Membrane Fusion Activity of the Influenza Virus Hemagglutinin

THE LOW pH-INDUCED CONFORMATIONAL CHANGE*  

(Received for publication, August 22, 1984)

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Influenza A viruses penetrate their host cells by membrane fusion. After binding to the cell surface, virus particles are internalized and transported to endosomes and lysosomes. The acidic environment in these organelles activates fusion between the viral and host cell membranes (1–3). The viral factor responsible for this fusion activity is the hemagglutinin (HA') (4–6), a spike glycoprotein that also binds the virus to cell receptors is located in the globular head region of the HA1 (X), shows a roughly rectangular 13.5-nm long trimer with globular head domains composed entirely of HA1 resting on top of a fibrous stem consisting chiefly of HA2. The cleavage point between HA1 and HA2 is in the stem region close to the viral membrane (15).

The site in the HA which mediates virus binding to host cells is localized to the globular head region of the HA1 (16). The hydrophobic amino-terminal peptide of HA2, in the stem region of the molecule, is thought to be involved in HA's membrane fusion activity (17). However, the precise role of this peptide has not been defined and the fusion mechanism is far from understood. A recent study by Skehel et al. (18) demonstrated that a major conformational change takes place in BHA at acid pH. The BHA becomes amphiphilic, two previously hidden tryptic cleavage sites are exposed, and changes are observed in its spectral (18) and antigenic (19–21) properties. On the basis of these results it has been suggested that acid treatment exposes the hydrophobic N terminus of HA2, thereby imparting amphiphilic properties to the HA ectodomain (18).

Here, using biochemical, immunological, and morphological methods we have further analyzed the changes in BHA. We have determined the kinetics of the conformational change, studied the interaction of acid-treated BHA with liposomes of various compositions, and confirmed the role of HA2 as the principal amphiphilic subunit. Furthermore, we have demonstrated that the changes in BHA reflect the behavior of HA as it resides in the viral membrane. The results are discussed in terms of a model for HA's fusion activity.
Materials and Methods

Results

Acid Conversion of BHA and HA—Our experiments were aimed at two aspects of HA’s response to acid pH, i.e. the conformational change in the protein and the nature of its subsequent interaction with added membranes and other amphiphiles. The studies on the conformational change were performed using both radioactively labeled and unlabeled BHA and intact X:31 virus in the absence of added membranes or detergents. To obtain a quantitative assay for the conformational change, the susceptibility of 125I-BHA to proteases was tested after incubation at different pH values. It was found that acid-treated BHA was susceptible to digestion with proteinase K, a nonspecific bacterial protease (Fig. 1). Both of the glycopolypeptide chains, HA1 and HA2, were digested (Fig. 1, inset), and greater than 85% of the radiolabel in 125I-BHA became trichloroacetic acid-soluble. Trichloroacetic acid precipitation and direct quantitation of the HA band after SDS-PAGE showed that the conversion into the protein kinase sensitive form began at pH 5.8, reached half-maximal values at pH 5.3, and was essentially completed at pH 4.8.

When 125I-labeled BHA was treated at various pH and digested with proteinase K, the pH dependence of digestion was nearly indistinguishable from that observed for unlabeled BHA. Furthermore, little or no differences were observed between radioiodinated BHA, unlabeled BHA, and unlabeled viruses. In each case the conversion appeared to be irreversible judging by the fact that incubation at neutral pH for up to an hour after the initial low pH treatment did not affect the fraction of protease-sensitive BHA.

The kinetics of conversion was next determined at two fixed pH values, 5.0 and 5.3. A solution containing BHA was acidified at room temperature and at various times aliquots were removed, neutralized, digested with proteinase K, and precipitated with trichloroacetic acid. At pH 5.0 75–80% of the BHA converted within the first minute, with another 5–10% of the label becoming protease-sensitive over the next hour (Fig. 3). The rate of conversion of the isolated protein was thus quite similar to that previously observed for the kinetics of fusion between influenza viruses and liposomes (35).

