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Stability of HA2 Prefusion Structure and pH-Induced Conformational Changes in the HA2 Domain of H3N2 Hemagglutinin

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Abstract

Influenza hemagglutinin is the fusion protein that mediates fusion of the viral and host membranes through a large conformational change upon acidification in the developing endosome. The “spring-loaded” model has long been used to describe the mechanism of hemagglutinin and other type 1 viral glycoproteins. This model postulates a metastable conformation of the HA2 subunit, caged from adopting a lower-free energy conformation by the HA1 subunit. Here, using a combination of biochemical and spectroscopic methods, we study a truncated construct of HA2 (HA2*, lacking the transmembrane domain) recombinantly expressed in *Escherichia coli* as a model for HA2 without the influence of HA1. Our data show that HA2* folds into a conformation like that of HA2 in full length HA and forms trimers. Upon acidification, HA2* undergoes a conformational change that is consistent with the change from pre- to postfusion HA2 in HA. This conformational change is fast and occurs on a time scale that is not consistent with aggregation. These results suggest that the prefusion conformation of HA2 is stable and the change to the postfusion conformation is due to protonation of HA2 itself and not merely uncaging by HA1.

Graphical Abstract

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Author Contributions

M.W.E. and H.M.H.S. carried out the experiments and analyzed the data. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biochem.1c00551>.

Photolysis of photoacid *o*-NBA, antibody binding assay, assessing HA2* stability by temperature-dependent spectroscopy, suitability of detergents for FTIR, and binding of sarkosyl to HA2* (PDF)

Accession Codes

The HA2 sequence is derived from H3N2 hemagglutinin with UniProt accession number P03438.

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Influenza hemagglutinin (HA) is an integral viral membrane glycoprotein that mediates viral uptake by and viral membrane fusion with the host cell.¹ HA is a homotrimeric protein with each HA monomer having two subunits: HA1 and HA2. HA is expressed as one chain as the inactive HA0 form. HA0 is activated by proteolytic cleavage into two subunits, HA1 and HA2, which are connected by a disulfide bond. Each subunit plays a different role in the viral infection process. HA1 binds sialic acid receptors on the cell surface.² Binding triggers a pathway that results in the virus being endocytosed by the host cell. HA2 drives fusion of the viral membrane with the host cell membrane in the maturing endosome.

Acidification of the maturing endosome causes a conformational change in HA that drives the membrane fusion process. In particular, the HA2 region undergoes a large conformational rearrangement. HA2 consists of four domains: the endodomain, the transmembrane domain (TMD), the highly conserved stalk region, and the hydrophobic fusion peptide (FP). Upon acidification, the stalk region undergoes a large conformational change that somehow drives membrane fusion by overcoming the barriers of the fusion process.³ The FPs insert into or interact with the host membrane. In the final postfusion conformation, the FPs and the TMDs are in the proximity of each other. It is thought that the FPs and TMDs interact to facilitate and stabilize fusion pore formation and expansion.⁴ The fusion process is cooperative, requiring triggering of several HA trimers.³

The molecular mechanism of the HA2 conformational rearrangement and how the free energy release from this change overcomes the barriers of membrane fusion are not known.³ Structures of the prefusion neutral-pH state and the postfusion low-pH state are known from X-ray crystallography,^{5,6} but the structures of the intermediate states are unknown. The classically accepted mechanism is described by the spring-loaded model. In the spring-loaded model, prefusion HA2 is in a metastable state at neutral pH. HA2 is held in this conformation by the surrounding HA1 subunit, like a loaded spring. Following acidification, the HA1 domain swings out of the way, allowing the HA2 domain to undergo a conformational change to a lower-energy state, releasing energy from the spring. The S2 loop (the B-loop) of the stalk is postulated to fold into an α -helix resulting in the formation of an extended coiled-coil structure. This extends the FPs out toward the host membrane.

This extended coiled-coil structure is also metastable and collapses on itself as the S5 region zips up, bringing the FP and TMD into the proximity to form the stable postfusion structure. In this model, there is a delicate kinetic balance between the transitions away from the two metastable conformations. Refolding to the final state before the FPs interact with the host membrane results in inactivated HA.

Recently, simulation-based studies have suggested that the folding of the B-loop into the extended coiled coil is not a downhill process and that only the folding of the first half of S2 is energetically favorable.^{7–9} This result supports a different mechanism. In this mechanism, the FPs are released from the core of the trimer upon acidification. The S2 region then partially forms a helix. This resulting intermediate is more disordered. The flexible, disordered intermediate allows the FPs to insert into the viral membrane, as well as the host membrane. The S2 helix eventually fully folds, and during the refolding process, S4 and S5 stabilize the S2 helix in the postfusion structure.

The metastable nature of HA2 in prefusion HA is the crux of the spring-loaded mechanism. The Wiley lab compared bromelain-cleaved HA (BHA)⁵ and TBHA₂, which is residues 38–175 of HA2 (missing the FP and TMD) bound via a disulfide linkage to residues 1–27 of HA1.⁶ X-ray crystallography shows BHA in the prefusion conformation⁵ and TBHA₂ in the postfusion conformation.⁶ TBHA₂ exhibited increased thermostability compared to that of BHA, leading to the suggestion that HA2 is metastable at neutral pH and adopts a more stable conformation at low pH,⁶ though the lack of FP and different interactions with HA1 may influence the stability of the postfusion state. They reported additional evidence for the metastability of HA2 by studying the soluble domain of HA2¹⁰ and full length HA2 with a maltose binding protein tag¹¹ recombinantly expressed in *Escherichia coli* without HA1. The results suggested that both HA2 variants adopt a structure at neutral pH very similar to that of TBHA₂, which is taken to be the postfusion state.^{10,11} The findings were based on indirect methods rather than the crystallography of the BHA and TBHA₂ studies. They suggested that these results showed that acidification is not necessary for HA2 to adopt the fusion active structure and that acidification causes changes in the HA1 domain that allow HA2 to access the more stable state. These studies, however, report on the proteins only at neutral pH and do not probe any changes that may occur upon acidification. It is also unclear how the maltose binding protein tag would affect the full length HA2.

If there were no additional changes upon acidification, then one would expect HA2 to be either completely incapable of driving membrane fusion processes (if the energy release from refolding is required to drive membrane fusion) or fusion active at neutral pH (assuming it adopts a fusogenic conformation). The Chernomordik lab showed that an HA2 construct consisting of amino acids 1–127 (FHA2) recombinantly expressed without HA1 drives membrane hemifusion in a pH-dependent manner; lipid mixing occurs at low pH but not at neutral pH.¹² They also showed that the full soluble ectodomain of residues 1–185 (HA2*) drives content mixing in a pH-dependent manner. When HA2* was exposed to low pH prior to the addition of membranes, it lost fusogenic activity, suggesting that this difference does not arise simply from the final protonation state of the protein.¹³ These results show that recombinantly expressed HA2 is not fusion active at neutral pH but has fusogenic properties at low pH consistent with native HA. There is clearly a functionally

relevant change in HA2 structure upon acidification. One explanation that has been offered for this behavior in FHA2 is the interaction of multiple FHA2 trimers with each other through the kinked region (residues 105–113) at low pH but not neutral pH.¹⁴ It has been proposed that these interactions are necessary for the membrane fusion process,¹² which could explain the pH dependence, though it is not clear why acidification prior to addition of membranes would lead to inactivation in this case.

