Conformational Aspects of the Acid-induced Fusion Mechanism of Influenza Virus Hemagglutinin

CIRCULAR DICHROISM AND FLUORESCENCE STUDIES

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Circular dichroism and tryptophan fluorescence spectroscopy have been used to investigate the structures of the influenza virus membrane glycoprotein hemagglutinin, acid-treated hemagglutinin, and fragments of hemagglutinin derived by proteolysis. The conformational change in hemagglutinin which occurs at the pH of membrane fusion (pH 5–6) was associated with a significant change of the environment of tyrosine residues, a change in the environment of tryptophan residues, but no changes in secondary structure. Tryptic digestion of the hemagglutinin in its low pH conformation which releases one of the subunit polypeptides (HA₁) caused minimal changes in tryptophan tryptophan environments but a small secondary structural change in HA₁. The secondary structure of the remainder of the molecule (HA₂) was very similar to that predicted from the known x-ray crystallographic structure of the native molecule. However, fluorescence spectroscopy indicated a tertiary change in structure in the coiled coil of α-helices which form the fibrous central stem of the molecule. These results are consistent with a conformational change required for membrane fusion which involves a decrease of HA₁/HA₁, HA₁/HA₂ interactions and changes in tertiary structure not accompanied by changes in secondary structure.

The major surface membrane glycoprotein of influenza virus, the hemagglutinin (HA), is a trimer of identical subunits, each consisting of two disulfide linked glycopolypeptides, HA₁ and HA₂. The three-dimensional structure of the ectodomain of HA, bromelain-released HA (BHA), has been determined by x-ray crystallography to 3Å resolution (reviewed by Wiley and Skehel, 1987), and a schematic diagram of a subunit is shown in Fig. 1A. HA is responsible for binding influenza virus to sialic acid containing receptors on host cell surfaces and for the membrane fusion activity of the virus. In the initial stages of infection, virus-receptor complexes are taken into endosomes which are subsequently acidified. At a pH specific for each strain of virus (between 5.0 and 6.5) a conformational change which is required for fusion activity is triggered and fusion of the virus membrane with the endosomal membrane occurs (reviewed by White et al., 1983). As a consequence, the transcriptase complex is transferred into the cytoplasm and virus replication begins.

The low pH conformational change in HA and the resulting membrane fusion activity have been the subject of a number of studies involving biochemical, biophysical, and immunological techniques (Huang et al., 1981; Skehel et al., 1982; Whiting et al., 1982; Daniels et al., 1983a, 1983b, 1985; Graves et al., 1983; Sato et al., 1983; Doms et al., 1985; Stegmann et al., 1985; Getting et al., 1986; Ruigrok et al., 1986a, 1986b; Wharton et al., 1986; Wharton, 1987) aimed at understanding the role of HA in membrane fusion. In this study we use circular dichroism (CD) and tryptophan fluorescence spectroscopy to compare the conformational properties of native and low-pH BHA and proteolytic fragments of low-pH BHA. Changes in the secondary and tertiary structure are discussed in relation to the known structure of the molecule determined at neutral pH.

MATERIALS AND METHODS

Viruses—Influenza A virus X-31 (H₃N₂) and a mutant containing a G→D substitution at HA₅ 112 which fuses membranes at pH 6.1, 0.5 higher than wild type (Daniels et al., 1985), were grown in the allantoic cavity of embryonated eggs as described elsewhere (Skehel and Schild, 1971).

Proteins and Protein Fragments—The proteins and protein fragments used in this study are listed in Table I and illustrated in Fig. 1B. Concentrations were calculated using extinction coefficients derived from their aromatic amino acid composition as previously described (Ruigrok et al., 1986a).