Antigenic Change in BHA and HA—The differential sensitivity to proteinase K provided an easy method for discriminating between the neutral and the acid conformations of HA and BHA. Another method used to monitor the conformational change employed monoclonal antibodies. Mice were immunized with acid-treated and neutral BHA, and hybridomas were selected for the production of antibodies capable of immunoprecipitating either the acid or the neutral BHA (or both) under non-denaturing conditions. Whereas most of the monoclonals obtained were able to precipitate both forms of BHA (Table I), one monoclonal, designated H3C10, precipitated only the neutral form. Another, H2D10, specifically precipitated the acid form. To determine whether the latter recognized a determinant on HA1 or HA2, we took advantage of a finding by Graves et al. (33) that reported that reduction of acid-treated influenza A virus with dithiothreitol dissociates HA1 from HA2. We found that the BHA could also be dissociated in this way, and that immunoprecipitation with H2D10 only brought down the HA1 chain, as determined by SDS-PAGE (Fig. 7, lane 5, Miniprint). Therefore, the epitope recognized by this antibody resided somewhere in the HA1 chain, but was only accessible in the acid-treated form of BHA.

The antibodies were next tested for their ability to interact with HA molecules of intact X:31 virus before and after the pH 5.0 treatment. The results in Table I indicate that the specificities were the same as observed for BHA. Thus, the antigenic changes occurring in BHA were comparable to those of HA in the virus itself. These results confirmed and extended the findings of Webster et al. (19) and Daniels et al. (20) who have shown that out of four well characterized epitopes on HA1, two are modified or lost after acid conversion, and that at least one new epitope is exposed. Our results indicated that a new epitope was exposed in HA1, and that BHA and HA displayed the same antigenic changes. The
antigenic change, and the identical proteinase K sensitivity of BHA and HA indicated that BHA responded to acid in a manner very similar to intact viral HA.

**pH Dependence of X:31 Membrane Fusion**—Having shown that BHA and the HA in intact virus undergo a similar pH-induced change, we determined the pH dependence of X:31 virus-induced membrane fusion. The fusion activity of the virus was measured by quantitating the extent of cell-cell fusion induced by added virus particles. After binding viruses at 0 °C, cell monolayers were warmed to 37 °C and acidified for 1 min. The average number of nuclei/cell was determined by microscopy (31). Fusion became detectable at pH 5.2 and was complete by pH 5.0 (Fig. 2). Half-maximal activity occurred between pH 5.0 and 5.1. The pH dependence of fusion was thus much steeper and shifted 0.25 pH units lower than the pH of conversion of the total BHA population to its proteinase K-sensitive form (Fig. 1). Half-maximal fusion was thus recorded at a pH where 80% of the viral HA was converted into the low pH conformation suggesting that the fusion activity may be a cooperative effect involving more than single HA molecules.

**Association of BHA with Liposomes**—One of the most dramatic consequences of the acid-induced conformational change in BHA is its change from a water-soluble protein to an amphiphilic molecule capable of attaching to liposomes, nonionic detergent micelles, or to itself in the form of oligomeric “protein-micelles” (18). Since this change in BHA’s solubility is likely to reflect the role of HA in fusion, we characterized the interaction of BHA with liposomes in some detail. The pH dependence, kinetics, morphology, and chemical nature of the interaction were determined as well as its dependence on lipid composition, divalent cations, and temperature.

The attachment of BHA to liposomes was assayed as follows. Liposomes containing PC, PE, SPH, CHOL, and PA (1:1:1:1:5:0.3) with a trace amount of 32P-labeled phospholipid were mixed with 125I-labeled BHA and the pH was adjusted by addition of acid. After 15 min at 37 °C, the mixture was neutralized, made 50% (v/v) with sucrose, placed in the bottom of a centrifuge tube, overlayed with a 0.5-ml sucrose step gradient, and subjected to ultracentrifugation. Owing to the high lipid-to-protein ratio used (>10,000 phospholipids/BHA), the resulting BHA containing liposomes were of low density and floated to the top of the gradient together with BHA-free liposomes. Unbound BHA remained in the bottom fractions (Fig. 8, Miniprint).