There is evidence that, in HA, HA2 undergoes some pH-dependent changes prior to the dissociation of HA1, suggesting that there may be more at play than HA1 holding HA2 in place. Study of the reversibility of pH-driven conformational changes in HA showed that the FPs of HA2 are exposed at a step prior to the irreversible dissociation of HA1.¹⁵ The authors suggest the existence of a “primed conformation” in which HA2 has been activated but is prevented from fully refolding.¹⁵ It has been shown that an antigen binding fragment (FAB) that binds to the receptor binding region of HA1 stabilized the HA1 trimer and prevented the full conformational change to the fusion active state but did allow the release of FP from HA2.¹⁶ These results are consistent with a mechanism in which the FPs can be released without large HA1 reorganization.

HA is a representative type 1 viral glycoprotein, and the spring-loaded mechanism has been considered the canonical mechanism for protein-mediated membrane fusion.¹⁷ Many questions remain, and additional insight could have implications for understanding the fusion machinery of many viruses. To understand the properties of HA2 and its role in the fusion process, it is useful to study HA2 independent of HA1. In this study, we describe a truncated HA2 (HA2*, comprising residues 1–185 of H3N2 HA2, lacking the TMD and endodomain, with a hexa-His tag) expressed in *E. coli* as a model system for the study of pH-dependent HA2 conformational change. We report an optimized protocol for expression and purification, highlighting the importance of the detergent. We show that at pH 7.4 HA2* forms trimers and folds into a structure consistent with the prefusion structure of HA2 in full length HA. We demonstrate that HA2* undergoes a conformational change upon acidification and that this change is consistent with a transition from a prefusion-like to a postfusion-like HA2 conformation. This pH-dependent change may be associated with the release of the FPs. Stopped-flow pH jump kinetics monitored by tryptophan fluorescence reveals submillisecond dynamics of this conformational change and demonstrates HA2* as a promising model system for studying HA2 dynamics. Additionally, we demonstrate the ability of soluble HA2* to be anchored to lipid bilayers, enabling study of HA2 in membrane environments. Taken together, these results suggest that HA2 is not trapped in a metastable state by the HA1 domain but rather undergoes a pH-triggered transition from a prefusion structure to a postfusion structure independent of HA1.

MATERIALS AND METHODS

Materials.

Mini-PROTEAN precast gels, Lamelli buffer, native sample buffer, and HRP enzyme substrate are obtained from Bio-Rad Laboratories. BL21(DE3), Rosetta (DE3) PLYS, and Color Prestained Protein Standard are obtained from New England Bio-Laboratories. Ultracel-3 regenerated cellulose membranes and Amicon Ultra-0.5 centrifugal Filter Units

are from Millipore Sigma. *o*-Nitrobenzaldehyde, bovine serum albumin lyophilized powder, 96% (agarose gel electrophoresis), LB broth, ampicillin, kanamycin, *N*-lauroylsarcosine sodium salt, and Triton X-100 are from Sigma-Aldrich. *n*-Decyl β -D-maltopyranoside (DM) is from Anatrace. All lipids are from Avanti Polar Lipids. Goat anti-human IgG (H+L) HRP is purchased from Invitrogen. Nonfat dry milk is obtained from Lab Scientific. CR8020 is a generous gift from M. Crank at the National Institute of Allergy and Infectious Diseases (NIAID).

Protein Expression and Purification.

The plasmid containing the DNA sequence of the H3N2 HA2_{1–185} ectodomain with a six-His tag at the C-terminus (HA2*) is synthesized (Genescript). The sequence is derived from hemagglutinin with UniProt accession number P03438. The pET24a(+) plasmid is transformed into BL21 (DE3) competent cells on an agar plate. After overnight growth, a single colony is transferred into LB broth with 100 μ g/mL ampicillin and shaken overnight at 30 °C and 200 rpm. The culture (3.3 mL) is then added to 1 L of LB-ampicillin culture medium and incubated at 37 °C and 200 rpm for 5 h. To induce protein expression, IPTG (final concentration of 0.5 mM) is added and the culture is incubated at room temperature for 6 h at 200 rpm. The culture is centrifuged (Beckman Coulter Avanti JXN-26) at 4 °C for 10 min at 5000*g*, followed by harvesting of the cell pellet and storage at –80 °C. The cell pellet is lysed for 1 h in protease inhibitor containing lysis buffer [50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 0.5% *N*-lauroylsarcosinate, and 0.5% Triton X-100 (pH 8)] at 4 °C, followed by sonication on ice. The lysate is then centrifuged for 15 min at 20000*g* and 4 °C, and the supernatant is retrieved and passed through a 0.2 μ m filter before being injected into a His-trap FF column (GE Healthcare Life Sciences) on an FPLC instrument (ÄKTA pure, GE Healthcare Life Sciences). The purified protein is stored at 4 °C in storage buffer [50 mM NaH₂PO₄, 300 mM NaCl, and 0.5% *N*-lauroylsarcosinate (or 0.17% decyl maltoside for protein intended for IR measurements)]. The protein is used in experiments within 4 days of purification.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is used to verify the molecular weight of the expressed protein. Briefly, HA2* is first mixed with Laemmli buffer in a 1:1 ratio, subjected to boiling at 99 °C for 1 min, and then incubated on ice for 30 min. The samples are loaded onto 12% Mini-PROTEAN precast gels (Bio-Rad) and run in an electrophoresis cell with SDS running buffer at 120 V for 30 min. Native PAGE is performed to study the molecular weight of HA2* oligomers under a nondenaturing condition. HA2 is first mixed with native sample buffer (Bio-Rad) in a 1:1 ratio and transferred to a 10% Mini-Protean TGX gel (Bio-Rad). The assay is run in Tris/glycine buffer at 100 V for 1 h.

Size Exclusion Chromatography (SEC).

SEC is performed with an FPLC instrument (AKTA pure, GE Technologies) equipped with a size exclusion column (Sephadex 200 Increase 10/300, GE Technologies), a 0.5 mL/min flow rate, and A₂₈₀ detection. The column is first equilibrated with 50 mM sodium phosphate buffer (pH 7) without protein. The HA2* protein samples are exchanged into 10 mM sodium phosphate buffer (pH 7) via dialysis with a 10 kDa molecular weight cutoff

(MWCO) dialysis membrane. Dialysis buffer is replaced each day for a total dialysis time of 3 days. The protein concentration is adjusted with a concentrator and then verified with A_{280} using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific). The HA2* is loaded into the column at a concentration of 10 μM . A series of mass standards consisting of vitamin B₁₂ (1.3 kDa), myoglobin (16.7 kDa), and bovine serum albumin (66.5 kDa) (Sigma-Aldrich) are loaded into the column to generate a calibration curve.

Antibody Binding.