BHA—Hemagglutinin was isolated from virus by digestion with bromelain as previously described (Brand and Skehel, 1972). Bromelain removes the carboxyl-terminal membrane anchor of HA₅, producing the soluble fragment BHA. After removal of the virus by centrifugation, BHA was purified in a sucrose gradient as previously described (Skehel et al., 1982). Low-pH BHA (BHA₅) was prepared by dialysis of BHA at pH 4.9 (0.15 M NaCl, 10 mM sodium phosphate). In some cases, BHA₅ was prepared by direct addition of citric acid to a solution of BHA. The experiments with BHA₅ were performed in the low-pH solution or after dialysis into phosphate-buffered saline (PBS), pH 7.4. All buffers contained 0.01% azide.

HA (28–328)–HA (28–328) was isolated from low-pH-treated virus by digestion with trypsin (virus/trypsin = 120:1, w/w) for 30 min at room temperature. Digestion at pH 7.4 resulted in cleavage at HA (27 and HA (234). The polypeptides HA (28–224) and HA (225–328), which are disulfide-linked, are released from the virus by this treatment (Skehel et al., 1982). If the digestion is carried out at pH 5, cleavage at residue 224 does not occur and HA (28–328) is released as a single polypeptide. The trypsin digestion was stopped by addition of an equal weight of soybean trypsin inhibitor (Worthington). After
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TABLE I

Composition of the BHA species used in this study

| Species     | HA    | HA1  | Nm  | Ntrp | Ntyr |
|-------------|-------|------|-----|------|------|
| BHA7/BHA5   | 1-328 | 1-175| 503 | 9    | 16   |
| HA, 28-328  | 28-328| 300  | 6   | 10   |
| BHA aggregates | 1-27 | 1-175| 202 | 1    | 6    |
| TBHAa       | 1-27  | 38-175| 163 | 1    | 5    |
| Rosettes    | 1-328 | 1-221| 549 | 12   | 17   |

BHA5 or virus incubated at low pH and are, therefore, in the low pH conformational state. In deriving values for the fragments from the x-ray structure we have assumed that protease digestions cause no changes in secondary structure. The agreement between the x-ray and CD-derived values of secondary structure (see Table II) is good except in the case of HA, 28-328 where the CD estimate of \( \beta \) structure is considerably higher than the value obtained by x-ray crystallography. We, therefore, examined the possibility that protease treatment does, in fact, affect the secondary structure. Small changes in the CD spectrum of BHA5 were observed on incubation with trypsin (40:1, w/w) at 37 °C (Fig. 2A). These changes were reproducible, and no changes were observed in a control experiment where BHA7 was incubated with the same concentration of trypsin. CONTIN analysis of the trypsin-treated BHA5 (Table II) showed that the change detected after protease digestion appears to correspond to a small increase in \( \beta \)-sheet content of some 5–7%; this value was, however, quite variable from experiment to experiment. This increase corresponds to some 25–35 residues of the whole molecule, and if the structural change responsible for this increase were confined to HA, 28-328 it would amount to an increase of 8.5–11.5% in \( \beta \)-sheet content and would, at least in part, explain the high \( \beta \)-sheet content predicted from the CD spectrum of HA, 28-328. However, it is also possible that the changes in CD spectra may result from a rearrangement of the random coil portion of the polypeptide as such changes could influence the CD signal.

Near UV CD Spectra of BHA and Proteolytically Derived Fragments

Near UV CD spectroscopy was used to compare the environment of aromatic residues in BHA7, BHA5, and in the proteolytically derived fragments, BHAa aggregates, HA, 28-328, and TBHAa.

