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Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion

(virus glycoprotein/lipid binding/detergent binding)

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ABSTRACT **A conformational change in the hemagglutinin glycoprotein of influenza virus has been observed to occur at pH values corresponding to those optimal for the membrane fusion activity of the virus. CD, electron microscopic, and sedimentation analyses show that, in the pH range 5.2–4.9, bromelain-solubilized hemagglutinin (BHA) aggregates as protein–protein rosettes and acquires the ability to bind both lipid vesicles and nonionic detergent. Trypsin treatment of BHA in the pH 5.0-induced conformation indicates that aggregation is a property of the BHA_2 component and that the conformational change also involves BHA_1. The implications of these observations for the role of the glycoprotein in membrane fusion are discussed.**

Viruses such as influenza that contain lipid membranes appear to enter cells during infection by a process involving the fusion of the viral membrane with a cellular membrane. The results of recent investigations have indicated that, for a number of viruses, fusion occurs optimally over narrow ranges of pH, in the case of influenza viruses between pH 5.0 and pH 5.5 (1–4), and it has been proposed that this correlates with the pH at the site of cell entry in intracellular vesicles such as lysosomes (5).

Evidence that the hemagglutinin (HA) glycoprotein is involved in influenza virus-mediated fusion includes the observations that post-translational cleavage of a precursor HA, HA_0, to HA_1 and HA_2 is required for both virus infectivity (6, 7) and *in vitro* virus-mediated fusion (4, 8) and that the hydrophobic amino-terminal sequence of HA_2 is analogous to that of the amino terminus of the F_1 component of Sendai virus fusion glycoprotein (9–11). Furthermore, the findings that the amino-terminal sequence of HA_2 consists of 10 uncharged hydrophobic amino acids (9) and is the most highly conserved sequence in the hemagglutinin (12) suggest that the terminal region may be directly involved in the membrane-fusion reaction. Analysis of the three-dimensional structure (12) of bromelain-released HA (BHA), which lacks the carboxyl-terminal hydrophobic region through which the complete HA is associated with the lipid membrane of the virus particle, suggests that a conformational change may be required before this can occur.

In this investigation, a conformational change of BHA from X-31 (H3N2) influenza virus has been observed, which occurs at pH values corresponding to those optimal for the *in vitro* membrane-fusion activity of the virus. CD, sedimentation, electron microscopy, and proteolytic susceptibility studies have been used to characterize the pH 5.0-induced transition. The experiments lead to the conclusion that, after incubation at low pH, BHA can form hydrophobic associations with other BHA molecules, with lipid vesicles, or with nonionic detergent micelles. These results are discussed with reference to the three-dimensional structure of HA and in relation to its possible role in virus-mediated fusion.

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METHODS

Virus and HA Purification. X-31 (H3N2) influenza virus (13) was grown in embryonated hens' eggs and purified as described (14). The HA was released from purified viruses by digesting them with bromelain (Sigma) at 37°C for 16 hr in 0.1 M Tris·HCl, pH 8.0/0.05 M 2-mercaptoethanol at an enzyme/virus protein ratio of 1:2. It was purified by density-gradient centrifugation (15) using 5–20% sucrose in phosphate-buffered saline (0.15 M NaCl/0.01 M sodium phosphate, pH 7.3) centrifuging for 16 hr at 20°C and 35,000 rpm in an SW 41 Beckman rotor.

Tryptic Digestion and Analysis of Products. BHA solutions (≈1 mg/ml) were incubated at various pH values (obtained by adding 0.1 M citric acid), adjusted to pH 7.3 by using 1.0 M Tris·HCl, and digested with trypsin (*N,N'*-dicyclohexylcarbodiimide-treated, Sigma) at a BHA/trypsin ratio of 20:1 (wt/wt) for 10 min at 20°C. Soybean trypsin inhibitor (Worthington) in weights equal to those of trypsin was used to stop the reactions. The tryptic products were analyzed by polyacrylamide gel electrophoresis (16) using 16.5% acrylamide gels and by sucrose density-gradient centrifugation as described above. The components of "soluble" and "aggregated" fractions (see Fig. 5) were reduced and alkylated as described (9) and separated by HPLC in 6 M urea/0.2 M formic acid on two I-125 columns (Waters) in series (17).

