Quaternary Structure of Influenza Virus Hemagglutinin after Acid Treatment

ROBERT W. DOMS AND ARI HELENIUS*

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510-8002

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Hemagglutinin (HA), a trimeric spike glycoprotein of influenza virus, mediates fusion between the viral envelope and the membrane of an endosome during virus entry. Fusion is triggered by low pH, which induces an irreversible conformational change in the protein. Several studies have indicated that intersubunit contacts along the trimer interfaces may be broken during this alteration. To determine whether HA dissociates into individual subunits as a consequence of the conformational change, we used velocity gradient sedimentation in the presence of Triton X-100. We also determined the resistance of acid-treated HA to dissociation by sodium dodecyl sulfate, a property of the HA trimer. At pH 7.0, isolated HA sedimented as a 9S trimer and gave the characteristic trimer pattern after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After acidification the HA remained trimeric irrespective of whether it was exposed to acid in intact virus particles or in solubilized form. Only when very low concentrations of HA were acidified did a fraction dissociate to dimers and monomers. In contrast, the water-soluble ectodomain fragment of HA (BHA) readily dissociated under a variety of conditions. Negative-stain electron microscopy supported the notion that HA molecules in virus particles do not dissociate upon acidification and may form larger oligomeric structures in the plane of the viral membrane. Taken together, the results suggested that it is the trimeric HA, or higher-order structures thereof, that are active in the acid-induced fusion reaction. Further, the results emphasized the role of the transmembrane anchors of HA in preventing dissociation of the trimer.

Hemagglutinin (HA) mediates attachment of influenza virus to the cell surface (10, 23) and induces fusion of the viral envelope with the membrane of endocytic vesicles (11, 17, 31, 34). As a result of the fusion reaction, the nucleocapsid gains entry into the cytoplasmic compartment. We studied the fusion activity of influenza virus because it represents an essential step in infection and because its properties may shed light on the mechanism of biological membrane fusion in general.

HA is a trimeric transmembrane glycoprotein (three 84-kilodalton subunits), in which each subunit consists of two disulfide-linked glycopolypeptides, HA1 (58 kilodaltons) and HA2 (26 kilodaltons) (see reference 15 for a review). A mildly acidic pH causes an irreversible conformational change which triggers the fusion activity of HA and exposes previously buried hydrophobic groups in the ectodomain (2, 6, 9). We have hypothesized that the trimer binds to the target membrane via the hydrophobic moieties and in this way brings the viral and cellular membranes into close proximity (6, 32). Biochemical and immunochemical data have also suggested that the domains at the top of the spike dissociate from each other (2, 6, 32). In a recent report, Nestorowicz et al. (21) showed evidence that isolated trimeric bromelain-solubilized fragments of HA (BHA), devoid of the C-terminal membrane anchors, dissociate to monomers when exposed to acid pH. Although contradicted by another study on BHA (24), the finding raises the possibility that the fusion-active form of intact HA in the virus is a monomer and not a trimer as previously postulated (2, 32).

Since the quaternary structure is of importance for understanding how HA brings about membrane fusion, we determined whether intact HA dissociates into its subunits when exposed to conditions that activate membrane fusion. We used centrifugation techniques and sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) to determine the quaternary structure of HA and BHA before and after acid treatment. The results show that whereas BHA dissociates, HA remains trimeric. This indicates that while the ectodomains of HA separate from each other during the acid-induced conformational change, the trimer is held together by the transmembrane anchors present at the C termini of HA2.

MATERIALS AND METHODS

Preparation of virus and radioactively labeled HA and BHA. The X:31 recombinant strain of influenza virus (14) bearing the HA from the A/Aichi/68 strain was propagated in embryonated eggs and isolated as described previously (6). To obtain ^{125}I -labeled HA, the intact virus (200 μg of protein) was labeled with 1 mCi of ^{125}I (Amersham Corp.) by the method of Fracker and Speck (8). Under these conditions, HA is the only viral protein that is extensively labeled (6). After labeling, the virus was solubilized in 1.0% Triton X-100 (TX100) for 30 min at 37°C, and the ^{125}I -HA was purified by velocity gradient centrifugation on a 5 to 25% (wt/vol) sucrose density gradient containing 1.0% TX100. An SW40 rotor (Beckman Instruments, Inc.) was used at 40,000 rpm for 16 h at 4°C. The ^{125}I -HA in the pooled peak fractions was greater than 95% pure as judged after SDS-PAGE and autoradiography.