Fig. 2B shows the near UV CD spectra of the BHA preparations and fragments listed in Table I with \( \Delta \) calculated on the basis of mean residue weight. Each species shows a major negative band centered at approximately 282 nm with a significant shoulder at about 290 nm. The position of the band suggests that the major contribution to the optical activity is from tyrosine residues. The difference between the spectra of BHA7 and BHA5 suggests that the low pH conformational change is accompanied by a significant change in the environment of one or more tyrosine residues. If all the signal did, in fact, arise from tyrosine the \( \Delta \)/tyrosine would be 0.98, 1.40, 0.77, and 1.83 for BHA7, BHA5, BHAa aggregates, and HA, 28-328, respectively. The spectrum of TBHAa (1 trypthphan, 5 tyrosine) is similar to that of BHAa aggregates (3 trypthphan, 6 tyrosine), though slightly more intense which also indicates that trypthphan makes a relatively small contribution to the near UV CD intensity.

A summation (with appropriate weighting for the number
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Fig. 1. A, schematic diagram of an X31 hemagglutinin subunit. HA is striped. The viral membrane would be at the bottom of the diagram and the trimeric interface would be to the left of the molecule. • denotes the residues at which cleavage by trypsin occurs when the molecule is in its low pH form. The positions of tryptophan 92 on HA1 and 222 on HA2, which are discussed later, are shown as ▲. The diagram was produced by a computer program written by Lesk and Hardman (1982). B, flow diagram of the proteolytically derived fragments of HA. The structures shown are those derived from the native structure of BHA; it is not intended to imply that these structures are entirely preserved in the proteolytic fragments. The carboxyl and amino termini of HA1 and HA2 (Cα1N1 and Cα2N2) are shown for all polypeptides. BHA2 aggregates are aggregates of 8 ± 1 molecules which remain trimeric (Ruigrok et al., 1986b). TBHA2 are trimeric molecules, and HA2 28–328 exists as monomers.

Table II
Secondary structure evaluated from far UV CD spectra compared with estimates from the x-ray structure of BHA

|  | α  | β  | Other |
|---|---|---|---|
| BHA7 | 22 | 28 | 50 |
| CD | 22 | 34 | 44 |
| BHA5 | 22 | 34 | 44 |
| HA1 28–328 X-ray | 7 | 36 | 57 |
| CD | 8 | 55 | 37 |
| BHA2 aggregates X-ray | 46 | 16 | 38 |
| CD | 52 | 16 | 32 |
| TBHA2 X-ray | 56 | 16 | 28 |
| CD | 64 | 18 | 28 |

Effect of trypsin on BHA5

| Species | α  | β  | Other |
|---|---|---|---|
| X31 Minus trypsin | 21 | 34 | 45 |
| Plus trypsin | 22 | 41 | 37 |
| 1a Minus trypsin | 20 | 34 | 46 |
| Plus trypsin | 22 | 39 | 39 |

Fluorescence Studies on Hemagglutinin

Fluorescence spectroscopy was used to study the environment of tryptophan residues in the various preparations of hemagglutinin.

Fluorescence Spectroscopy of the Conformational Change of Hemagglutinin—The membrane fusion activity of HA requires a conformational change in the molecule which is triggered on incubation at pH 5.