Triton X-100 and Lipid Binding. To estimate the amount of Triton X-100 bound at pH 5.0, BHA solutions (1 mg/ml) in phosphate-buffered saline were adjusted to pH 5.0 in the presence of 1% Triton X-100 and centrifuged as above through 5–20% sucrose gradients in 0.1 M NaCl/0.02 M Tris·HCl, pH 7.5/0.05% [^{3}H]Triton X-100 (New England Nuclear; 0.2 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) as described (18). BHA association with lipid at pH 5.0 was determined by mixing BHA with lipid vesicles containing phosphatidylethanolamine/phosphatidylcholine/sphingomyelin/cholesterol (1:1:1:1.5) and ^{32}P-labeled baby hamster kidney cell lipid prepared as described (19) and adjusting the pH to 5.0 with 0.5 M acetic acid. The mixtures were made 50% in sucrose, overlayed with 4.0 ml sucrose gradients from 0 to 45% and centrifuged for 4 hr at 45,000 rpm, 4°C, in an SW 50 rotor.

CD. Spectra were recorded on a Jasco J41C spectropolarimeter equipped with a model J-DPY data processor as described (20).

Abbreviations: HA, hemagglutinin; BHA, bromelain-released HA.

RESULTS

Aggregation of HA at Low pH. When the pH was decreased from 7.3 to 5.0, BHA was observed to aggregate, as judged by its sedimentation properties in sucrose density gradients (Fig. 1), and to acquire the ability to agglutinate chicken erythrocytes. The sedimentation coefficient of the aggregates was determined in separate experiments (data not shown), with reference to 28S ribosomal RNA, to be ≈30S. This agrees with the observations of rosette formation (protein–protein micelles) in the electron microscope, in which complexes of 6–10 molecules were detected (Fig. 2).

Detergent and Lipid Binding Properties of HA at Low pH. If the changes in pH to pH 5.0 were made in solutions containing the nonionic detergents Brij 36T (0.5%) or Triton X-100, BHA aggregation was not observed (Fig. 1). By using [^{3}H]Triton X-100, BHA was shown to bind detergent at pH 5.0 (0.39 mg/mg of BHA). Experiments with lipid vesicles (Fig. 3) indicated that, at pH 5.0, BHA also binds quantitatively to lipid bilayers.

