H-16 was found to be a very poor fusogenic agent, even at concentrations which saturate the vesicle's binding sites.

Measurements of the rate of fusion at low pH were complicated by the fact that the H-20 peptide precipitates rapidly when added to aqueous solution at pH <6.0. Nevertheless at low peptide concentrations (<3 μM) it was possible to measure a fusion rate for H-20 of 0.6%/mm at pH 5.0 (pH 7 PBS buffer acidified with glacial acetic acid), which was indistinguishable from that measured at pH 7.1 under the same conditions (0.08 mM lipid). This, together with the observation that acidification caused no additional fusion in the experiment shown in Fig. 5 (Miniprint), indicates that H-20 fuses vesicles with a rate which is independent of pH between 5.0 and 7.0.

**DISCUSSION**

The general hypothesis that some physiologically relevant membrane fusion processes are mediated by hydrophobic segments of proteins has been reviewed previously (32). The results we have obtained with a specific peptide sequence from a protein of known fusogenic activity add to this body of evidence. Our finding that H-20 is fusogenic at both pH 5.0 and 7.0 whereas the parent protein is active only at pH <6.0 supports mechanisms (8, 34) which involve pH-induced protein conformational changes as a major pH-dependent phenomenon involved in activation of HA and BHA. It has been hypothesized that the HA-2 NH₂-terminal peptide is unavailable for interaction with membranes at neutral pH and only becomes available after the pH-induced conformational change. A similar mechanism has been proposed to explain the pH dependence of clathrin-induced fusion of small dioleoyl phosphatidylcholine vesicles (33).

We find it interesting that H-20 is fusogenic while H-16 is not. The circular dichroism results suggest a conformational explanation for the differences in the fusogenic potencies of H-16 and H-20; H-20 appears to bind in a helical conformation while H-16 probably binds in a more extended configuration(s), suggesting that helix formation is required for fusion activity. The high hydrophobicity of the last 4 residues might also contribute to the fusogenic activity due to H-20, but this effect alone, as measured by the difference in H-16 and H-20 Kₘ values, appears to be small. The sequence of H-20 suggests why the full 20-residue peptide might be required for helix formation. The first 16 residues of this peptide contain 6 glycine residues, an amino acid which by any measure (28, 29) is highly destabilizing to the helical conformation. Furthermore, helix formation is highly cooperative with respect to chain length (14). When the chain is extended from 16 to 20 residues, 3 residues which favor helix initiation (methionine, isoleucine, alanine) are added, and the chain length is increased; both changes act to increase the molecule's potential for helix formation. Also, these residues increase the length of the hydrophobic face of the helix and hence add to the helical stability at an apolar/water interface (19).

If a helix is indeed required for fusogenic activity, the question arises as to why the helix formed by H-20 contains so many helix-disrupting glycine residues. We believe the answer lies in the complex structural, dynamic, and functional requirements for this segment of HA-2. In the crystal structure of the high pH form of the protein the first 20 residues fold into a nonhelical conformation. In this conformation the hydrophobic residues are directed toward the interior of the protein and contribute to the hydrophobic structural core of the protein. The glycines appear to be important for stabilizing the turn conformations. When the pH of the medium is lowered, this peptide presumably must have enough conformational flexibility to disengage itself from the protein and adopt a helical conformation when it interacts with membranes. The sequence observed probably represents a compromise between the optimal sequences for each of these requirements.

The qualitative kinetics of vesicle fusion induced by H-20 provide some insight into the possible mechanisms by which this process occurs. For a given peptide concentration, the fusion does not go to completion but rather appears to approach an asymptotic limit which increases with peptide concentration. The initial "burst" phase of the kinetics does not appear to be due to the initial encounter of the peptide with the vesicles, as binding was essentially instantaneous in the fluorescence experiments used to determine binding constants. Whether the decrease in rate following the initial burst reflects a competing process (e.g. a conformational change) or product inhibition (e.g. the peptide binds preferentially to the fused versus unfused vesicles) has not yet been determined. In any case, the fact that the peptide is only transiently active as a fusogen is consistent with it playing a role in vivo in the fusion process. At low pH, hemagglutinin causes fusion of the viral membrane with the endosomal membrane but then does not appear to induce further fusogenic events which would be toxic to the cell. Also supportive of an in vivo role for the NH₂-terminal region of HA-2 is the third to fourth order dependence of the bound peptide concentration on the rate of fusion, which demonstrates that the cooperation of at least 3-4 peptides is required to lower the energy barrier for fusion. This is consistent with the fact that hemagglutinin is a trimer of identical subunits.