The attachment of 125I-BHA to liposomes was highly efficient and the pH dependence was almost identical to that observed for BHA’s acid conversion (Fig. 2). Binding was apparently irreversible because return to neutrality overnight at room temperature did not reduce the amount of BHA attached to the liposomes. The kinetics of binding at various pH values (pH 5.0, 5.3, and 5.6) shown in Fig. 3 was also similar to that already observed for influenza fusion (35) and for BHA’s conversion to the proteinase K-sensitive form (indicated for pH 5.0 in Fig. 3 by open circles). When BHA/liposome mixtures treated at an intermediate pH (pH 5.3) were fractionated by flotation on sucrose gradients and subjected to proteinase K digestion, >90% of the BHA that had associated with the liposomes was digestable, compared to less than 10% of the non-lipid associated BHA (data not shown). Taken together, these results indicate that when converted to the low pH form, BHA rapidly and quantitatively associates with liposomes. For maximal binding, liposomes and BHA had to be present together at the time of acidification. If, in the standard assay (which contained only a trace amount of 125I-BHA) the liposomes were added 1 min after acidification to pH 5.0, 50% of the 125I-BHA bound; if they were added after 5 min, only 25% bound. Thus, the acid form of BHA retained its ability to bind to liposomes for a relatively short time. The rate of inactivation increased when increasing concentrations of unlabeled BHA were present. For example,
addition of 5 \( \mu \text{g} \) of unlabeled BHA reduced the amount of bound BHA from 50 to 28\% when liposomes were added 1 min after acidification. The inactivation was probably a consequence of the irreversible aggregation occurring between BHA molecules in the low pH form as reported by Skehel et al. (18). We confirmed that protein aggregation was occurring as judged by a higher sedimentation rate in sucrose velocity gradients and by negative staining, which revealed rosettelike protein complexes similar to those described by Skehel et al. (18).

**Effect of Lipid Composition, Temperature, and Membrane Fluidity**—The liposomes used up to this point consisted of a mixture of natural phospholipids and cholesterol (PE:PC:SPH:PA) in a molar ratio of 1:1:1:0.3. Liposomes with this composition were chosen because they are good target membranes for fusion with influenza virus (35). To determine if any of the lipids were crucial for BHA attachment, liposomes with different compositions were tested. As seen in Table II, all liposomes tested, irrespective of composition, proved equally efficient as targets. Complete pH dependence curves were determined for liposomes lacking PA and for liposomes consisting of egg PC alone; they were identical to that observed for the standard liposomes (Fig. 2). Interestingly, the inclusion of gangliosides (17\%) which can serve as receptors for HA did not influence the extent of binding at acid pH, although a slight increase in binding was observed at neutral pH. These findings are consistent with the observed fusion activity of an influenza A virus (fowl plague virus) which is also largely independent of the phospholipid head groups, the cholesterol content, and the presence of gangliosides (35). One interesting difference between viral-membrane fusion and the binding of BHA to liposomes was observed. Whereas fusion with the influenza virus is inhibited by 50\% when PE is omitted (35), attachment of BHA to liposomes was unaffected by the PE content.

To determine whether temperature was an important factor in the attachment of BHA to liposomes, BHA and liposomes of the standard composition were incubated for 5 min at pH 5.0 at a variety of temperatures. Under these conditions, BHA bound with equal efficiency over the entire 0–37 °C range. However, after only 2 min at pH 5.0, the binding was less efficient at temperatures lower than 37 °C, suggesting that binding occurred more slowly at lower temperatures. Since virtually all (90\%) of the BHA is converted to its acid form after 2 min at pH 5.0 (see Fig. 3), this difference most likely reflects a slower rate of attachment of acid BHA to liposomes at low temperatures, as opposed to a slower rate of conversion. This observation is reminiscent of the slower rate of fusion observed between fowl plague virus and liposomes at reduced temperatures (35).