^{35}S -methionine-labeled HA was prepared by infecting confluent CV-1 cells (a monkey kidney cell line) with 5 μg of purified virus. At 6 h after infection, the cells were placed in a methionine-free medium containing 1 mCi of ^{35}S -methionine (Amersham). Infection was allowed to proceed for another 18 h, after which the cells were solubilized with 1.0% TX100 in a buffer containing 30 mM MES (morpholineethanesulfonic acid), 20 mM Tris, and 100 mM NaCl (MNT buffer) with 2 mM phenylmethylsulfonyl chloride (Sigma Chemical Co.). The lysate was centrifuged for 5

* Corresponding author.

FIG. 1. Sedimentation of HA at pH 7.0 and 4.8 (A) $^{125}\text{I-HA}$ (20 μg of protein per ml) was placed on top of a 5 to 25% sucrose density gradient in MNT-0.1% TX100 buffer, pH 7.0. After centrifugation, fractions were collected from the bottom, and the amount of ^{125}I activity was determined in each. Additional samples from the indicated fractions were prepared for SDS-PAGE (inset). (B) $^{125}\text{I-HA}$ at a concentration of 20 $\mu\text{g}/\text{ml}$ was incubated at pH 4.8 for 15 min at 37°C in MNT-0.1% TX100 buffer. The solution was neutralized, and the protein was placed on top of a sucrose density gradient and centrifuged as above. Samples of the protein taken before centrifugation and subjected to proteinase K digestion confirmed that all of the protein had converted to the acid conformation. (C) $^{125}\text{I-HA}$ was incubated at pH 4.8 for 15 min in the absence of cold carrier (the final concentration of HA was 1.0 $\mu\text{g}/\text{ml}$) and was centrifuged and analyzed for ^{125}I activity as above. Samples from the trimer, dimer, and monomer peaks were subjected to SDS-PAGE (inset).

min at $2,000 \times g$ to remove nuclei, and the supernatant was passed over a ricin affinity column (6). After the column was washed with MNT buffer, the bound HA precursor (HA0) was eluted with 0.2 M galactose in MNT buffer with 0.1% TX100. The fractions containing the HA0 were pooled and concentrated in a filtration cell (Amicon Corp.) with an XM-50 filter. Additional purification was achieved by centrifugation on a 5 to 25% sucrose gradient in MNT-0.1% TX100 buffer for 16 h in an SW40 rotor at 40,000 rpm and 4°C. HA was generated from HA0 by digestion with 5 μg of L-1-tosylamide-2-phenylmethyl chloromethyl ketone-trypsin (Sigma) per ml for 10 min at 37°C. Digestion was stopped by the addition of a 10-fold weight excess of soybean trypsin inhibitor (Sigma).

BHA was prepared as previously described (6) by a 16-h bromelain digestion. A sample was taken 4 h after digestion to obtain virus particles which possessed an intermediate spike density. These particles were examined by negative-stain electron microscopy (see below).

Velocity gradient sedimentation. The BHA or HA in a volume of 300 μl of MNT-0.1% TX100 buffer was placed on

top of a continuous 5 to 25% sucrose gradient in MNT-0.1% TX100 buffer, unless otherwise noted. The samples were centrifuged in an SW40 rotor for 16 h at 40,000 rpm at 20°C. The gradients were fractionated from the bottom, and the amount of ^{125}I or ^{35}S activity in each fraction was determined. Sucrose concentrations were determined by using a refractometer. Proteins with known $s_{20,w}$ values were centrifuged in parallel gradients, and their positions were determined by protein assay. The standards used were urease, porcine thyroglobulin, bovine serum albumin, catalase, and glutamic dehydrogenase, all from Sigma. Human immunoglobulin G was from Dako Corp. The $s_{20,w}$ values of BHA and HA were determined for the peak fractions by the methods of both McEwen (20) and Martin and Ames (19).