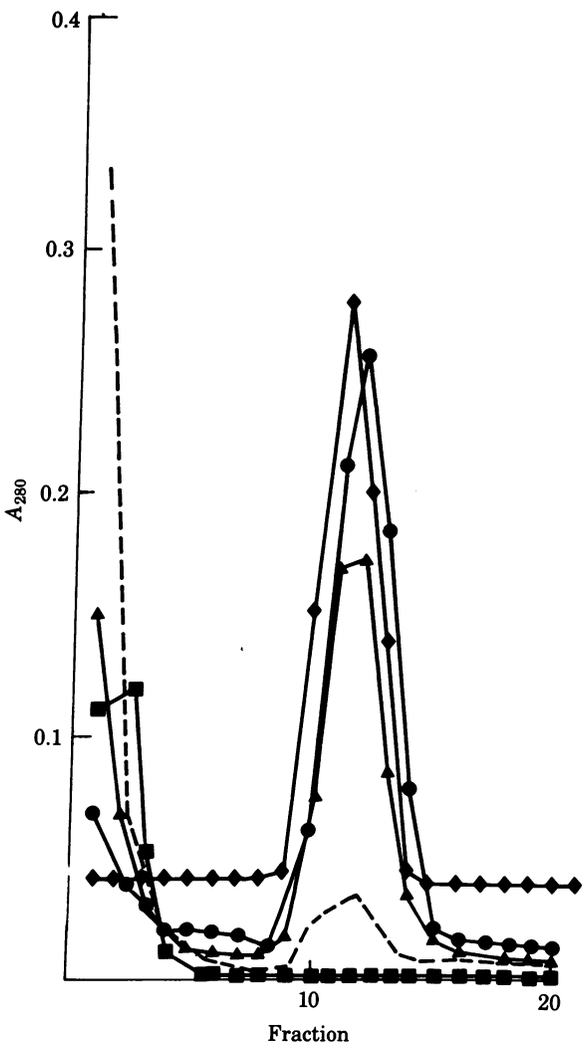

FIG. 1. Sucrose density gradient centrifugation of BHA after incubation at various pH values. X-31 BHA solutions (1 mg/ml) in phosphate-buffered saline were adjusted to various pH values by adding 0.1 M citric acid. They were then layered onto 5–20% sucrose density gradients and centrifuged at 35,000 rpm and 20°C for 16 hr. pH: ●, 6; ▲, 5.5; ---, 5.25; ■, 5.0. ◆, BHA solution made 0.5% in Brij 36T before adjustment to pH 5.0. Fraction 1 is the bottom of the gradient and fraction 12 contains the native form of BHA, 9S trimeric spikes.

Conformational Changes at Low pH. *Tryptic digestion of BHA.* To obtain information on the extent of change in the conformation of HA at low pH, BHA solutions were adjusted to various pH values below 7.3 and then returned to pH 7.3 and treated with trypsin. The products of digestion were analyzed by polyacrylamide gel electrophoresis. The results indicate that, both in the presence and the absence of Brij 36T, BHA became susceptible to tryptic digestion after incubation below pH 5.5 and that the samples that had been adjusted to pH ≈5.0 were cleaved most extensively under these conditions (Fig. 4).

CD. CD spectra in both the near and far UV have been reported previously for the HA (22). The effects of pH variation on the structure of the molecule were therefore assessed by CD analyses in both regions of the spectrum and the results are shown in Fig. 5. In the near-UV (aromatic) region, decreasing the pH to 5.0 resulted in significant changes in the CD spectrum of BHA and an increase in the intensity of the signal between 270 and 280 nm. These effects were also observed when the adjustments in pH were made to solutions containing 0.2% Brij 36T. They were, therefore, interpreted as resulting from a changed conformation of BHA at low pH. The spectra do not revert when the pH is readjusted to pH 7.3 confirming that the conformational change is not readily reversible.

Products of Tryptic Cleavage. It was concluded from the results in Fig. 4 that, of the two polypeptide components of BHA, BHA_1 and BHA_2, only the former appeared to be digested by trypsin. Three products were detected of apparent $M_r \approx$ 40,000, 25,000, and 15,000. Further analyses of the products of digestion obtained in the absence of detergent involved their separation by sedimentation in sucrose density gradients. As shown in Fig. 6*A*, two major fractions (aggregated and soluble) were obtained and the polypeptide composition of each was determined by polyacrylamide gel electrophoresis. In the presence of reducing agent, the fraction containing aggregated material appeared to consist exclusively of the BHA_2 polypeptide (apparent M_r 20,000; Fig. 6*B*, lane b) while the fractions from the top of the sucrose gradients contained three polypeptides (lane a). Electrophoretic separation of the polypeptides in the two fractions following their dissociation without reduction of disulfide bonds showed only a single component in each fraction. The component of the aggregated fraction had an apparent M_r of 25,000 and that of the soluble fraction had an apparent M_r of 40,000. These observations suggest that the BHA_2 polypeptide is not the only component in the aggregated fraction and that the two smallest polypeptides in the soluble fraction are linked by disulfide bonds and are derived from the largest polypeptide component (apparent M_r 40,000).

To determine the sites of tryptic cleavage, the amino-terminal amino acid sequences of the tryptic fragments were determined. The polypeptide components of both aggregated and soluble fractions were separated by HPLC (17) and the sequences of the isolated polypeptides were determined automatically. The results indicate that the three polypeptides of the soluble fractions were generated by tryptic cleavage at residues 27 and 224 of BHA_1 and that only the amino-terminal sequence of BHA_2 was present in the aggregated fraction (Table 1 and Fig. 7). [The amino terminus of BHA_1 is a blocked pyrolidone carboxylic acid residue (23)].