Fig. 3A compares the fluorescence emission spectra of BHA7 in pH 7 buffer and BHA5 in pH 5 buffer and indicates a small decrease in intensity and a small blue shift. The spectrum of BHA5 in pH 7 buffer is identical to that in pH 5 buffer indicating that the pH-induced fluorescence change is irreversible. Similar, though somewhat larger, changes in fluorescence are seen on lowering the pH of a solution of HA rosettes (data not shown). Small changes in the fluorescence of HA rosettes on lowering the pH have also been observed by Sato et al. (1983). Adjustments to pH were made in these experiments by addition of concentrated (0.15 M) citrate buffer (pH 3.5) to the HA solution in the cuvette. Control experiments (data not shown) showed that addition of the same amount of 0.15 M citrate (pH 7.0) had no effect on the fluorescence spectrum. The change in fluorescence of BHA7 on lowering the pH is unlikely to be due to scattering artifacts resulting from glycoprotein aggregation since similar results of residues) of the spectra of BHA2 aggregates and HA1 28–328 is shown in Fig. 2B. Since this is essentially identical to the spectrum of BHA5, tryptic cleavage of BHA5 does not lead to substantial changes in tertiary structure detected by CD of the aromatic amino acids. This was confirmed directly by incubating BHA5 with trypsin (50:1, w/w) when no changes in the near UV CD spectrum were observed.
Comparison of the Fluorescence Changes of X31 BHA and a Mutant Which Fused Membranes at a Higher pH—To investigate the possibility that changes of tryptophan fluorescence upon lowering the pH resulted from an effect of protonation of histidine or other residues in the molecule, we compared the fluorescence properties of X31 BHA with those of a mutant which contains a single amino acid substitution, HA\(_{28-328}\) 112D\(\rightarrow\)G (mutant 1a), and undergoes the conformational change at 0.5 pH unit higher than X31 (Daniels et al., 1985). Fig. 3B shows the effects of titrating solutions of X31 and the mutant BHAs with small aliquots of citrate buffer (0.15 M, pH 3.5) and monitoring changes in fluorescence emission at 335 nm. The changes in fluorescence closely parallel the pH-induced changes in fusion activity occurring 0.5 pH unit higher for the mutant than for X31. Therefore, taken together with the far UV CD results, the fluorescence changes appear to arise from the relative movements of structural domains occurring during the conformational change rather than from a direct effect of protonation of histidine or other residues.

Effect of Trypsin on Fluorescence Emission—As a result of

are obtained with BHA\(_7\) at one-hundreddth the concentration used above (results not shown). Furthermore, HA rosettes, which do not aggregate at low pH (Ruigrok et al., 1986b), showed similar changes in fluorescence upon incubation at pH 5. The pH-induced change in the fluorescence emission spectrum is consistent with the observations that changes are detected in the near UV CD spectrum which also indicates that there is a change in tertiary structure. Since exposed tryptophan residues fluoresce with a maximum around 350 nm and buried tryptophans fluoresce at a shorter wavelength, nearer 330 nm, the ratio of fluorescence at 330-350 nm is a measure of the degree of exposure of the tryptophan residues. This ratio changes from 1.02 in BHA\(_7\) (1.00 in rosettes at pH 7) to 1.11 in BHA\(_5\) (1.10 in rosettes at pH 5). The residues quenched in the pH-induced transition, therefore, are predominantly those that are exposed in the native form, i.e., the emission difference spectrum is that of exposed tryptophans (long wavelength emission, low 330/350 intensity ratio).

Comparison of the fluorescence changes of BHA and a proteolytically derived fragments of BHA5. All proteins were between 1.0 and 1.5 mg/ml in 10 mM phosphate buffer, pH 7.4, and near UV CD spectra were recorded at 25 °C.}

**Fig. 2.** Circular dichroism spectra of hemagglutinin and proteolytically derived fragments. A, effects of trypsin digestion upon the far UV CD spectra of acid-treated hemagglutinin. The far UV CD spectrum of BHA5 (0.1 mg/ml) in 10 mM phosphate buffer, pH 7.4, was recorded (A), and the sample was then incubated with trypsin (40:1, w/w) at 25 °C for 20 min and the spectrum rerecorded (●). B, near UV CD spectra of BHA, BHA5, and proteolytically derived fragments of BHA5. All proteins were between 1.0 and 1.5 mg/ml in 10 mM phosphate buffer, pH 7.4, and near UV CD spectra were recorded at 25 °C. ΔE was calculated on the basis of mean residue weight. ΔE, BHA\(_7\) ●, BHA\(_5\) ○, HA, 28–328; ▲, BHA\(_2\) aggregates. The summation of HA, 28–328 and BHA\(_2\) aggregates correcting for the number of residues.