Proteinase K digestion. Digestion with proteinase K provides an easy method to differentiate between the neutral and low-pH forms of both BHA and HA. As previously described in detail, the acid forms of BHA and HA are digested to trichloroacetic acid-soluble fragments, whereas the neutral forms are resistant (6). Portions of BHA and HA were digested with 0.2 mg of proteinase K per ml in phosphate-buffered saline for 30 min at 37°C, after which the samples were precipitated with 10% (final concentration [wt/vol]) trichloroacetic acid. The trichloroacetic acid-soluble and -precipitable radioactivity was then determined.

Electron microscopy. Negative staining was carried out as previously described (6). Samples were applied to carbon-coated mica, stained with 1% sodium silicotungstate (pH 7.0), and examined with a JEOL 100CX II at an accelerating voltage of 60 kV. Virions with reduced spike density were prepared as described above.

RESULTS

Sedimentation of HA before and after acid treatment. Centrifugation in sucrose velocity gradients containing 0.1% TX100 was used to determine whether intact $^{125}\text{I-HA}$ dissociates as a result of the acid-induced conformational change. Without prior acidification, we found that the HA trimers sedimented as homogeneous particles, with $s_{20,w} = 9.0$ (Fig. 1A) as determined by the methods of Martin and Ames (19) and McEwen (20). To convert the protein to the acid form, HA was incubated at pH 4.8 in the presence of 0.1% TX100 for 15 min and then reneutralized by the addition of base. Samples were subjected to proteinase K digestion to confirm that all HA had undergone the acid-induced conformational change (6). When subjected to centrifugation, the acidified HA sedimented as a broad peak. It was recovered in the bottom third of the gradient, and the estimated sedimentation coefficients ranged from 9.0 to $>14 s_{20,w}$ (Fig. 1B). This suggested partial aggregation of HA in spite of the detergent present. There was no evidence for dissociation of the trimeric HA molecules; the amount of radioactivity in the top half of the gradient, where HA monomers would have sedimented, was low.

We found the same sedimentation patterns for HA when intact viruses were acid treated before TX100 solubilization. The distribution of HA in the velocity gradients was, in this case, determined by SDS-PAGE and Coomassie blue staining of individual gradient fractions (not shown). This indicated that the lack of dissociation upon acidification was not merely a property of solubilized HA-TX100 complexes, but also of HA in the viral membrane. The sedimentation pattern of acidified HA (Fig. 1B) was not affected by the inclusion of 0.5 M NaCl in the gradients or by changing the pH of acidification to 5.2. In addition, no differences were ob-

served if gradients were made with pH 4.8 buffer and the proteins were kept at pH 4.8 throughout centrifugation.

Evidence for the dissociation of TX100-solubilized HA could, however, be obtained in very dilute solutions of ^{125}I -HA. When trace-labeled ^{125}I -HA was acid treated and used without added cold carrier, it sedimented as three peaks with $s_{20,w}$ values of 11S, 7.7S, and 4.3S (Fig. 1C). That the three peaks corresponded to trimers, dimers, and monomers, respectively, was shown by their SDS sensitivity (see below).

SDS-PAGE analysis of HA dissociation. In agreement with the observation of Laver and Valentine (16) that morphologically intact, trimeric HA can be extracted from viruses by SDS, we found that only a fraction of the trimeric HA was dissociated to monomers when exposed to this denaturing detergent at room temperature. Instead of the normal single band at the position of the HA monomer that is routinely observed when boiled samples are subjected to SDS-PAGE, three bands were seen (Fig. 2, lane 1). These corresponded to the apparent molecular weights of the trimer ($M_r \sim 210,000$), dimer ($M_r \sim 140,000$), and monomer ($M_r \sim 70,000$) as determined by molecular weight markers. If reducing agents were included, the HA trimers, dimers, and monomers dissociated readily, giving two bands corresponding to the molecular weights of monomeric HA1 and HA2 (Fig. 2, lane 5). The uncleaved trimeric precursor of HA, called HA0, also gave the trimer, dimer, and monomer bands, but, unlike HA, this pattern was observed regardless of whether the samples had been reduced (Fig. 2, lane 4). The trimeric, neutral forms of HA and its precursor HA0 were thus found to be remarkably resistant to dissociation by SDS. This property was next used to monitor the quaternary structure of acidified HA.