Supported lipid bilayers are prepared in 96-well glass (#1 coverglass) bottom plates (Cellvis) to anchor HA2 for antibody binding assays. Lipids presolvated in chloroform are mixed in 2 mL gas chromatography vials [2 μmol of POPC and 0.5 μmol of DGS-NTA(Co)]. Chloroform is evaporated under a stream of nitrogen, and the lipid cakes are dried by lyophilization overnight. Dried lipid cakes are dissolved in 1 mL of assay buffer [50 mM phosphate and 100 mM NaCl (pH 7.0)]. Lipid vesicles (~100 nm) are prepared by sonication of this solution until it becomes clear.

Well plates are treated with 5 M NaOH (2 h) and 1 N H₂SO₄ (30 min). Following treatment of the glass, wells are washed three times with 300 μL portions of assay buffer. Co²⁺-NTA-doped POPC vesicles are added (75 μL) and diluted with assay buffer (75 μL) to a final lipid concentration of 1.25 mM (20% Co²⁺-NTA headgroup doping). Bilayers are allowed to form over 1 h, and the wells are washed three times with assay buffer.

HA2* (0.2 μg in 40 μL of assay buffer) is added and incubated for 1 h. In negative control wells, 40 μL of assay buffer is added instead. To anchor HA2* to the membrane, Co²⁺ headgroups are oxidized to Co³⁺ with 10 mM H₂O₂ (20 μL , 15 min), and wells are washed three times with assay buffer. The ligand exchange rate of Co(III) is slow, essentially locking down HA2* through hexa-His tag binding.^{18,19} Wells testing neutral pH (prefusion) are incubated with 300 μL of assay buffer for 1 h. Wells testing low pH (postfusion) are incubated with dilute HCl (1 mM). After incubation, wells are washed three times with assay buffer.

Prior to the addition of antibodies, 300 μL of the blocking solution (5% milk in assay buffer) is added for 1 h, and the wells are washed three times with assay buffer. The primary antibody (CR8020) is added as 0.4 μg in 40 μL of 1% milk and incubated for 5 min, after which the wells are washed six times with assay buffer. The secondary antibody (HRP goat anti-human) is added as 3 μg in 40 μL of 1% milk and incubated for 5 min followed by six washes with assay buffer. The HRP enzyme substrate (50 μL) is added. After 5 min, the reaction is stopped by addition of 50 μL of 1 N H₂SO₄. The absorbance at 450 nm of each well is recorded using a plate reader (Biotek Cytation 5).

Buffer Exchange and Sample Preparation.

For the preparation of samples for fluorescence and transmission FTIR, the protein stock in storage buffer is concentrated to 100 μL in an Amicon Ultra-0.5 Centrifugal Filter Unit (Milipore) with a 3 kDa MWCO by spinning at 10K RCF for 10 min. The 100 μL sample is diluted to 500 μL with the relevant buffer. For fluorescence samples, the buffer consists of 10 mM sodium phosphate (pH 7.4) and 100 mM NaCl. For FTIR samples, the solvent consists

of 100 mM NaCl and 0.17% DM; no buffer is used to minimize the buffering capacity in the FTIR phototitration measurements. After dilution, the sample is concentrated to 100 μL . This process is repeated five times for a total of six rounds of buffer exchange. The protein concentration is determined using a calculated extinction coefficient at 280 nm of $31960 \text{ cm}^{-1} \text{ M}^{-1}$ (ExPASy) and A_{280} as measured by a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific). The concentration is adjusted by addition of a relevant solvent. Samples for FTIR are aliquoted and lyophilized. Following lyophilization, three rounds of H/D exchange are performed by hydrating the samples in D_2O , incubating, and then lyophilizing. After the final round, the samples are stored at -20°C .

For CD, the protein stock is first exchanged into 10 mM sodium phosphate buffer (pH 7) via dialysis with a 10 kDa MWCO dialysis membrane. Dialysis buffer was replaced each day for a total dialysis time of 3 days. The protein concentration was adjusted with a concentrator and then verified with A_{280} using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific).

Circular Dichroism.

Information about the secondary structure of HA2* is obtained from CD spectra. Spectra are acquired with a CD instrument equipped with a temperature controller (J-1500 series, JASCO). Spectra are obtained for HA2* (6 μM) in 10 mM sodium phosphate buffer at pH 4, 5, 6, and 7 in a 1 mm path length quartz cuvette. The scanning range of the instrument is set between 190 and 260 nm at a rate of 200 nm/min with a 2 s response time and a 1 nm spectral bandwidth.

Helical contents in HA2* are calculated using the mean residue ellipticity at 222 nm:

$$\text{MRE} = \frac{\theta M}{ncl} \quad (1)$$

where θ is the observed ellipticity (millidegrees), c is the protein concentration, l is the cuvette path length, M is the molecular weight of the protein, and n is the number of peptide bonds. Percent helicity is calculated as follows:^{20,21}

$$\% \text{ helicity} = \left(\frac{\text{MRE}_{222} - 3000}{33000 - 3000} \right) \times 100 \quad (2)$$

where MRE_{222} is mean residue ellipticity at 222 nm, 3000 is the mean residue ellipticity of β -character and random coil at 222 nm, and 33000 is the mean residue ellipticity of pure helical character at 222 nm.²¹

Fluorescence.

Equilibrium native tryptophan fluorescence spectra of HA2* (6 μM) at pH 7 and 4, as well as temperature-dependent fluorescence spectra, are acquired using a FluoroMax spectrophotometer (Horiba Scientific) equipped with a temperature controller (Peltier, PerkinElmer). Spectra are collected using a 1 cm fluorescence cuvette with 2 nm excitation and emission slit widths. Samples are excited at 280 nm, and emission is collected from 285 to 550 nm with an integration time of 10 s.

FTIR Phototitration.

FTIR spectra are recorded using a modified Varian 660 FT-IR spectrometer. For transmission FTIR, the IR beam is picked off through an external sample chamber to an external detector. The sample is loaded into a sample cell consisting of a 75 μm PTFE spacer between two CaF_2 windows (Harrick) held in a copper housing. Rather than preparing HA2* at multiple pH values and collecting FTIR spectra for each sample, we use a phototitration method making use of *o*-NBA as a caged proton. A saturated solution of *o*-NBA is prepared by adding 1 mL of D_2O to an excess of *o*-NBA in an amber GC vial. The vial is vortexed, heated at 50 $^\circ\text{C}$ for approximately 10 min, vortexed again, and then allowed to cool slowly to room temperature. The sample is transferred to a 1.5 mL centrifuge tube and centrifuged at 1K RCF for 5 min. The saturated supernatant is transferred to another amber vial. The concentration of this solution is measured using an extinction coefficient of $8300\text{ cm}^{-1}\text{ M}^{-1}$ at 268 nm²² and is ~8 mM. The lyophilized HA2* is rehydrated using this saturated *o*-NBA to a final protein concentration of 1.4 mg/mL (61 μM). The pD of the sample (7.4) is measured to ensure that the sample was starting at the prefusion pD.

All transmission FTIR spectra are the average of 200 scans. All HA2* spectra are ratioed to the spectrum of unphotolyzed saturated *o*-NBA in D_2O . Prior to any illumination, initial spectra are obtained. The sample is illuminated at 351 nm with a Nd:YLF laser (Crystallaser) for a set length. Following illumination, spectra are obtained, and the process is repeated.