The effects of membrane fluidity on BHA binding were determined after incubation at pH 5.0 for 30 min with egg PC or dimyristoylphosphatidylcholine (DMPC) liposomes. While egg PC, like the standard mixture, remains fluid throughout the 0–40 °C temperature range, DMPC bilayers are only fluid above the major transition temperature of 33 °C. Below 23 °C, the lipid molecules of the bilayer are in a crystalline array, with little lateral or rotational mobility (36). The results in Fig. 4 show that BHA binding to egg PC liposomes remained virtually unchanged over the entire temperature range whereas attachment to DMPC liposomes was gradually depressed at temperatures below 37 °C. Below the transition temperature, no further decrease in binding occurred. Thus, although low pH BHA binds to a greater extent to “fluid” bilayers, it can also bind to a significant degree (about 40\%) to membranes where the lipids exist in a crystalline array.

**Lipid:Protein Ratio**—The effect of the lipid:protein ratio on BHA attachment to liposomes was studied by floation of BHA-liposome complexes in continuous sucrose gradients. The effects of membrane fluidity on BHA binding were determined at low temperatures, as opposed to a slower rate of conversion.

![Temperature dependence of 125I-BHA binding to DMPC and egg PC liposomes](image)

**Figure 4.** Temperature dependence of 125I-BHA binding to DMPC and egg PC liposomes. 125I-BHA was incubated with egg PC or DMPC liposomes at 37 °C. Aliquots were placed in a water bath and the temperature was slowly lowered to the desired value. After 5 min at the indicated temperatures, samples were acidified for 30 min, neutralized, and the extent of binding was determined by flotation as in Fig. 8.
the resulting proteo-liposomes was approximately 700 phospholipids to every BHA trimer. This corresponds to approximate protein-to-lipid ratios) at pH using high concentrations of urea (6 50%, made 60% with sucrose, and placed at the bottom of 5-30% sucrose gradients. After centrifugation, fractions were taken from the bottom and assayed for the presence of 125I-BHA (O_--O) and 32P-phospholipid (O_--O). The buoyant density was determined by refractometry (A_--A).

Nature of BHA's Interaction with Liposomes—The low pH-induced attachment of BHA to liposomes was not affected to any large extent by high or low ionic strength (1 or 0.001 M NaCl) or by metal chelators such as EDTA and EGTA (1 or 10 mM). Attempts to elute bound BHA from the liposomes using high concentrations of urea (6 M) or potassium iodide (1 M, a chaotropic agent) also failed, confirming that the BHA was quite firmly attached. The only condition which eluted the BHA (besides treatment with detergents) was elevation of the pH to 10.0-12.5 (which is below the pH required to hydrolyze peptide bonds or lipids (40)). Under these conditions, up to 75% of the bound BHA came off. When the pH was subsequently returned to neutrality, the eluted BHA quantitatively reattached to the lipids, and was susceptible to proteinase K digestion. The elution of BHA from liposomes by base proved to be independent of ionic strength.

Thus, in many respects the binding properties of BHA resembled those of integral membrane proteins hydrophobically anchored to lipid bilayers (41). The reversible elution by base suggested, however, that the BHA can undergo a second pH-dependent conformational change at high pH which allows it to detach. Such a phenomenon is not usually observed for integral membrane proteins (41), and it may suggest that the BHA is interacting with the bilayers more superficially.