When intact, trimeric HA was acidified and then exposed to SDS in the absence of reducing agents, the same three bands in the same proportions were observed as with HA exposed only to neutral pH (Fig. 2, lane 2). This suggested that the HA remained trimeric even after acidification. The apparent lack of sensitivity to SDS after acidification was not unique to TX100-solubilized HA. When intact ^{125}I -labeled viruses were acidified, solubilized with SDS under nonreducing conditions at room temperature, and subjected to SDS-PAGE, the three characteristic HA bands were seen again (Fig. 2, lane 3). The relative resistance of acid HA to SDS thus provided independent evidence that HA (both in the intact virus and in isolated, detergent-solubilized form) did not dissociate during the acid-induced conformational change.

The reliability of SDS in distinguishing between oligomeric forms of HA was confirmed by using the fractions of sucrose gradients containing dilute samples of either pH 7- or pH 4.8-treated ^{125}I -HA. Whereas the 9S peak obtained with neutral HA (Fig. 1A) gave the characteristic three-band pattern on the SDS gels (see inset), the three peaks obtained with acidified HA (Fig. 1C) each had a different pattern (see inset). The broad 11S peak gave the normal three bands, the 7.7S peak gave the two lower bands, and the 4.3S peak gave only the lowest band (which corresponds to monomeric HA). We concluded that the SDS-PAGE pattern was, indeed, determined by the oligomeric state of the protein and that the three peaks in the gradient (Fig. 1C) corresponded to the trimer, dimer, and monomer.

These results, taken together with the sucrose density-gradient data, indicated that HA remains trimeric after acidification. The trimers may aggregate to form larger complexes, but dissociation into dimers and monomers can

FIG. 2. SDS-PAGE analysis of HA and HA0. ^{125}I -HA isolated from virus was incubated at pH 7.0 or 4.8 for 15 min at room temperature. Protein was precipitated with 10% (final concentration [wt/vol]) trichloroacetic acid and prepared for SDS-PAGE at 37°C. Alternatively, virus was incubated at pH 4.8 for 15 min, after which it was solubilized with SDS and prepared for SDS-PAGE (lane 3). Lanes: 1, HA (pH 7); 2, HA (pH 4.8); 3, HA acidified while in the viral membrane; 4, HA0 (pH 7) with dithiothreitol; 5, reduced HA (pH 7); 6, reduced HA (pH 4.8).

occur only in extremely dilute solutions. Since dissociation was not observed when intact viruses were acidified, we can conclude that it is not likely to play an important role in HA-catalyzed membrane fusion.

Electron microscopy of acid-treated virus. To determine if structures larger than trimers might be involved in virus-membrane fusion, we examined acid-treated viruses by negative-stain electron microscopy. Figure 3A shows influenza virus at neutral pH. The HA spikes are well-ordered, rectangular structures projecting approximately 13 nm from the virus membrane (see also Fig. 3C and D). Their shape and dimensions correspond quite closely to the known X-ray structure of BHA trimers (33). When viewed head on, the spikes appear to have a diameter of approximately 5 nm. After incubation at pH 5.0, the well-ordered appearance was lost, and individual spikes were difficult to discern (Fig. 3B) (5, 24, 30). In general, the length of the spikes was increased by approximately 10% after acid treatment, as observed by Ruigrok et al. (24).