ATR-FTIR.

ATR spectra are obtained using an ATR trough plate accessory with a 10-bounce Ge crystal (HATR, Pike Technologies) in the internal cavity of the same Varian 660 instrument described above. The polished Ge crystal is treated with 1 M H_2SO_4 following glow discharge cleaning. An SLB is formed on the resulting hydrophilic surface. All solutions are added in 1 mL volume by micropipette. Vesicles are prepared by sonication of 20% Co-NTA-doped POPC lipid cakes hydrated in phosphate buffer [100 mM phosphate and 100 mM NaCl (pH 7.0)] to a concentration of 1.5 mM. The vesicle solution is incubated on the crystal for 1 h followed by three washes with H_2O to remove excess lipids. Bovine serum albumin (BSA, 100 μM) in phosphate buffer is added for 10 min to block any exposed hydrophilic regions of the crystal, and excess BSA is removed by washing. HA2* (7.5 μM) in phosphate buffer is added, and the mixture incubated for 10 min. Co^{2+} headgroups are oxidized by addition of H_2O_2 to a final concentration of 10 mM. Unbound HA2* is removed with a buffer wash. Acetate buffer [100 mM acetate and 100 mM NaCl (pH 4.5)] is added to acidify the sample. FTIR spectra are recorded after every step of the process and are the average of 1000 scans.

Stopped-Flow Fluorescence.

Stopped-flow fluorescence pH jump studies are conducted using a Biologic SFM2000 two-syringe stopped-flow mixer with a microvolume cuvette. Light from a Xe lamp (HPX-2000 Mikropack) is fiber-optically coupled to the SFM instrument. Emitted light is detected at a 90 $^\circ$ angle by an amplified photomultiplier detector (PMM02 Thorlabs) with a dc power supply (MDC01 Tacklife). The detector output is digitized by a PCIe-6321 board (National Instruments), and data are collected in the BIO-KINE software package (Biologic). For

tryptophan fluorescence, excitation light is filtered by a 290/10 nm bandpass filter (Edmund Optics Techspec) and emission light by a 340/26 nm bandpass filter (Brightline).

Syringe 1 contains 50 μ M HA2* in 10 mM Na phosphate (pH 7.4) and 100 mM NaCl. Syringe 2 contains 22.5 mM HCl. Each shot is 75 μ L from each syringe for a final concentration of 25 μ M HA2* at pH 4.8. The flow rate of each syringe is 14 mL/min. The temperature is held at 37 °C by a circulating water bath (RTE7 Thermo). The final pH after mixing is verified by mixing 75 μ L of each solution in a microcentrifuge tube and then measuring the final pH with a pH probe.

Data collection is started 20 ms before hard stop valve closure to ensure observation of the complete mixing event. Time resolution is one point every 10 μ s for 2000 points. The first two shots are discarded to ensure that the system has been adequately washed and equilibrated. Average traces are computed from 16 shots. Correction for the background signal is achieved by subtracting a 16-shot average trace of HCl shot against the phosphate buffer from the HA2*. Background-corrected average traces are smoothed using a boxcar smoothing algorithm (Igor Pro 5, Wavemetrics) with a total boxcar width of 11 points (five on each side). Time zero is taken to be the point after valve closure at which the fluorescence intensity begins to change, and the traces are adjusted to reflect this point as time zero.

RESULTS

Protein Expression and Purification.

HA2* is based on a previously reported construct developed from the H3N2 hemagglutinin of the 1968 Hong Kong Flu.¹³ The sequence of HA2* and the structure of the regions of HA corresponding to HA2* are shown in Figure 1. Our attempts to purify the protein by solubilization without any detergent or with non-ionic detergents resulted in very poor yields (<0.3 mg of protein/L of expression medium). The best yields were obtained using the anionic detergent sarkosyl (*N*-lauroylsarcosinate) to solubilize the cell mass and in the purification buffer. Using the optimized method, yields of ~0.6 mg of protein/g of cell mass were obtained for yields of ~4 mg/L of expression medium. Because sarkosyl has a carboxylate group, it cannot be used in FTIR studies due to the overlap of bands. Similar, but slightly lower, yields were obtained when solubilizing with sarkosyl and using *n*-decyl β -D-maltopyranoside (DM) in purification buffer. To determine if a different cell line could further improve the yield, a second cell line, Rosetta (DE3) pLysS, was tested. The original BL21 (DE3) line yielded ~15% more protein.

Gel Chromatography.

SDS–PAGE shows a distinct band at a mass just under 25 kDa (Figure 2A). This is indicative of the successful expression and purification of HA2*. The mass of individual monomers of HA2* is between 22 and 23 kDa. It is expected that HA2* would be fully monomeric under the denaturing conditions of an SDS gel. The slight smearing of the band to higher molecular weights is consistent with HA2* tightly bound by sarkosyl detergent molecule(s). We observe that sarkosyl interacts strongly with the protein (Figure S5) and suspect that sarkosyl may strongly bind to the FPs.

The results of the native PAGE gel (Figure 2B) are more complex. The band around 25 kDa shows the presence of monomeric HA2*. The band around 75 kDa is consistent with trimeric HA2*. The higher-molecular weight bands are consistent with higher-order oligomers of HA2*. These results show that, in solution, HA2* exists in a monomer–trimer equilibrium with some higher-order oligomers present.

Size Exclusion Chromatography.

The elution profile of dialyzed HA2* shows a dominant peak at 8.2 mL, smaller features at 5.0 and 11.2 mL, and a less defined shoulder around 9 mL as shown in Figure 2C. A calibration curve (Figure 2D) is generated by injecting BSA, myoglobin, and vitamin B₁₂ at the same flow rate and in the same buffer. Using the calibration curve, the dominant peak at 8.2 mL is determined to be at a mass of ~93.4 kDa, which is assigned to trimeric HA2*. The 11.2 mL peak is assigned to monomeric HA2*, and the shoulder ~9 mL is assigned to dimeric HA2*. The peak at 5.0 mL is assigned to larger oligomers/aggregates of HA2*. The peak areas indicate a monomer:trimer ratio of 1:9. These results are for HA2* at neutral pH. SEC was not performed with HA2* at low pH, to avoid damage to the column caused by protein aggregation.

Antibody Binding.

CR8020 is a broadly neutralizing antibody that binds a structural epitope on the stem region of properly folded hemagglutinin in the neutral-pH structure and prevents the transition to the fusogenic form upon acidification.^{25,26} CR8020 has been shown to explicitly bind H3N2 hemagglutinin at a site on HA2 near the membrane.^{25,26} Binding of this antibody to HA2* is used to evaluate if HA2* has the same neutral-pH structure observed for HA2. The intrinsic hexa-His tag of HA2* is used to immobilize HA2* in the well plates for testing antibody binding. Interaction of the His tag with Co-NTA-labeled lipid headgroups anchors HA2* to SLBs prepared on the well surface. HA2* bound to Co²⁺ becomes essentially irreversibly bound when Co²⁺ is oxidized to Co³⁺.^{18,19} This allows unbound HA2* to be thoroughly washed away without appreciably reducing the amount of HA2* bound to the surface.