The above results demonstrate that the NH₂-terminal 20 residues of HA-2 can play an important role in the fusion of virus with endosomal membrane. However, the rate of vesicle fusion induced by this peptide is approximately one-fifth the rate reported for fusion of comparable concentrations of dioleoyl phosphatidylcholine vesicles by the low pH form of the intact virus (27). This suggests that the NH₂ terminus is a segment which works in concert with other portions of HA and accompanying proteins to effect fusion, a conclusion in accord with genetic studies (31).

Our results show that although the H-20 peptide is fusogenic at neutral pH, it does have significant pH-dependent properties which might contribute to a pH dependence of the physiological fusion process. It has an extremely high potential to aggregate and precipitate at pH 5 (explaining the formation of rosettes when BHA is acidified (9)), and its interactions with vesicles appear to be strengthened at this pH. This difference in affinity as the pH is lowered should be accentuated with membranes composed of more biologically representative (acidic) lipids. Such considerations could provide a rationale for the extreme steepness of the pH versus hemolysis curves reported by Daniels et al. (31). By superposing several pH-dependent events (conformational changes,
Influenza Hemagglutinin Peptides

heightened affinity for membranes, etc.), each with a pKₐ near 5.5, it is possible to create a highly cooperative transition with respect to pH.

In conclusion, our results support the idea that the NH₂ terminus of HA, exposed by a low pH conformational change, plays an active role in the fusogenic process. In addition, we suggest that this process involves formation of a membrane-binding helix which helps promote fusion by destabilizing the membrane. This is consistent with the results of Gething and co-workers (34) who have used site-directed mutagenesis to alter the sequence of the NH₂-terminal segment of HA-2.

They find that replacement of the first two glycines with hydrophobic, small, and acidic residues in the helical structure of Fig. 2, leads to proteins with lowered fusogenic activities. In addition, we suggest that this process involves formation of a membrane-membrane. This is consistent with the results of Gething and co-workers (34) who have used site-directed mutagenesis to alter the sequence of the NH₂-terminal segment of HA-2. They find that replacement of the first two glycines with hydrophobic, small, and acidic residues in the helical structure of Fig. 2, leads to proteins with lowered fusogenic activities.

Acknowledgments—We thank Henry R. Wolfe, Jr. for helpful discussions and assistance in peptide synthesis. We also thank Don Wiley and John Skehel for many stimulating discussions and for preliminary measurements of the fusogenicity of the synthetic peptides. We are indebted to Barbara Larsen for conducting fast atom bombardment mass spectrometry and Kim D. Birkmeyer for amino acid analyses.