After trypsinization, no changes in CD were recorded in the far (200- to 260-nm) UV. However, in the near (250- to 340-nm) UV in the absence of detergent, trypsin treatment led to partial reversal of the spectrum at pH 5.0 toward that obtained at pH 7.3, suggesting partial disaggregation by trypsin of the complex formed at low pH (Fig. 5*A*). The CD characteristics of both the soluble fraction and the aggregate obtained after trypsinization were also determined. In the far UV, the fractions were quite

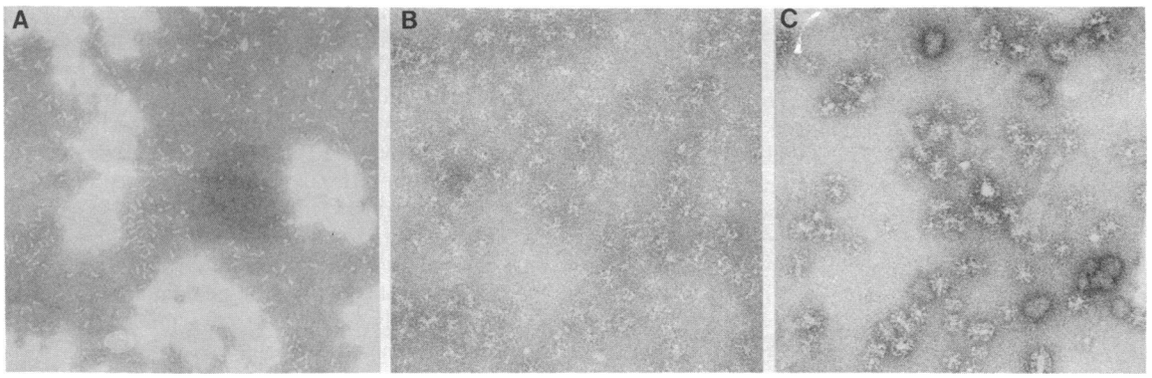

FIG. 2. Electron micrographs of BHA after incubation at pH 7.3 (*A*) and pH 5.0 (*B*) and of the aggregate fraction obtained by tryptic digestion of BHA after incubation at pH 5.0 (*C*). (1% sodium silico-tungstate negative stain; ×150,000.)

distinct (Fig. 5*B*), and estimates of α-helix content indicate that the aggregate contained ≈47% α-helix, while the soluble fraction was rich in β-structure and contained only ≈4% α-helix. These values are consistent with the cleavage pattern shown in Fig. 7 and the retention by the fractions of essentially the same secondary structure as observed in the three-dimensional structure of the intact BHA trimer (12).

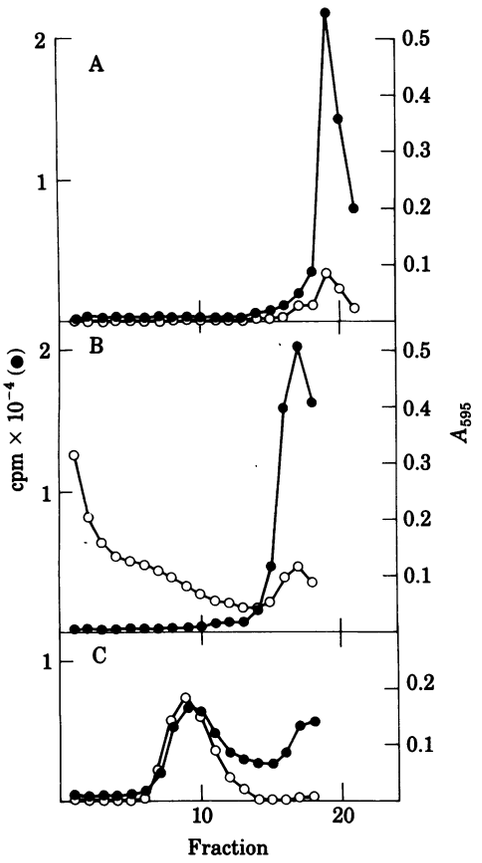

FIG. 3. Association of BHA with lipid vesicles at pH 5.0 ^{32}P-labeled lipid vesicles were mixed with BHA solution and the pH was adjusted to 5.0. Vesicles and protein were made 50% in sucrose and overlayed with a 0–45% sucrose gradient, and the vesicles were then purified by flotation in a 4-hr centrifugation. Protein concentration (○) was measured by the method of Bradford (21) and recorded as A_{595} (lipid reacts slightly with this reagent; note fractions 17–19 in *A* and *B*). (*A*) Gradient contained lipid vesicles but not BHA. (*B* and *C*) Gradients contained lipid vesicles and BHA mixtures that had been incubated at pH 7.0 and 5.0, respectively. Only pH 5.0-treated BHA bound to lipid vesicles.