Binding of CR8020 to HA2* is tested by a colorimetric assay, using a secondary antibody (HRP goat anti-human) that binds to CR8020. The wells are initially colorless upon addition of the HRP substrate. As 3,3',5,5'-tetramethylbenzidine (TMB) is oxidized in the presence of HRP, color is generated. Initially, a blue color develops as a charge transfer complex of the radical cation forms. Addition of H₂SO₄ stops the reaction and fully oxidizes the partially oxidized TMB. The resulting yellow diimine oxidation product can be monitored by absorption at 450 nm.²⁷

Water blanks are used to correct for background in absorption measurements. Binding of CR8020 at neutral pH (prefusion) and at decreased pH (postfusion) is tested. Binding of CR8020 to HA2* was compared to CR8020 nonspecifically binding to the SLB well substrate. A qualitative visual assessment suggests that CR8020 binds HA2* at neutral pH but not low pH (Figure S2). *A*₄₅₀ is shown in Figure 3. A higher absorbance means a larger amount of HRP present. At neutral pH (7), the + wells have a higher average absorbance than the – wells, suggesting binding of CR8020 to HA2* beyond nonspecific interactions.

At low pH (~3), there is not a difference between the absorbance of both the + and – wells, suggesting that, at low pH, CR8020 does not bind to HA2*. This is also supported by the difference in absorbance between the neutral- and low-pH + wells. An acid-induced conformational change in HA2* is expected to be irreversible over the time scale of these assays.²⁸ These differences in the low- and neutral-pH absorbances are consistent with a conformational change in HA2* upon acidification that abolishes the structural epitope of CR8020.

Circular Dichroism (CD) Spectroscopy.

CD spectroscopy shows that HA2* adopts an α -helical secondary structure at pH 7, with characteristic negative bands at 222 and 208 nm, and has 44% α -helicity at pH 7. As the HA2* is titrated against buffers of decreasing pH, there is a gradual decrease in signal intensity, which we attribute to the aggregation of HA2* at acidic pH due to exposure of the hydrophobic FPs in an environment with most of the SRC detergent removed (Figure 4A).¹³ SRC is removed for the CD measurements (replaced with DM) because it has a strong absorbance in the region of 220 nm. The results of this study show that HA2* is stable and not aggregated without SRC at neutral pH. Partial loss of the CD signal upon acidification is consistent with a pH-induced conformational change that leads to aggregation and supports the claim that HA2* is stable and not aggregated under perfusion conditions. Significant aggregation is observed only when HA2* is acidified. The ratio between the CD signal at 208 and 222 nm indicates, qualitatively, whether the protein exists as an isolated helix or as multiple helices that form a coiled coil. A 222 nm:208 nm ratio of >1.1 indicates a coiled-coil state.^{29–31} In HA2*, the 222 nm:208 nm ratio increases from 0.9 at pH 7 to 1.8 at pH 4, passing an inflection point at pH 5.5 (Figure 4B), clear evidence for the refolding of HA2* to form a helical coiled-coil state. This pH-dependent transition in the secondary structure is consistent with what is observed for HA (in the context of HA1), with a midpoint for the conformational change at pH ~5.5 and formation of an extended helical coiled-coil conformation.^{13,28}

Fluorescence.

HA2* has four Trp residues at positions 14 (FP), 21 (FP), 92 (S3), and 185 (S5) (Figure 1). The Trp fluorescence is quenched as the pH is decreased from neutral to below pH 6 (Figure 4C); unlike the CD data, the pH dependence of the fluorescence is not well fit to a single sigmoidal transition (Figure 4D). It is unclear if this is because of a multiphasic transition (insufficient resolution to fit a higher-order sigmoid) or if it is due to noise. The Trp emission is sensitive to the local environment of the individual residues, and Trp fluorescence can be used to probe local and global protein conformational changes. If HA2* undergoes a conformational change from a perfusion-like HA2 to a postfusion-like HA2, then one would expect a change in the environment of most, if not all, of the Trp residues (Figure 1). In particular, exposure of the FPs is expected to lead to a significant change in the environment of W14 and W21. With such a conformational change, W185 may also become more exposed and W92 less exposed.

As shown in Figure 4C, upon acidification of HA2* from pH 7 to 4, the emission intensity decreases by ~40%. The observed shift in the emission maximum is minimal, from 335.2

to 334 nm. This decrease in the emission intensity and minimal shifting is consistent with previous work on Trp emission in BHA,³² with a larger decrease in the case of HA2*. Because one would expect a 10–15 nm red-shift for a transition that exposes a fully buried Trp to solvent, the small observed shift indicates a more complex situation. The FP Trps are likely partially solvent exposed in the neutral-pH structure when HA1 is not present, consistent with the position of the Trp emission maximum at neutral pH. There is also residual SRC detergent present in these samples that likely interacts with the hydrophobic FP once it is released by the pH-induced conformational change. Therefore, the net change in solvent exposure of the FP Trps in the pH-induced transition is likely small. In addition, there are four Trp residues distributed throughout the HA2* structure, all of which contribute to the fluorescence spectra. In addition to changes in the environments of Trp residues, the decrease in fluorescence intensity upon acidification may be partially attributable to aggregation of HA2* and crashing out of solution, similar to what was observed in the CD experiments. The stopped-flow fluorescence results discussed below, however, indicate that aggregation and loss of HA2* cannot explain the entirety of the change in the fluorescence signal. Thus, while it is difficult to interpret the fluorescence changes in terms of a specific conformational change, it is clear that there is a conformational change that is induced by the acidification of HA2*.

To investigate the stability of HA2* as a function of temperature, HA2* is subjected to gradual heating from 20 to 80 °C and monitored by Trp fluorescence and CD (Figure S3). The intensity of tryptophan fluorescence decreases, and the emission maxima red-shift by only 3 nm as the temperature increases. Temperature-dependent CD spectra reveal a gradual loss of signal. These data show that HA2* has a T_m of >60 °C.

FTIR Phototitration.

The dominant features in FTIR spectra of proteins are the amide I and amide II bands, also called amide I' and amide II', respectively, if the solvent is D₂O. Amide I' is predominantly the C=O stretch of the protein backbone amide bond. Amide II' is the N–H bending and C–N stretching of the protein backbone. Both C=O and N–H bonds participate in hydrogen bonding, and thus, the amide I' and amide II' bands are sensitive to the differences in hydrogen bonding in different protein secondary structures. In particular, the amide I' band is very useful as a probe for protein secondary structure.^{33–35}

From crystal structures, prefusion HA2 has significant α -helical and random-coil content (Figure 1). In the postfusion structure, there is an extended coiled-coil structure as S2 folds from a random coil to form an α -helix and part of S5 goes from partially helical to more structured. Using STRIDE to calculate the helical content of the pre- and postfusion structures, we predict an overall increase in helical content from ~50% to ~60%.³⁶ Additionally, coiled coils have IR signatures different from those of normal α -helices.³⁷ Thus, it should be possible to determine the pH-dependent changes in secondary structure from the pH-dependent FTIR difference spectra.