Binding Occurs through the HA2 Chain—The observation of Graves et al. (33) that reduction of acid-treated influenza virus allows the dissociation of the HA1 and HA2 chains from each other was used to determine which of the subunits of BHA is responsible for the attachment to liposomes. Liposomes with bound 125I-BHA were incubated with dithiothreitol and subjected to separation in a sucrose flotation gradient. The material which remained in the sample zone and the material which bound to the liposomes was analyzed by SDS-PAGE and fluorography. As shown in Fig. 7 (lane 6), HA2 remained bound while HA1 had dissociated. The result was confirmed by detergent binding analysis. Bordier (34) has shown that Triton X-114-solubilized proteins, which have a hydrophobic intramembranous moiety, preferentially partition into the detergent phase formed above the detergent's cloudpoint, whereas soluble proteins and peripheral membrane proteins partition into the aqueous phase. As shown in Table III, the neutral pH form of BHA behaved as a water-soluble protein. After acid treatment, it partitioned preferentially into the detergent phase confirming its conversion to an amphiphilic form. However, if the acid-treated BHA was reduced prior to partitioning, only a fourth of the 125I activity was found in the detergent phase. SDS-PAGE of both phases showed that 93% of the HA1 was in the aqueous phase, compared to 33% of the HA2. The HA1 retained its antigenic reactivity with a variety of monoclonal antibodies including the antibody specific to the acid HA (Fig. 7). The remaining 7% of the HA1 and 67% of the HA2 were in the detergent phase. These results strongly suggested that the attachment of BHA to liposomes and detergent is through the HA2 chain.

Lastly, to test the possibility that BHA might have phospholipase activity, BHA was incubated with egg PC vesicles at pH 5.0 for 30 min at a protein:lipid ratio of 1:750. The sample was neutralized and dried under vacuum, and the lipid

Table III

| Sample | pH | DTT | Aqueous phase | Detergent phase | Amount in detergent phase |
|--------|----|-----|---------------|----------------|--------------------------|
| 1      | 7  | -   | 106           | 18             | 5                        |
| 2      | 5  | -   | 22            | 11             | 84                       |
| 3      | 5  | +   | 86 (total)    | 30 (total)     | 26                       |
|        |    |     | 74 (HA1)      | 6 (HA1)        | 8                        |
|        |    |     | 12 (HA2)      | 24 (HA2)       | 67                       |
was separated by thin layer chromatography (42). No decrease in the amount of PC was observed, and no lysophosphatidylcholine was detected.

**Morphological Analysis**—Electron microscopy of Influenza virus after negative staining showed the HA molecules on the virus surface as densely packed rectangular projections perpendicular to the membrane (Fig. 6A). Their apparent length (13 nm) and overall shape was consistent with the x-ray crystallographic structure of BHA (15). After acid treatment and reneutralization, the spikes on the virus surface became disorganized, and the individual HA molecules became difficult to distinguish as spikes (Fig. 6C). Isolated BHA molecules had a compact rectangular shape very similar to that of the HA spikes on the virus (Fig. 6B). When mixed with liposomes at neutral pH they showed no preferential attachment, but after a brief pH 5.0 incubation they were seen attached to the liposome surface. If the lipid-to-BHA ratio is low (2000:1) the aggregated fraction of BHA was seen in the background (Fig. 6D). The proteinaceous layer on the liposomes extended 10–15 nm from the membrane surface, but individual BHA molecules were difficult to resolve because they seemed to have lost their compactness and defined shape. While thin, wiry connections were seen within the structure, the overall impression was that of a molecule which could assume many conformations.

**DISCUSSION**

Among the viral spike glycoproteins known to possess membrane fusion activity (1), the influenza HA is the best characterized. To be active in fusion the HA must be in its mature (cleaved) form (6, 9, 10), be anchored in one of the membranes to be fused (6), and be exposed to a pH value below a critical threshold which varies from 5.1 to 5.8 depending on the virus strain (43). In this study we have used quantitative assays to characterize the changes in the isolated ectodomain of the HA in response to acid treatment, specifically its conformational change and the concomitant attachment to liposomes and detergent micelles. We have validated that the behavior of BHA at low pH is a faithful reflection of the behavior of HA molecules in the viral membrane, characterized the conformational change in some detail, and probed the nature of the interaction between BHA and liposomes.