To remove a fraction of the HA molecules and thus allow visualization of individual spikes, virus particles were digested with bromelain. After digestion, approximately 5% of the virus population contained spikes at an intermediate density, while the other particles had lost either all or none of their surface projections (Fig. 3C and D). The partially digested viruses were gradient purified and treated at pH 5.0 for 15 s before negative staining. The short acidification time was used to reduce aggregation between virus particles and to ensure 85% conversion of HA to the acid form (6). In addition, influenza virus fuses with a $t_{1/2}$ of approximately 15 to 30 s (22, 28, 30). Figure 3E shows a large virus particle,

FIG. 3. Electron microscopy of influenza virus at acid and neutral pHs. Virus was incubated at pH 7.0 (A) or pH 5.0 (B) for 15 min at 37°C. Samples were placed on carbon-mica films and stained with sodium silicotungstate, pH 7.0. The virus was digested with bromelain for 4 h to obtain virions with reduced spike density. The virus was gradient purified after digestion and incubated at pH 7.0 (C and D) or pH 5.0 (E, F, and G) for 15 s at 37°C. $\times 100,000$.

perhaps formed by the fusion of two or more virions. Structures composed of a central nexus with six or seven radiating "arms" were seen. The arms were approximately 9 ± 1.0 nm in length and were approximately one-third the thickness of HA trimers (1.5 nm) in the neutral conformation. It is clear that these rosettelike aggregates must constitute a complex of several HA proteins. If each arm corresponds to one HA1 subunit, then each rosette would consist of two to three trimers. In addition, HA spikes which remained in the pH 7 conformation are clearly visible on the surface. Figures 3F and G show additional acid-treated virus particles with similar spike aggregates on their surface. The images suggest that acidified HA molecules can associate with each other laterally in the plane of the membrane and raise the possibility that an HA complex rather than individual spikes may be involved in fusion.

Sedimentation analysis of acid BHA. The soluble trimeric ectodomain of HA released from the virus by bromelain digestion (BHA) lacks the membrane-spanning domain, a 6-kilodalton C-terminal fragment of HA2 (1). The X-ray structure has been determined by Wilson et al. (33). BHA has been extensively used by us and others to characterize the changes involved in the acid triggering of the fusion

activity of HA (5, 6, 9, 12, 21, 27; F. Boulay, R. W. Doms, and A. Helenius, UCLA Symp. Mol. Cell. Biol. in press).

Two studies addressed the subunit structure of BHA after acidification and arrived at opposite conclusions. Nestorowicz et al. (21) reported that the BHA dissociates to monomers by velocity and sedimentation equilibrium centrifugation, whereas Ruigrok et al. (24) found that it remains trimeric as determined by analytical centrifugation and negative-stain electron microscopy. In an attempt to resolve this contradiction, we subjected BHA to acid pH and gradient centrifugation under the same conditions used above for intact HA. Whereas the neutral BHA sedimented as a homogeneous particle, with $s_{20,w} = 8.9$ (Fig. 4A) as previously shown by Skehel et al. (27), the acidified BHA was recovered in either two or three peaks depending on the BHA concentration and the pH used for acidification (Fig. 4B and Table 1). Three peaks ($s_{20,w}$ values of 11S, 7.7S, and 4.3S, respectively) were observed when relatively large amounts (20 $\mu\text{g/ml}$) of BHA were acidified to pH 4.8 and applied to the gradients. All three peaks consisted of intact BHA polypeptide chains as judged by SDS-PAGE. When trace amounts of ^{125}I -BHA were used, only the 7.7S and 4.3S particles were present (Fig. 4C). It is clear that the degree of

dissociation is highly concentration dependent, which may explain why Ruigrok et al. (24), who used very high concentrations of BHA, may not have observed dissociation.

We found that all forms of BHA, irrespective of whether they were acid treated, were dissociated to monomers by SDS. This precluded the use of relative SDS sensitivity as a criterion for quaternary structure analysis of the various forms of BHA. The results of Nestorowicz et al. (21), Ruigrok et al. (24), and our unpublished hydrodynamic data indicate, however, that the 11S complexes are trimers, the 7.7S complexes are dimers, and the 4.5S complexes are monomers. The similarity in sedimentation between acid BHA and dilute samples of acid HA supports this interpretation. Moreover, the binding of TX100 to the acidified BHA only amounts to about 10 molecules per HA (Doms and Helenius, unpublished data), which is not enough to greatly influence the partial specific volume and the sedimentation behavior of the solubilized molecules.