Due to the intrinsic difficulties of properly matching titrated protein and reference samples at different pH values and the relatively high concentrations of protein required for FTIR measurements, we opted for a light titration method in which we controlled the release

of protons into the sample via illumination of a photoacid (*o*-NBA) rather than preparing samples at many different pH values. The release of protons can be followed directly through changes in the FTIR spectra. As *o*-NBA is photolyzed (Figure S1), there are bleaches of the NO₂ and the aldehyde absorbances as well as positive features corresponding to the appearance of the NO group and carboxylate (Figure S6). The strongest feature, the NO₂ bleach, falls in a region where there is no overlap with protein features and is used to follow proton release. The concentration of proton release can be calculated by comparing the magnitude of the NO₂ bleach to the total NO₂ magnitude of saturated *o*-NBA.

Figure 5A shows the initial spectrum of the protein with *o*-NBA overlaid with a spectrum of saturated *o*-NBA. The two bands present in the protein spectrum not seen in the *o*-NBA spectrum are amide I' (~1645 cm⁻¹) and amide II' (1457 cm⁻¹). The amide I' band is fairly broad, which is consistent with a prefusion HA2-like structure consisting of α -helical and random-coil content. Figure 5B shows the difference spectra (ratioed to the initial protein spectrum) of HA2* with an increasing illumination time. As the sample is illuminated longer, more *o*-NBA photolyzes, more protons are released, and the characteristic *o*-NBA NO₂ and aldehyde bands are bleached. The *o*-NBA photoproduct carboxylate band grows in, but the photoproduct NO band is obscured by changes in the protein bands.

Figure 5C shows the difference spectrum for illuminated HA2* overlaid with the difference spectrum of *o*-NBA illuminated for the same time and with essentially the same degree of *o*-NBA photolysis. The calculated concentration of protons released in these samples is ~1.5 mM. To clearly see changes in the protein spectrum apart from the *o*-NBA changes, the difference spectra are scaled to have exactly the same magnitude NO₂ bleach, and then a double difference spectrum is computed (Figure 5D).

The bleach at 1564 cm⁻¹ corresponds to the loss of HA2* COO⁻ groups (Glu and Asp) (band c), and the positive feature at 1725 cm⁻¹ corresponds to the growth of COOH (band d). This shows that the acidic protein side chains are being protonated by the protons released from the *o*-NBA photoproduct. The bleach at 1644 cm⁻¹ is assigned to the loss of random coil (band a). The positive features at 1617 and 1690 cm⁻¹ are characteristic of β -sheet secondary structure (band b).³³ Taken together, the data indicate that HA2* undergoes a conformational change as the side chains are protonated. This conformational change leads to a decrease in the random-coil content.

The formation of β -sheet is not expected for the transition of prefusion-like to postfusion-like HA2; instead, it is consistent with the formation of protein aggregates. We attribute the partial loss of intensity of the CD and fluorescence spectra to a decrease in HA2* concentration due to aggregation and crashing out of solution. Transmission FTIR is performed in buffer with some detergent. DM is used to stabilize HA2* rather than SRC because the carboxylate of SRC spectrally overlaps the amide I band; DM is not sensitive to pH and is silent in the amide region (see Figure S5). While this detergent stabilizes HA2*, there is no membrane-like environment into which the FPs might insert. With a conformational change in HA2* that exposes the fusion peptides upon acidification, the hydrophobic FPs likely seed aggregation and formation of β -sheet structure. Bleaching of the random-coil band and formation of β -structure support the conclusion that HA2*

undergoes a conformational change that releases the FPs upon acidification. Additionally, β -structure is observed only upon acidification of HA2*. This further shows that HA2* is not aggregated at neutral pH and forms aggregates only upon acidification.

ATR-FTIR of Supported Lipid Bilayer (SLB)-Bound HA2*.

ATR-FTIR is used to evaluate HA2* on a membrane by anchoring it to a supported lipid bilayer. We added the system components sequentially by passing them into the ATR flow cell and monitoring the ATR-FTIR spectrum. Formation of an SLB by flowing POPC vesicles over the hydrophilic Ge ATR crystal is followed by monitoring the IR absorbances of the lipid tail C–H stretching and the C=O stretch of the fatty acid headgroups (Figure 6A). The intensity of the C–H peak is consistent with a lipid bilayer.³⁸ The quality of the SLB coverage is assessed by binding of BSA. BSA binds to exposed regions of the hydrophilic Ge crystal of the ATR cell. This also prevents nonspecific binding of HA2* to the crystal, which is added subsequent to the BSA.³⁹ BSA binding is monitored by the growth of amide I and II bands upon addition of BSA. We observe negligible growth of the amide I and II absorbances, meaning BSA binding is minimal and the SLB coverage on the crystal is complete. Binding of HA2* to POPC lipids with Co²⁺-NTA headgroups (20% doping level in the POPC SLB) is also monitored using the amide I and II protein bands. Oxidation of Co²⁺ to Co³⁺ causes a baseline shift above 2000 cm⁻¹ (Figure 6B). When the sample is acidified (Figure 6C), a loss of absorbance is observed at 1100 cm⁻¹ due to the switch from phosphate buffer to acetate buffer.

There are clear pH-dependent changes to the protein FTIR bands evident in the difference spectrum (pH 4.0–7.4) shown in Figure 6D. As expected, the Glu and Asp carboxylate (COO⁻) stretch is observed to bleach (band 9) and the carboxylic acid (COOH) stretch (band 8) appears as a positive absorbance, consistent with protonation of these groups with a decrease in pH. A strong positive band at 1650 cm⁻¹ (band 6) is assigned to an α -helix, consistent with an increase in helical structure content induced at low pH. In contrast to the phototitration FTIR measurements (in the absence of a membrane), there is no evidence for β -sheet structure in the difference spectrum (Figure 6D), which should give rise to bands at 1610 and 1690 cm⁻¹. This could be due to several factors. With the SLB, the hydrophobic FPs have a membrane with which to interact, which may prevent seeding of aggregation. Additionally, anchoring HA2* by metal–His chemistry restricts protein movement. Because HA2* is anchored to the surface, the mobility and orientation are restricted and HA2* trimers cannot associate as easily to cause aggregation. Another possibility is that tying up the His tag residues with the Co headgroups changes the aggregation behavior. With HA2* free in solution, His tag residues are free to take up protons while the His tag residues in SLB anchored HA2* are less likely to take up protons. It is possible that positive charge on the His tag may influence the HA2* conformation, but it is unlikely that this could cause the observed aggregation, because an increasing positive charge should strengthen repulsion rather than promote aggregation.

Stopped-Flow Fluorescence pH Jump.

Equilibrium fluorescence measurements showed a decrease in fluorescence intensity caused by a conformational change following acidification or by aggregation and loss of protein.