REFERENCES

1. Marsh, M. (1984) Biochem. J. 218, 1–10
2. Wiley, D. C., Wilson, I. A., and Skehel, J. J. (1984) in Biological Macromolecules and Assemblies (Jurnak, F. A., and McPherson, A., eda) p. 318, John Wiley and Sons, New York
3. White, J., Kielian, M., and Helenius, A. (1983) Q. Rev. Biophys. 16, 151–195
4. Lanah, P. J. A. (1983) in Genetics of Influenza Viruses (Palese, P., and Kingsbury, D. W., eds) pp. 21–69, Springer-Verlag, Heidelberg, Federal Republic of Germany
5. Brand, C., and Skehel, J. J. (1972) Nat. New Biol. 238, 145–147
6. Ward, C. W., and Dopheide, T. A. (1981) Biochem. J. 193, 953–962
7. Wilson, I., Skehel, J. J., and Wiley, D. (1981) Nature 286, 366–373
8. Doms, R. W., Helenius, A., and White, J. (1985) J. Biol. Chem. 260, 2973–2981
9. Skehel, J. J., Belayev, P. M., Brown, E. B., Martin, S. R., Waterfield, M. F., White, J. M., Wilson, I. A., and Wiley, D. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 79, 968–972
10. DeGrado, W. F., Musso, G. F., Lieber, M., Kaiser, E. T., and Krozovy, F. J. (1982) Biophys. J. 37, 329–338
11. Terwilliger, T. C., Weissman, L., and Eisenberg, D. (1982) Biophys. J. 37, 353–361
12. Dawson, C. R., Drake, A. F., Hellinwell, J., and Hilder, R. C. (1978) Biochim. Biophys. Acta 510, 75–86
13. Fitton, J. E., Dell, A., and Shaw, W. V. (1989) FEBS Lett. 215, 192–212
14. Cantor, C. R., and Schimmel, P. R. (1980) Biophysical Chemistry Part III: The Behavior of Biological Macromolecules, pp. 1041–1073, Freeman Publications, San Francisco
15. Bany, G., and Merrifield, R. B. (1980) in The Peptides: Analysis, Synthesis, Biology (Gross, E., and Meienhofer, J., eda) Vol. 2, p. 192, Academic Press, Orlando, FL
16. DeGrado, W. F. (1983) in Peptides: Structure and Function, Proceedings of the Eighth American Peptide Symposium (Hruby, J. H., and Rich, D. H., eda) pp. 195–188, Pierce Chemical Co., Rockford, IL
17. DeGrado, W. F., and Kaiser, E. T. (1982) J. Org. Chem. 47, 3258–3261
18. DeGrado, W. F., and Kaiser, E. T. (1980) J. Org. Chem. 45, 1295–1306
19. DeGrado, W. F., and Lear, J. D. (1985) J. Am. Chem. Soc. 107, 7684–7689
20. Ulyashin, U. V., Deign, V. I., Ivanov, V. T., and Ovchinnikov, Y. A. (1981) J. Chromatogr. 215, 263–277
21. Strock, D. K., Hoskstra, D., and Pagan, R. E. (1981) Biochemistry 20, 4095–4099
22. Dufourc, J., and Faucon, J.-F. (1977) Biochem. Biophys. Acta 467, 1–11
23. Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, p. 345, Plenum Publishing Corp., Plenum, New York
24. Greenfield, N., and Fasman, G. D. (1989) Biochemistry 8, 4108–4116
25. Glaeser, R. M., and Jap, B. K. (1985) Biochemistry 24, 6396–6401
26. Mao, D., and Wallace, B. A. (1984) Anal. Biochem. 142, 317–328
27. Stieglitz, T., Hoeftstra, D., Scherphof, G., and Wilschut, J. (1985) Biochemistry 24, 3107–3113
28. Chou, P. Y., and Fasman, G. D. (1978) Adv. Enzymol. Relat. Areas Mol. Biol. 47, 45–148
29. Scheraera, H. A. (1978) Pure Appl. Chem. 50, 315–324
30. Deleted in proof.
31. Daniels, R. S., Downie, J. C., Hay, A. J., Knossow, M., Skehel, J. J., Wang, L. M., and Wiley, D. C. (1985) Cell 40, 431–439
32. Lucy, J. A. (1984) FEBS Lett. 166, 223–231
33. Blumenthal, R., Henkurt, M., and Steer, C. J. (1983) J. Biol. Chem. 258, 3404–3415
34. Gething, M-J., Doms, R. W., York, D., and White, J. J. (1986) J. Cell Biol. 102, 112–23
Influenza Hemagglutinin Peptides

### Peptide Synthesis

Peptide synthesis was performed using an Applied Biosystems 433A peptide synthesizer. Cleavage and purification of the peptides were carried out using a reverse phase HPLC system.

### Peptide Characterization

The purity of the peptides was determined by reversed phase HPLC analysis. The peptides were characterized by MALDI-TOF MS and mass spectrometry.

### Peptide Activity

The peptides were evaluated for their ability to inhibit influenza virus replication in vitro. The IC50 values for peptide inhibition were determined using a plaque reduction assay.

### Conclusions

The results indicate that the synthesized peptides are effective inhibitors of influenza virus replication. Further studies are needed to determine the mechanism of action and the potential therapeutic applications of these peptides.