DISCUSSION

The conformational transition in influenza virus HA reported here is a result of incubation at pH 5.0 and is directly reflected in changes in the susceptibility of the glycoprotein to trypsin, in its aggregation, in its ability to bind lipid and detergent, and in its CD properties in the near-UV (aromatic) region of the spectrum. Identification of the regions of the molecule involved in these changes and assessment of the nature of the structural modifications that occur at pH 5.0 can be made be referring to the three-dimensional structure of the molecule determined at pH 7.5 (12).

Examination of the sites of tryptic cleavage at residues 27 and 224 of BHA_1 indicate that, at pH 7.5, the peptide bond at residue 27 is buried behind residues 32 and 33, near the trimer interface and 15 Å from the amino terminus of BHA_2. The peptide bond at residue 224 may be inaccessible to trypsin due to its proximity to the trimer interface and the carbohydrate side chain at residue 165 of the adjacent subunit. A conformational change affecting subunit interactions could, therefore, render both residues susceptible to tryptic attack.

The protein aggregation that results from the conformational change at pH 5.0 appears to involve hydrophobic interactions because it does not occur in the presence of nonionic detergents and because, after incubation at pH 5.0, BHA acquires the ability to bind to lipid vesicles and nonionic detergent micelles. The

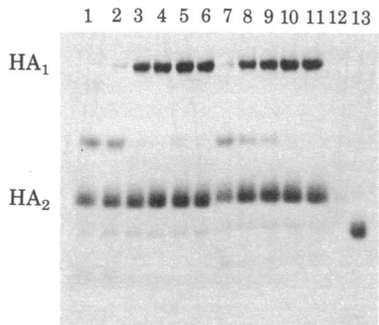

FIG. 4. Susceptibility of BHA to tryptic digestion after incubation at low pH. BHA solutions with and without 0.5% Brij 36T were adjusted to various pH values and then readjusted to pH 7.3 and digested with trypsin. Lanes: 1–6, digestion products obtained after BHA incubation at pH 4.85, 5.01, 5.22, 5.52, 5.98, and 6.72, respectively, in the absence of detergent; 7–11, products obtained after digestion at pH 5.05, 5.2, 5.55, 5.95, and 6.7, respectively, in the presence of detergent; 12, trypsin; 13, trypsin inhibitor. Electrophoresis was at 7.5 V/cm for 16 hr. Digestion of HA_1 is observed for pH values in the 4.8–5.2 range.

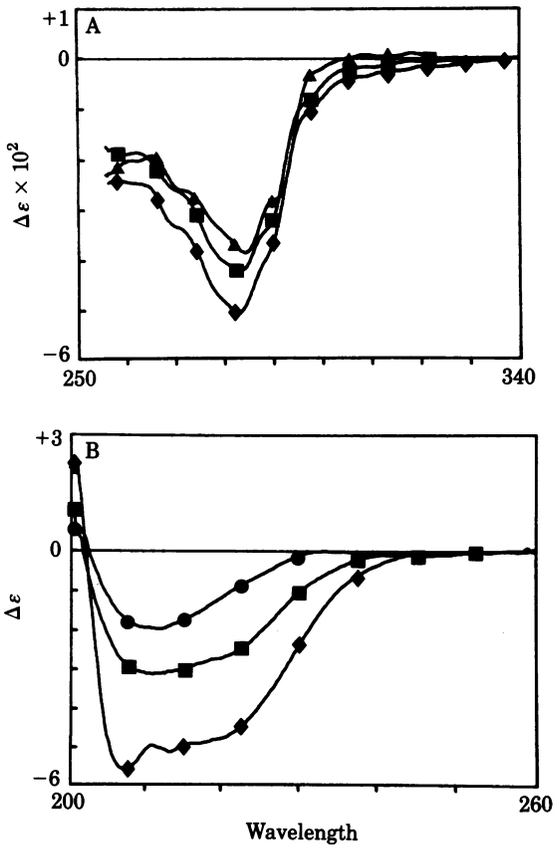

FIG. 5. CD spectra of BHA and BHA tryptic fragments in the near (250- to 340-nm) (*A*) and far (200- to 260-nm) (*B*) UV. (*A*) Spectra at pH 7.5 (▲), at pH 5.0 (◆), and following tryptic digestion after incubation at pH 5.0 and readjustment to pH 7.3 (■). (*B*) Spectra of BHA at pH 7.5 (■) and of the aggregate (◆) and soluble fractions (●) obtained by tryptic digestion.