Stopped-flow fluorescence allows us to probe the kinetics of the fluorescence change, and thus the underlying conformational change. Stopped-flow kinetics data are shown in Figure 7 as a boxcar-averaged (boxcar width of 11 points), background-corrected, average of 16 shots. The data are fit to a double exponential; fitting to a triple exponential does not appreciably improve the fit. The fast phase ($\tau_1 = 270 \pm 20 \mu\text{s}$) dominates the signal ($A_1 = 0.159 \pm 0.007$). This submillisecond phase is too fast to be explained by association of multiple HA2*'s to form large aggregates. Instead, the origin of the fast fluorescence response is likely a change in the Trp environment caused by a conformational change of the protein induced by protonation of the Asp and Glu side chains. The smaller ($A_2 = 0.054 \pm 0.007$) slow phase ($\tau_2 = 250 \pm 50 \text{ ms}$) is more ambiguous. It is difficult to say whether this is due to further changes in the protein conformation or if it is due to aggregation. In summary, the dominant kinetic phase is too fast to be attributed to aggregation and thus represents a protein conformational change, as expected from all of the spectroscopic observations.

The amplitude of the decrease seen in the kinetics does not account for the entirety of the change seen in the equilibrium fluorescence experiment. One possible origin of this discrepancy is aggregation at low pH in the equilibrium experiment that is not observed in the kinetic experiment because it occurs on a longer time scale. Another is the limited time resolution of the measurement, because the time constant of the fast phase is approaching the estimated dead time of the stopped-flow mixer ($200 \mu\text{s}$), and it is not possible to observe faster events using this system. The "missing" fluorescence amplitude may be due to early events that are not captured within the time resolution of the experiment. An experimental approach with a higher time resolution, such as a laser-induced pH jump, is needed to obtain a complete picture of the dynamics of HA2*.

DISCUSSION

This study describes the characterization of the recombinantly expressed His-tagged ectodomain of HA2 (HA2*) with biochemical and spectroscopic methods. Significant findings of this study are as follows. (1) At pH 7.4, HA2* folds into a conformation like that of prefusion HA2 and forms trimers. (2) HA2* undergoes a conformational change upon acidification. This conformational change is consistent with a change from pre- to postfusion HA2. (3) The conformational change in HA2* is fast and is not due to aggregation.

HA2* Folds Properly.

A key assumption of the spring-loaded mechanism is that HA2 is metastable; that is, the prefusion structure of HA2 is not stable, even at neutral pH, but HA2 is caged in that conformation by HA1. Without a metastable HA2 spring caged into the higher-energy conformation by HA1, there is no spring-loaded mechanism. According to this model, without HA1, HA2 folds into the postfusion conformation, even at neutral (prefusogenic) pH. Our results do not support this model because they show that HA2*, recombinantly expressed without HA1, folds into a stable, trimeric state that resembles HA2 of full HA at the prefusion pH. Antibody binding assays have previously been used to evaluate the proper folding of HA constructs.^{40,41} Here, antibody binding assays show that CR8020, which binds only to a structural epitope on the properly folded stalk region of prefusogenic H3

HA, binds to HA2* at neutral pH. This shows that this structural epitope is present in HA2* at neutral pH. Furthermore, the observation of significantly weakened binding of CR8020 to HA2* at low pH indicates a pH-induced conformational rearrangement that removes the binding epitope, as expected if HA2* adopts the postfusion structure after acidification.

CD spectroscopy has been used extensively to probe the secondary structure of proteins,⁴² including previously reported HA2 truncations.^{43–45} CD spectra of HA2* at pH 7 showed that HA2* adopts an α -helical structure with 44% helicity, which is in agreement with the estimated 50% helicity for prefusion HA2 (estimated for the HA2 portion of 1HGF using STRIDE).³⁶ This falls within the range of percent helicity reported in the literature from 24% for a previous study with HA2*¹³ to 64% in a study of a similar HA2 construct.⁴⁵

Native PAGE and SEC show that HA2* exists as a mixture of a monomer, a trimer, and higher-order oligomers at neutral pH. This ratio is likely dependent on protein concentration and may depend on detergent properties and concentration.⁴⁵ The fact that an only minimal amount of dimer is seen with either methodology shows that there is not significant disulfide cross-linking between monomers and the intramolecular disulfide bonds must be properly formed. We performed SEC without cross-linking, which gives a more accurate sampling of the solution phase oligomeric equilibrium. The molecular weights calculated from retention times using the calibration model described are larger than the known values of HA2* oligomers. This discrepancy likely arises from two sources. The first is that there is certainly detergent tightly bound to the protein. The amount bound is difficult to quantify. We show that buffer exchange and dialysis does not eliminate all sarkosyl (Figure S5), and previous SEC work with different HA2 constructs highlights the binding of detergent,⁴⁵ though the number of detergent molecules in this case would have to be very large (~80 per trimer). The second and more likely reason is that the shape of HA2 (and thus HA2*) is rod-like and very different from that of the globular size standards used in SEC calibration. The rod-like structure of HA2* likely changes its retention time on the column compared to a globular protein with a similar MW, leading to an overestimate of its size from the calibration.⁴⁶ In summary, these results show that, while HA2* does form some degree of large aggregates at neutral pH, most is not aggregated, even without SRC. Catastrophic aggregation is observed only when HA2* is acidified, as discussed below.

HA2* Undergoes an Acid-Induced Conformational Change.

Additional evidence against a high-energy metastable HA2* caged by HA1 is the substantial conformational change observed upon acidification of HA2*. If prefusion HA2 is unstable at neutral pH, then there should be no conformational changes upon acidification as HA2 would have already adopted the more stable conformation. The substantial conformational changes reported here, which seem to be related to the release of the fusion peptides, are not consistent with such a model of metastable HA2.

Antibody binding assays show that the pH-dependent conformational change abolishes the ability of CR8020 to bind HA2*, just as in the case of full HA, evidence that the conformational change is likely similar to that experienced by the stalk region in HA. CD spectroscopy shows a change in the 222 nm:208 nm ratio from 0.9 at pH 7 to 1.8 at pH 4 with an inflection point at pH ~5.5 for HA2*. This change is indicative of the formation

of an extended coiled-coil state, with the transition centered around the expected midpoint for HA, providing additional evidence that HA2* undergoes a conformational change like that of HA2 in full length HA. The structural origin of the quenching seen in the Trp fluorescence assays is difficult to assign precisely, but it is consistent with solvent exposure of Trp residues that are at least partially buried initially. This is consistent with the release of the partially shielded fusion peptides, and it is not consistent with the interaction of multiple HA2* trimers through the kinked region, due to the location of the Trp residues. FTIR pH dependence shows that HA2* undergoes a loss of random-coil character as it is acidified. The double difference spectrum shows characteristic bands of β -sheets. While this is not the extended coiled-coil structure expected to be produced in this transition, the presence of β -sheet character is informative. It indicates formation of β -aggregates on the long time scale (minutes) of the FTIR experiment. Aggregation is also noted in other methods used in this study. Aggregation can be indicative of release of the FPs in the absence of a membrane-like environment.

Another possibility is that, upon protonation of the acidic residues, the decreased surface charge, and thus weakened electrostatic repulsion, favors HA2* aggregation. HA2* anchored to a SLB does not form β -aggregates, however, which suggests that the changes are due to exposure of the FPs rather than changes in surface charge. In the ATR experiments, the His residues of the His tag are coordinated to Co and thus not likely protonated even at pH 4. It is possible that this effect is partially responsible for differences between ATR and transmission FTIR results. While different than the natural TMD attachment of HA, anchoring of HA2* to a metal headgroup-containing bilayer is promising for future studies in an environment more like the viral surface.