**Fig. 3.** Tryptophan fluorescence characteristics of hemagglutinin. All excitations were at 288 nm, and slit widths were set at 5 nm. A, effect of the conformational change upon the tryptophan fluorescence of BHA. The fluorescence spectrum of 0.1 mg/ml BHA in PBS was recorded (O). The pH of the solution was then lowered to 5.0 by the addition of 60 μl of citrate buffer and the spectrum rerecorded (●) taking into account the effects of sample dilution. The difference spectra (●) multiplied by a factor of 2.5 is also shown. B, comparison of the dependence upon pH of the fluorescence properties of X31 BHA and a BHA which undergoes the conformational change onto a higher pH. BHA (0.1 mg/ml in PBS) was incubated at various pH values for 5 min at 37 °C and the fluorescence intensity at 335 nm recorded and plotted against pH. ▲, X31; ●, higher pH mutant 1a. C, effect of trypsin digestion upon the fluorescence of BHA5. The fluorescence spectrum of 0.1 mg/ml BHA 5 was recorded (O). The BHA was then incubated with trypsin (40:1, w/w) for 20 min at 25 °C and the fluorescence spectrum rerecorded (●). The difference spectrum multiplied by a factor of 2.5 is also shown (▲).

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the pH-induced conformational change, BHA becomes susceptible to proteolysis by trypsin at residues HA, 27 and HA, 224 and as a consequence residues 28-328 of HA, are released from the remainder of the molecule as monomers. The effect of trypsin digestion on the fluorescence emission spectrum of BHA5 was investigated by incubating a solution of BHA5 with trypsin (40:l, w/w) for 20 min at pH 7.0. Fig. 3C shows that trypsin digestion leads to a small reduction in the intensity of the emission spectrum. Similar results were obtained when a solution of HA rosettes which had been incubated at pH 5 was digested in the same way (not shown). Incubation of BHA7 with trypsin did not result in any change in the spectrum. However, the changes in the 330/350 intensity ratio after trypsin digestion are very different from those accompanying the pH-induced change. The ratio of 1:11 for BHA5 (1.10 for pH 5 rosettes) changes to 1:05 on trypsin digestion (1.04 for rosettes). This result plus the fluorescence difference spectrum (Fig. 3C) suggests that the tryptophan residue involved was not exposed in the intact molecule.

**Relative Fluorescence and Quenching Properties of BHA and Proteolytically Derived Fragments**—We compared the relative fluorescence intensities and determined the quenching constants (K_q) of BHA and proteolytically derived products in an attempt to localize the fluorescence effects to specific regions of the molecule and to examine further the effects of removing HA, from HA, due to trypsin digestion of BHA5. In addition to the products of trypsin digestion, HA, 28-328 and BHA, aggregates, we also analyzed TBHA, the soluble fragment which is formed from BHA, aggregates by thermolysin treatment. TBHA, lacks the amino terminus of HA, and is of particular interest since it contains only one tryptophan residue at HA, 92.

In order to compare fluorescence intensities for the different protein fragments we calculated normalized fluorescence intensities, Fn, using the equation,

\[
Fn = \frac{\text{measured intensity}}{(\text{C} \cdot N_{cw})}
\]

where C is the concentration of the fragment (µM) and N_{cw} is the number of tryptophan residues that it contains. Values of Fn (at 335 nm) are given in Table III; the values of N_{cw} used are listed in Table I. As a check on these values Fn for BHA was calculated from a weighted sum of values for HA, 28-328 and BHA, aggregates using the equation

\[
Fn(BHA,calc) = \frac{(6 \cdot Fn \text{ HA}, 28-328 + 3 \cdot Fn(BHA, aggregates))}{9}
\]

The calculated value of Fn(BHA, calc) was 7.45, which is very close to the value obtained for trypsin-treated BHA5, 7.70.

HA, 28-328, BHA, aggregates, TBHA, and BHA, all of which are produced from the low pH form of BHA, have the same fluorescence properties at pH 7 and 5.