DISCUSSION

A variety of biochemical, immunological, and genetic results have shown that major, irreversible modifications occur in the interface region between the top domains of the HA trimer due to the low-pH-induced alteration associated with membrane fusion (3, 6, 9, 12, 29). Interchain salt bridges, which stabilize trimeric HA at neutral pH, are broken. We and others have concluded that the trimeric ectodomains separate and that the hydrophobic amino termini of the HA2 subunits become exposed (2, 32). In the present paper, we addressed the question of whether HA

FIG. 4. Sedimentation of BHA at pH 7.0 and 4.8. ^{125}I -BHA was centrifuged at pH 7.0 (A) or after acid treatment (B and C). The concentration of BHA in both panels A and B was 20 μg of protein per ml, while in panel C the concentration was 0.1 $\mu\text{g}/\text{ml}$. Acid treatment was for 15 min at pH 4.8. The protein was then reneutralized and centrifuged as for HA (Fig. 1).

TABLE 1. Effects of pH and concentration on BHA dissociation^a

Concn ($\mu\text{g}/\text{ml}$)	pH	Dissociation (%)				Dimer/ monomer ratio
		Monomer	Dimer	Trimer	>Trimer	
0.3	4.8	21.5	46.6	20.7	4.4	2.3
0.3	5.0	8.7	70.5	9.5	1.1	8.1
0.3	5.2	7.4	81.3	1.0	0.1	10.9
3.0	4.8	11.1	16.0	23.4	33.0	1.4
3.0	5.0	9.3	11.8	23.5	35.2	1.3
3.0	5.2	9.1	13.6	29.4	34.8	1.5

^a ^{125}I -BHA incubated at the indicated concentration and pH was subjected to sucrose density gradient centrifugation. A 0.5-ml 67% sucrose cushion was included at the bottom of the gradient. After fractionation, the 11S, 7.7S, and 4.3S peaks were identified. The trimeric BHA was defined as BHA in the 11S peak ± 2 fractions. The sum of total ^{125}I activity in these fractions was expressed as the fraction of total ^{125}I activity recovered from the gradient. Similar calculations were performed for both the 7.7S (dimeric) and 4.3S (monomeric) materials. The ^{125}I activity in the bottom three fractions of each gradient was defined as BHA having a sedimentation coefficient of $\geq 14\text{S}$.

dissociates entirely at acid pH or whether dissociation is limited to the ectodomains.

Our results showed that intact HA, both in the virus and in the isolated TX100-solubilized form, remained trimeric after acidification. Significant dissociation was observed only when very low concentrations of isolated HA were incubated with acid. This suggested that the trimers are most likely to be the active form present during membrane fusion. The morphological demonstration of rosettelike aggregates in the viral membrane after acidification, as well as the tendency of acidified HA trimers to aggregate in detergent solution (Fig. 1B), raises the possibility that the fusion active form of HA may be a complex of several HA trimers. The structures seen by electron microscopy (Fig. 3) were probably composed of two to three HA trimers each. Their dimensions and shape suggested that the center was composed of HA2, with the individual HA1 subunits radiating outwards. Previous observations have implied that fusion activity may be a cooperative phenomenon requiring more than a single HA trimer. We have found that fusion occurs only when 80% or more of viral HA is converted to the acid conformation (6), and a relatively high concentration of HA molecules in the membrane is needed (7). A likely interpretation of these data is that the density of acid HA molecules in the membrane is critical for the fusion activity and that several HA trimers may have to come together to form some type of fusion complex. Whether the structures visualized here by electron microscopy represent such complexes, and whether such complexes are involved in fusion remain to be seen.