Our results indicate that the conformational change and acquired hydrophobicity of BHA reflect the response of HA in the viral membrane to acid pH. The changes in the protease susceptibility, morphology, and antigenic structure of BHA and viral HA are indistinguishable. Moreover, the acid-induced interaction of BHA with liposomes mirrors many of the functional characteristics previously observed for fusion between intact viruses and liposomes (35).

**The Nature of the Conformational Change**—The differences in structure and properties of neutral and acid-treated BHA and HA have been studied by biochemical, immunochemical, morphological, and spectroscopic methods in several laboratories including our own. The results available so far can be summarized as follows. 1) Acid BHA possesses two tryptic cleavage sites (located in HA1, positions 27 and 224) which are not accessible in neutral BHA (18). 2) Both the HA1 and HA2 subunits of BHA and HA become fully susceptible to protease K upon acidification (Fig. 1). 3) Two antigenic epitopes in HA1 (called B and D, Ref. 44) are lost or modified after acid treatment. The acid form of the protein has at least one unique epitope (19–21) which we have located at the HA1 chain (Fig. 7). 4) The interchain disulfide bond located in the stem of the molecule close to the viral membrane is accessible to reduction only after conversion to the acid form (33). We found that the reduction resulted in the dissociation of HA1 and HA2 in both BHA and intact virus (Table III) and that the HA2 was apparently responsible for the lipid-binding character of acid BHA. 5) Whereas the neutral HA and BHA have a compact rectangular morphology when viewed by electron microscopy, the acid forms have lost their compactness and defined shape (18) (Fig. 6). 6) While neutral BHA behaves like a soluble glycoprotein, the acid form is amphiphilic (18). 7) Dichroic spectra of neutral and acid BHA suggest differences in conformation (18). In addition we have recently found that polyclonal antipeptide antibodies against the C-terminal peptide of HA1 and the N-terminal peptide of HA2 bind specifically to the acid-treated form of BHA, suggesting a change in exposure and/or conformation of these moieties.

When projected against the known x-ray structure of neutral BHA (15), these observations indicate that the HA trimer is affected throughout its length, from the top of the HA1 domains to the very base where the interchain disulfide is located. At the same time, the available evidence suggests that the acid conversion does not lead to major denaturation; the sialic acid binding property of HA1 remains intact (18), most of the antigenic determinants on HA1 are retained, and most of the potential trypsin cleavage sites remain inaccessible (18). Our working hypothesis, based on these findings and the electron microscopic images, postulates that the trimer dissociates partially or completely along the trimer interface, thus exposing the surfaces between the three subunits. While retaining their overall tertiary structure, the top domains (HA1) may detach from each other and move away from the central axis of the molecule. Nevertheless they may remain flexibly attached to HA2 via the interchain disulfide bond. The stem and the hydrophobic N-terminal of the HA2 chains would thus be exposed and made accessible for attachment to a target membrane. In evaluating this and other models in terms of fusion activity one must, however, keep in mind that

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3 J. White and I. Wilson, unpublished results.
the “acid BHA” analyzed by us and others has usually been reneutralized after acid treatment. Its structure may therefore differ in important respects from that of nascent fusion active molecules present shortly after acidification.

**BHA’s Interaction with Liposomes**—The exposure of a hydrophobic moiety in HA is probably a key element in the attachment function has proved difficult to study with whole bilayer, thus explaining, in part, its ability to be eluted at elevated pH. It is likely that the hydrophobic determinant in HA2 corresponds to the N-terminal peptide because, apart from the membrane anchoring peptide, it is the only distinctly hydrophobic sequence in HA2 (23). In addition, it is highly conserved among HA molecules of different virus strains, and recent studies using site specific mutagenesis have shown that mutations in this region affect the fusion activity. The fact that BHA can be eluted off the liposomes by base suggests that it is less firmly attached than most integral membrane proteins. The relative shortness of the hydrophobic N-terminal peptide (10 residues) could mean that the protein only inserts into the outer leaflet of the bilayer, thus explaining, in part, its ability to be eluted at elevated pH.