From this data we can also calculate the apparent fluorescence intensity for individual tryptophan residues. Thus, the intensity from Trp-92 (in TBHA, aggregates) is 25.4, the average intensity from the six tryptophans in HA, 28-328 is 4.75, and the average intensity from Trp-14 and Trp-21 is 6.15. The latter value was obtained by comparing the Fn values for TBHA, and BHA, aggregates. We cannot, however, be sure that the environment of Trp-92 is the same in BHA, aggregates as it is in TBHA, aggregates. The fact that Trp-92 is highly fluorescent in TBHA, is indicative of the residue being buried in the molecule.

To get a more quantitative measure of the accessibility of the various tryptophan residues the acrylamide quenching constants for the various species were calculated, and the results are summarized in Table III. The low value obtained for Trp-92 in TBHA, confirms that this residue is, indeed, inaccessible to quencher. The high value obtained for HA, 28-328 on the other hand suggests, as does the low Fn value, that most of these residues are accessible to quencher.

**The Effect of Tryptic Cleavage at Position 224 upon the Fluorescence of HA, 28-328**—Tryptic cleavage of HA in the low pH conformation occurs first at HA, 27 and then at HA, 224. Furthermore, tryptic digestion at pH 5 rather than following pH reversal to pH 7.0 results in cleavage exclusively at HA, 27. We, therefore, determined the site of digestion involved in the fluorescence decrease by comparing the fluorescent properties of BHA5 digested at pH 5 and 7.

The fluorescence and quenching constants of these preparations are listed in Table III. The average tryptophan fluorescence was less in HA, 28-328 containing the secondary cleavage at HA, 224, and correspondingly the tryptophans appeared to be on average less exposed in uncleaved HA, 28-328. Incubation of uncleaved HA, 28-328 at pH 7 with trypsin (50:l, w/w) results in cleavage at position 224. This process was followed fluorimetrically and was found to result in a decrease in fluorescence in keeping with the above results. Thus, it appears that at least part of the decrease in fluorescence which occurs when BHA5 is incubated with trypsin results from effects of the cleavage at position HA, 224, which probably reflects a change in the environment of Trp-222 in HA, 28-328. In this connection it may be noted that both preparations of HA, 28-328 had identical near and far UV CD spectra.

### DISCUSSION

Between pH 5.0 and 6.5, depending on the particular virus strain, influenza virus hemagglutinins undergo conformational changes as a result of which the viruses can fuse with target membranes (Skehel et al., 1982; White et al., 1982; Huang et al., 1981; Sato et al., 1983). At low pH isolated BHA molecules aggregate into rosettes of 8 ± 1 trimERIC molecules (Skehel et al., 1982; Ruigrok et al., 1986b); alternatively the binding of lipids or detergents prevents rosette formation. The region of the HA involved in aggregation is the HA, amino terminus (Daniels et al., 1983a) which is translocated to the pH of fusion from its buried location in the center of the native molecule (Wilson et al., 1981). Although the low pH change does not involve detectable changes in protein structure, the change appears to be accompanied by a decrease or decrease in the intrinsic fluorescence at pH 7.

**TABLE III**

| Species       | N_{cw} | Fn   | K_q (effective) |
|---------------|--------|------|-----------------|
| BHA5          | 9      | 10.05| 3.75            |
| BHA5 (+ 6.5 M urea) | 9     | 8.85 | 3.59            |
| BHA7          | 9      | 8.70 | 3.70            |
| Rosettes (pH 7) | 12    | 5.75 | 3.50            |
| Rosettes (pH 5) |      | 5.10 |                 |
| BHA aggregates | 3     | 12.85| 1.76            |
| HA, 28-328    | 6      | 4.75 | 5.45            |
| Cleaved       | 6      | 5.48 | 4.78            |
| Uncleaved     | 6      | 5.48 | 4.78            |
| TBHA2         | 1      | 25.40| 1.56            |
changes arise from relative movements of structural domains during the conformational change rather than from the direct effect of protonation of histidine side chains close to tryptophan residues at several places in the native structure. This interpretation is supported by the observation that the fluorescence emission difference spectrum indicates the further exposure of residues that are already partly exposed in the native structure. As the three tryptophans of BHA, are buried in the native structure this change must be associated with the tryptophans in HA.