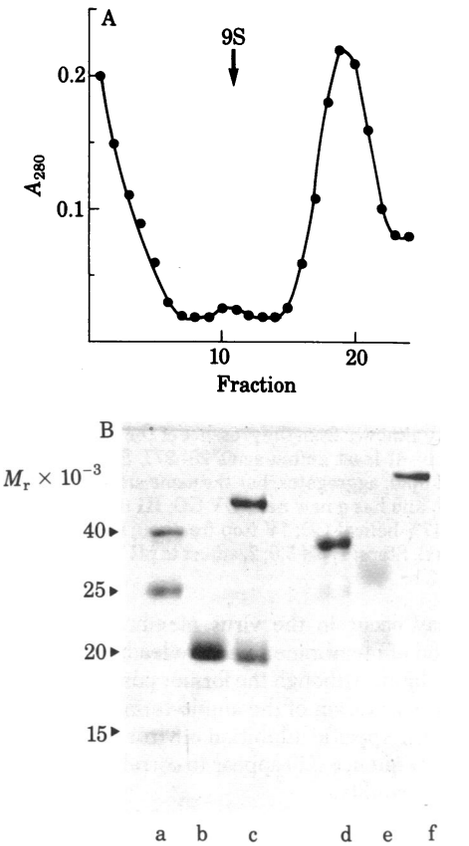

FIG. 6. Sucrose density-gradient fractionation (*A*) of the products of tryptic digestion of BHA after incubation at pH 5.0. BHA in phosphate-buffered saline was adjusted to pH 5.0 and then to pH 7.3 and treated with trypsin. The digestion products were separated on sucrose density gradients, and a soluble component was found in fraction 19 and an aggregated component was found in fraction 1. (*B*) The polypeptide components of fractions 1 and 19 from *A* were analyzed by polyacrylamide gel electrophoresis at 7.5 V/cm for 16 hr. Lanes: a, reduced fraction 19 (soluble; M_r, 40,000, 25,000, 15,000); b, reduced fraction 1 (aggregate; M_r, 20,000); c, reduced BHA (HA_1, M_r ≈50,000; BHA_2, M_r ≈20,000); d, unreduced fraction 19 (soluble; M_r, 40,000); e, unreduced fraction 1 (aggregate; M_r ≈25,000); f, unreduced BHA (HA_1/HA_2; M_r 70,000).

site involved in aggregation is in the stem region of the molecule (12) as shown by the composition of the soluble (BHA_1 residues 28–328) and aggregate fractions (BHA_2 and probably BHA_1 residues 1–27) obtained after tryptic removal from the molecule of essentially all of the BHA_1 (Fig. 7). It is possible that the hydrophobic interactions of aggregation and of lipid and detergent micelle binding involve the amino-terminal region of BHA_2, which has been implicated in membrane-fusion activity on the basis of analogies in sequence (9–11) and in post-translational modification (6, 7, 25) to Sendai virus fusion protein. Model building indicates that the amino-terminal sequence could be exposed to participate in hydrophobic interactions if the amino-terminal glycine, isoleucine-2, and phenylalanine-3 of BHA_2 were withdrawn from the cavity that they occupy near the trimer three-fold axis and the remainder of the amino-terminal region was peeled away from the surface of the molecule.

The aromatic amino acid residues involved in generating the CD in the near UV have not been identified and the structural correlates of the changes in the CD spectrum at pH 5.0 cannot currently be determined. In the far UV, the peptide bond region of the spectrum, the limited changes observed even after tryptic digestion argue against gross secondary structure modifications at pH 5.0; they are, however, not inconsistent with relative movements of molecular domains that independently maintain their tertiary structure.