In contrast to intact HA, BHA readily dissociated after acidification. The ratio of monomers, dimers, and trimers depended on protein concentration and pH. The results were essentially in agreement with those of Nestorowicz et al. (21), but the lack of dissociation reported by Ruigrok et al. (24) may be explained by the high BHA concentrations used. Whether the increased proportion of trimeric, acid BHA seen at high BHA concentrations (Fig. 4) reflects decreased dissociation or aggregation of dissociated subunits remains to be determined. The relative ease by which acid BHA dissociates, in contrast to acid HA, indicates that the transmembrane C-terminal peptides of intact HA are important in keeping the trimeric HA together. The dissociation of BHA is also consistent with the models referred to above, in which the ectodomain of HA is opened up during acid treatment. Not only do the HA1 subunits come apart,

exposing the central trimeric stem domain of the HA spikes, but the dissociation of BHA suggests that hydrophobic and polar interactions between HA2 subunits in the stem may also be radically weakened. Dissociation at acid pH represents the most important difference observed so far between BHA and HA, but it is not the only one. We found that BHA underwent certain aspects of the acid-induced conformational change at a higher pH (up to 0.5 U) than did the corresponding HA, as judged by the binding of several conformation-specific monoclonal antibodies. Moreover, we found, by using a hydrophobic photoactivatable probe, that both neutral and acid BHA possessed hydrophobic pockets that are not labeled in intact HA (Boulay et al., in press). These differences demonstrate that while similar to HA in many ways, the properties of BHA are sufficiently different to warrant caution in using it as a model for the acid-induced conformational changes in HA.

Neutral HA and HA0 trimers as well as acidified HA were only partially dissociated to dimers and monomers by the potent denaturing detergent SDS. This provided a convenient method for determining the subunit composition of various forms of the proteins. BHA, in contrast, was readily dissociated under the same conditions, suggesting again that the transmembrane portion of the intact molecules was, at least in part, responsible for the resistance. The lack of dissociation in SDS is not without precedent among hydrophobically anchored transmembrane proteins. An analogous effect has been described for the coat protein of bacteriophage f1 (18) and glycophorin, a major erythrocyte membrane protein (26). Glycophorin gives both dimer and monomer bands in SDS-PAGE, with the ratio between them being dependent on the protein concentration and ionic conditions (26). The region of glycophorin responsible for the SDS-resistance is the transmembrane domain which consists of a single membrane-spanning alpha helix (25).

The mechanism by which the membrane anchors confer SDS resistance to these proteins is not clear, though there are at least two possibilities. First, the three transmembrane segments of the HA spike may intercalate with each other to form a compact, stable oligomeric structure. Evidence for close interactions between membrane-spanning alpha helices comes from the recently solved structure of the *Rhodospseudomonas viridis* photosynthetic reaction center (4). The central part of this complex consists of two subunits which interact in large part by intercalating transmembrane alpha helices. While the structure of the membrane anchor of HA is not known, it is likely that the three membrane-spanning regions form alpha helices. That these are in close proximity to each other can be inferred from the crystalline structure of BHA (33). Second, it is possible that SDS is relatively inefficient in disrupting and denaturing structures rich in alpha helical configuration. SDS has been shown to induce alpha helix formation in a variety of proteins, whether or not the native protein originally contained alpha helices (13). Hydrophobic helices may actually remain unperturbed in SDS and serve as nuclei for detergent binding. The interacting transmembrane domains of HA spikes may thus constitute inherently SDS-stable structures.

In conclusion, we showed that the fusion active form of the influenza HA is a trimer or higher-order structure. While some acid-induced dissociation occurred, it did so only at low protein concentrations which do not reflect the situation in the intact virus. Furthermore, our findings are consistent with the hypothesis that acid pH disrupts intersubunit contacts, allowing the trimeric ectodomains to separate. However, the overall trimeric structure of HA was maintained by

the transmembrane or cytoplasmic domains at the base of the trimer. A consequence of the conformational change was that hydrophobic moieties, including the amino termini of HA2, were exposed. This confers an amphiphilic character to the ectodomains and results in the binding of a small but measurable amount of nonionic detergent (Doms and Helenius, unpublished data). Studies with fusion variants and transmembrane mutants will more fully characterize the molecular details of HA-catalyzed membrane fusion as well as examine the role of the transmembrane anchor in both trimer assembly and stabilization.

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