Far UV CD studies indicate that a small increase in β-sheet content occurs upon trypsin digestion. Analysis of the CD spectra of the digestion products showed that BHA, aggregates have a predicted secondary structure very similar to that computed from the x-ray data. This is remarkable in view of the observation (Ruigrok et al., 1986b) that the length of BHA, increases as a result of the conformational change. HA, 28–328, however, contains 25–35 more residues in the β-sheet configuration than is expected from an examination of the x-ray structure of BHA.

The pH-induced conformational change exposes trypsin-sensitive sites at Lys-27 (HA,) and Arg-224 (HA,) (Skehel et al., 1982). Cleavage at 224 is prevented by performing the digestion at pH 5. Digestion at Lys-27 results in the release of a monomeric fragment of HA, residues 28–328. It is possible that the two pendant strands of HA, residues 28–50 and 300–328 may acquire some extra sheet like structure when the constraints imposed by their interaction with HA, are relaxed (Fig. 1B). It has been observed before that upon heating although low pH HA, becomes susceptible to trypsin at 50 °C, HA, 28–328 only becomes susceptible at 63 °C (Ruigrok et al., 1986a). This also indicates that there is some additional structure in isolated HA, 28–328 which protects potential heat-induced tryptic sites when the pendant strands are freed from interaction with HA,.

Near UV CD spectra show that the spectrum of BHA, is well represented by the appropriately weighted summation of the spectra of HA, 28–328 and BHA, aggregates. Since trypsin digestion did not affect the spectrum, the process of trypsin cleavage does not appear to affect the environment of those aromatic residues which contribute to the near UV signal. This is consistent with the fact that there is only a single tyrosine in the pendant strands of HA, and none in HA, at its points of contact with HA,.
By contrast, fluorescence studies show that trypsin digestion resulted in quenching of the emission of buried tryptophan residues. Comparison of the spectra of cleaved and uncleaved HA, 28–328 indicates that about 50% of this change derives from trypsin cleavage at position HA, 224, probably due to a change in the environment of Trp-222 (HA,). If this interpretation is correct changes in fluorescence associated with disruption of HA,HA contacts appear to be small (about 5% of the total signal from BHAS), and this removal of HA, from HA, in BHAS does not significantly affect the environment of tryptophan residues.

In the crystal structure of BHAS, Trp-92 (HA,2) is very close to HA, Lys-307 of the same subunit and HA, Lys-310 of a neighboring subunit. Tryptic removal of the pendant chains would expose Trp-92 (HA,2) if it were still positioned as in the native structure (Fig. 4). Our results show that this residue is highly fluorescent and inaccessible to quenchers. This suggests either that the strand (residues HA, 58–74) connecting the HA, shorter helix with the longer central helix covers Trp-92 in the low pH form or that a structural change in the helical trimer results in Trp-92 being buried in the trimeric interface. It can be calculated that the exposed surface area of Trp-92 is 2.3 Å² in the native structure whereas if HA, were removed this value would increase to 75 Å². Totally exposed tryptophan residues have surface areas of 361 Å².

In conclusion, the results presented here from spectroscopic studies yield information in the acid-induced fusogenic conformational change of hemagglutinin. The exposure of tryptophan residues, changes in the environment of tyrosine residues, and the absence of a significant spectroscopic change after detaching two-thirds of the molecule by trypsin treatment support the conclusion that the conformational change in HA required for membrane fusion involves changes in HA,HA, and HA,HA contacts within the trimer without major changes in secondary structure.

Acknowledgments — We thank David Stevens, Rose Gonsalves, and Lesley Calder for assistance.

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