The connection of the conformational change in HA reported here to the processes of membrane fusion and virus penetration is based on the observations that HA is directly involved in these processes (4, 8) and that they all occur optimally at pH 5.0 (2–4). The relevance of the conformational change to the mechanism of virus-mediated membrane fusion is not known but two possibilities are suggested. It may be that, for HA to be active in fusion, a molecular rearrangement is necessary to allow direct interaction of a hydrophobic region of HA_2—e.g., the amino-terminal decapeptide—with cellular membrane lipid. The observations regarding detergent micelle and lipid binding by BHA after incubation at pH 5.0 may indicate direct correlation with such an interaction. Alternatively, HA–HA interactions analogous to BHA aggregation at pH 5.0 in the absence of de-

Table 1. Amino-terminal sequences of tryptic digestion products obtained after incubation of BHA at pH 5.0

Product apparent M_r	Sequence	Location
40,000	Thr-Ile-Thr-Asp-Asp	BHA_1 residue 28
25,000	Thr-Ile-Thr-Asp-Asp	BHA_1 residue 28
15,000	Gly-Leu-Ser-Ser-Arg	BHA_1 residue 225
20,000	Gly-Leu-Phe-Gly-Ala	BHA_2 residue 1

The sequences were located by reference to the reported amino acid sequences of X-31 (23, 24).

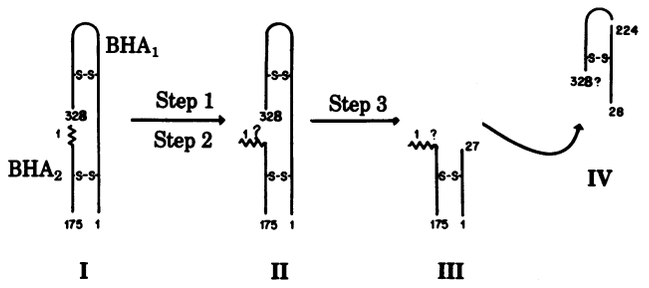

FIG. 7. Diagrammatic representation of sites of tryptic cleavage of HA after incubation at pH 5.0 and of products of the reaction. A single subunit (I) of the HA trimer is represented. [The results of cross-linking studies indicate that the components of the soluble fraction may be monomeric (unpublished).] The COOH terminus of the soluble fragment is unknown; from the presence of the disulfide bond, the fragment must be at least amino acids 28–277. Structures: II, binds detergent and lipid, aggregates, has the same antigenic activity and far-UV CD as I, and has a new near-UV CD; III (bottom fraction), aggregates, has 47% helical CD; IV (top fraction), soluble, β-conformation CD (4% helix). Steps: 1, pH 5.0; 2, adjust to pH 7.0; 3, treat with trypsin at 20°C for 2 hr.

tergent may occur in the virus membrane with consequent modification of membrane structure leading to the exposure of membrane lipid. Although the former possibility is favored, the observed conservation of the amino-terminal sequence of HA_2 (9, 12) and the specific inhibition of virus infection by peptides of identical sequence (26) appear to correlate more readily with the latter possibility.

Schmidt *et al.* (27, 28) have detected fatty acid bound to viral membrane glycoprotein, including the HA of influenza virus. In the 3-Å-resolution electron density map (12) of BHA, a 16-Å-long peak not currently interpreted as protein is found near conserved and primarily hydrophobic residues on the long helix of HA_2 (residues 90–103) and parts of HA_1 in the stem region. This site is near the tryptic susceptible site at HA_1 (lysine-27) shown at low pH and one helical turn from the amino terminus of HA_2. If this density is a fatty acid, it may supply a further hydrophobic interaction to promote fusion with target membranes or may indicate the presence of a "fusion enzymatic site"—e.g., a phospholipase such as A2 (29, 30).

Finally, the soluble fraction obtained from tryptic digests of BHA that had been incubated at pH 5.0 retain most of the antigenicity of intact HA in HA-inhibition blocking tests (unpublished). These findings are in accord with the CD results indicating the maintenance of local HA structure at low pH and suggest that the tryptic fragments could be used in HA immunochemical analyses.

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