CD results also indicate a conformational change consistent with the formation of an extended coiled coil rather than aggregation caused by the electrostatic effects of protonation. The loss of signal in the CD measurements suggests some aggregation does occur, but this is a small effect compared to the FTIR results due to the lower HA2* concentration. The temperature-dependent CD data (Figure S3) also show that the transition caused by heating is not the same as the transition caused by acidification; this can clearly be seen in the shape of the spectra. Heating clearly unfolds the protein, producing a disordered conformation, whereas acidification changes the helical signature to one characteristic of an extended helical coiled coil. This provides additional evidence of the specific nature of the acid-induced transition. Taken together, these results suggest that protonation of key residues triggers a conformational change involving the release of the fusion peptides and formation of an extended coiled coil. Without a membrane-like environment, and at the high protein concentrations required for FTIR measurements, the fusion peptides interact and seed aggregation due to their hydrophobic character.

One Component of the HA2* Conformational Change Is Fast.

The stopped-flow pH jump kinetics also provide evidence that the change induced in HA2* is more complicated than simple aggregation. Stopped-flow Trp fluorescence has been used to study HA dynamics previously, but these studies did not have access to improved time resolution and dead times.⁴⁷ For HA2*, biphasic kinetics are observed, and the fast phase

has a time constant of 270 μ s. This is certainly too fast for any aggregation event. To the best of our knowledge, this is the first report of any dynamics on a time scale that is this fast for any component of HA. There are also likely faster events that cannot be captured with stopped-flow methodology. Capturing such dynamics, which could lead to improved understanding of the mechanism of HA2, will require a method like laser-induced pH jump spectroscopy. HA2* is an ideal candidate for studies with these methods. The FTIR phototitration studies lay the groundwork for pH jump IR studies,^{22,48,49} and the four intrinsic Trp residues make it a candidate for pH jump fluorescence.⁵⁰

CONCLUSIONS

In this study, we report results suggesting that HA2 is not metastable at neutral pH, which is not in agreement with the long-held spring-loaded model. We recombinantly express HA2* in *E. coli*, a truncation of HA2 from a H3N2 virus that lacks the HA1 and transmembrane domains. Through a combination of biochemical and spectroscopic methodologies, we show that HA2* at neutral pH is in a conformation that is consistent with prefusion HA2. We also show that, upon acidification, HA2 undergoes a conformational change that is consistent with what is known about the transition of HA2 in full length HA. Specifically, we show that this conformational change results in the formation of an extended coiled coil and that this conformational change is coupled with the release of fusion peptides from a partially protected state. These results for HA2* provide new insight into the mechanism of acid-induced refolding of HA2 but may partially arise from the specific conditions of the experiments. Further studies under more biologically relevant conditions and kinetic studies investigating faster time scales are essential to better understand the mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

HA	hemagglutinin
FP	fusion peptide
TMD	transmembrane domain
DM	<i>n</i> -decyl β -D-maltopyranoside

CD	circular dichroism
FTIR	Fourier transform infrared
ATR-FTIR	attenuated total reflectance Fourier transform infrared spectroscopy
SLB	supported lipid bilayer

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Figure 1. HA2* model system. (A) Regions of HA present in the HA2* truncation. The prefusion structure is on the left, and the postfusion structure is on the right. PDB entries 1HGF²³ for prefusion and 1QU1²⁴ for postfusion. (B) Legend for the HA2* structure and sequence. The four Trp residues relevant for fluorescence studies are highlighted in red (W14, W21, W99, and W185). (C) Sequence of HA2*. HA2* is the first 185 residues of HA2 with a five-residue linker and a hexa-His tag.

Figure 2. Determination of the size of HA2* and oligomers. (A) SDS-PAGE gel of HA2*. (B) Native PAGE gel of HA2*. (C) Size exclusion chromatogram of HA2 at pH 7. (D) Calibration curve of size exclusion chromatography using three standards, BSA (64 kDa), myoglobin (16.7 kDa), and vitamin B₁₂ (1.3 kDa).

Figure 3. Antibody binding assay. The absorbance at 450 nm detects the diimine TMB oxidation product. Samples labeled + have HA2* present and samples labeled – are negative controls without HA2*. Data are the average of three wells. Error bars are one standard deviation.

Figure 4. Equilibrium spectroscopic characterization of HA2*. (A) pH-dependent CD spectra of HA2*. (B) pH-dependent 222 nm:208 nm ratio showing formation of a helical coiled coil (ratio of >1.1) at low pH. The solid curve is a fit to a two-state transition with a midpoint of pH 5.5. (C) pH-dependent tryptophan fluorescence spectra of HA2*. (D) pH-dependent change in the Trp emission intensity of HA2*. Data are not well fit to a two-state model (—).

Figure 5.

FTIR phototitration of HA2*. (A) Preillumination FTIR absorption spectra of saturated *o*-NBA (black) and HA2* in saturated *o*-NBA (red). (B) FTIR difference spectra of HA2* with *o*-NBA illuminated over time. Difference spectra represent the spectrum at the indicated illumination time minus the initial (dark) spectrum. (C) FTIR difference spectra of saturated *o*-NBA (black) and HA2* with *o*-NBA (red) illuminated for 25 s. The magnitude of the NO₂ bleach is approximately the same. (D) Double difference spectrum of spectra from panel C (HA2* minus *o*-NBA). The spectra are scaled so that the magnitude of the NO₂ bleach matches precisely before subtraction. Assignments: (a) random-coil bleach, (b) β-sheet, (c) COO⁻ bleach, and (d) COOH.

Figure 6.

ATR-FTIR of HA2* on a SLB. Relevant spectral features are labeled according to their assignments: (1) water bleach (OH str), (2) phosphate bands, (3) lipid tail bands (CH str), (4) lipid fatty acid headgroups (C=O str), (5) NTA headgroups, (6) amide I, (7) amide II, (8) $\nu(\text{C}=\text{O})$ COOH side chains, and (9) ν_{as} COO⁻ side chains. The letters in parentheses denote the label of the corresponding feature in Figure 5D. (A) Formation of the SLB and assessment of SLB coverage. (B) Binding of HA2* to Co²⁺ headgroups and oxidation of headgroups to Co³⁺. (C) pH-induced change in HA2* conformation. (D) Difference spectrum (pH 4.0 – pH 7.4).

Figure 7. Stopped-flow fluorescence pH jump. Integrated Trp fluorescence response ($\lambda_{\text{ex}} = 290 \text{ nm}$; $\lambda_{\text{em}} = 340 \text{ nm}$) of HA2* to a mixing-induced jump from pH 7.4 to 4.0 (red trace). The data are an average of 16 shots, and a boxcar average is applied in the time domain. A double-exponential fit is shown in black with the following parameters: $y_0 = 0.812 \pm 0.008$, $A_1 = 0.159 \pm 0.007$, $\tau_1 = 270 \pm 20 \mu\text{s}$, $A_2 = 0.054 \pm 0.007$, and $\tau_2 = 250 \pm 50 \text{ ms}$.