**HA’s Role in Membrane Fusion**—BHA binds avidly to membranes but it does not, itself, promote membrane fusion (6). Although informative in illuminating how acid BHA interacts with liposomes, our results do not explain how the membrane-bound, intact HA is able to elevate the otherwise extremely low fusion incidence between membranes. One major obstacle against fusion of membranes is the “hydration force,” a strong repulsive force opposing close approach of two hydrated polar surfaces (46). Studies by Rand and coworkers have shown that the hydration force becomes an important factor when the distance between the bilayers is 2 nm or less (for review see Ref. 47). We have suggested (1) that one of the functions of HA (and other fusion proteins) may be to help overcome this energy barrier. The HA molecules, which traverse the viral membrane, may do this by incorporating firmly into the target membrane via the hydrophobic N-terminus of HA2. They may, in this way, mechanically bring the two membranes so close that direct contact between the lipid molecules becomes possible. The fact that PE enhances the fusion activity of both Influenza and Sindbis viruses (and to a lesser extent Semliki Forest virus) (27, 35, 48) is consistent with this notion. Being less hydrated than most other phospholipids, PE-containing membranes are known to exhibit significantly lower hydration forces (47). The demonstration that BHA-binding to liposomes does not require PE suggests that it is not the lipid-protein interaction per se, but rather the interaction of two membranes, which is PE-dependent.

The HA may also be instrumental in the second and third stages of fusion: the coalescence and subsequent separation of the membranes (49). For fusion to occur the lipids must be locally disorganized; this may also require the presence of HA. Our data suggest that HA penetrates into the hydrophobic interior of the target bilayer. Whether this can explain the drastic change in lipid organization remains to be seen. PE may also have a role at this stage of fusion since it is more likely to adopt nonlamellar structures within membranes than other phospholipids (50). The fact that BHA can be incorporated at high concentrations into liposomes will allow studies on the BHA-lipid interactions by biophysical techniques. Such studies will be performed to determine whether BHA does, indeed, modify the organization of the membrane lipids and induce the non-lamellar configuration(s) needed for fusion.

Acknowledgments—We wish to thank Dr. John Skehel of the National Institute for Medical Research, Mill Hill, London, for his gift of the X31 virus inoculum. We also thank Dr. Don Wiley of Harvard University and Dr. Gary Rudnick for helpful discussions, Leola Z. Eugenio for secretarial assistance, Margie Moench for her art work, and Pam Ossorio for her photographic work. Finally, we thank Dr. Mark Marsh, Dr. Margaret Kielian, and Connie Copeland for critically reading the manuscript.

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Preparation of Virus

The 1:31 (A/Hong Kong/1968) recombinant strain of influenza was grown in the allantoic cavity of 11 day embryonated eggs infected with 0.01 hemadsorbing units. Allantoic fluid was collected 48 hours post-infection and clarified by centrifugation at 10,000 x g for 15 minutes in a Beckman J-60 centrifuge. Virus was then purified according to methods described by J. Smith (personal communication) as follows: Polyethylene glycol (6000 - Sigma) was added to a final concentration of 5% (w/v), the mixture stirred at room temperature for 30 minutes, and centrifuged at 10,000 x g for 30 minutes at 4°C. The pellet was resuspended in phosphate buffered saline (PBS) at a ratio of 1 ml PBS per gram of polyethylene glycol, disrupted by sonicate homogenization, applied to a 20% sucrose step gradient (30% / 50%) and centrifuged at 4°C for 1.5 hours at 80,000 x g in a Beckman SW 28 rotor. The virus was collected at the 30 - 60% interface, diluted approximately 4 times with PBS, and pelleted for 1.5 hours at 80,000 x g in the SW 28 rotor. The pellet was resuspended overnight in approximately 0.1 ml of cold PBS and applied to a 15 to 45% (w/v) continuous sucrose gradient in a Beckman SW 40 rotor. The virus band was collected following a 45 minute centrifugation at 80,000 x g and the protein concentration was determined by the method of Lowry (21). Aliquots were frozen in liquid nitrogen and stored at -80°C.

Preparation of BHA

The BHA was obtained by exhaustively digesting purified 3:31 virus with pronase (14). After digestion, the viral cores were pelleted at 100,000 x g for 1 hour and the BHA containing supernatant passed over a DE-52-Sephrose column (M. J. Gething, personal communication). After extensive washing with PBS, the BHA was eluted at pH 7.0 and the peak fractions stored at 4°C. In SDS-PAGE, BHA runs as a single band of Mw = 70,000 (Fig. 7, lane 4). Upon reduction, HA1 and HI each give separate bands at Mw = 58,000 and Mw = 21,000 respectively (Lane 3). The HA chain of BHA (lane 3) migrates somewhat faster than authentic HA2 (lane 1), owing to the loss of the 46 residue membrane spanning and cytoplasmic domain (23).

Radiolabeling

X31 and BHA were labeled with 125I using iodogen (Pierce) as described by Frazer and Steck (24). When intact virions were iodinated BHA was preferentially labeled (Fig. 7, lane 2). In BHA N2A was heavily labeled as well (Fig. 7, lane 3). While BHA could also be labeled with Bolton-Hunter reagent, this procedure gave rise to a variety of artefacts. The midpoint of BHA's conversion was shifted, for example, from pH 5.7 to pH 5.8. Thus, iodogen was routinely used to iodinate BHA.

Preparation of Liposomes

Liposomes were freshly prepared before each experiment as described by White and Helenius (25), except that 20% 2-Dimorpholinooxetane/cholesterol/dioleoyl PC (10/30/60 or 15/20/65) buffer (Mg-2+), adjusting to pH 7.0 was used. As a buffer, when necessary, trace amounts of 2-thiobarbital labeled lipids from BHA-21 cells were added to the lipid mixture to allow quantification. Phosphatidylyethanolamine (P) from bovine brain, phosphatidic acid (P) from egg yolk, synthetic dimyristoylphosphatidylcholine (DMPC), and cholesterol (Chol) were obtained from Sigma and used without purification. Phytmitinyl (from bovine brain sphingomyelin (SM)); phosphatidylethanolamine (PE) from soybean and egg yolk, and egg phosphatidylcholine (PC) were obtained from Sigma or Avanti. Dio- and tri-sialogangliosides were obtained from Supelco. Lipids were kept at -80°C in chloroforom:methanol (1:1) as lyophilized stocks. Concentration was determined by the method of Morrison (27).

Assay for HA Binding to Liposomes

Trace amounts (20 - 70 mg) of 125I-BHA in a volume of 5 µl were added to 4% of liposomes (90% phospholipid). Temperature was regulated with an ITCOM heat-flow oven (ITCOM Ltd., Ireland) in a small chamber containing such items as a heating mantle, a stirring rod, a stirrer, a temperature controller, etc. Samples were pulsed with sodium chloride (150 mM), sodium chloride (150 mM), EDTA (10 mM), and 1% sodium dodecyl sulfate (SDS) to disrupt the complexes. The assay was allowed to come to equilibrium at 4°C. The assay was performed as follows: 33 µl of 20000 or 100000 counts per minute (cpm) in 10 mM sodium phosphate buffer (pH 7.0) was added to 37° or 4°C buffer containing BHA. After 30 or 60 minutes, the mixture was 5% trichloroacetic acid (TCA) or 200 µl of 50% TCA filtered through a 0.45 µm filter and counted in a Beckman LS-12000 liquid scintillation spectrometer. Backgrounds were subtracted from each sample. The assay was performed in quadruplicate and each value is the average of four readings. Backgrounds were subtracted from each sample. The assay was performed in quadruplicate and each value is the average of four readings.