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A soluble domain of the membrane-anchoring chain of influenza virus hemagglutinin (HA_2) folds in *Escherichia coli* into the low-pH-induced conformation

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ABSTRACT **The extensive refolding of the membrane-anchoring chain of hemagglutinin (HA) of influenza virus (termed HA_2) in cellular endosomes, which initiates viral entry by membrane fusion, suggests that viral HA is metastable. HA_2 polypeptide residues 38–175 expressed in *Escherichia coli* are reported here to fold *in vivo* into a soluble trimer. The structure appears to be the same as the low-pH-induced conformation of viral HA_2 by α-helical content, thermodynamic stability, protease dissection, electron microscopy, and antibody binding. These results provide evidence that the structure of the low-pH-induced fold of viral HA_2 ($TBHA_2$) observed crystallographically is the lowest-energy-state fold of the HA_2 polypeptide. They indicate that the HA_2 conformation in viral HA before low pH activation of its fusion potential is metastable and suggest that removal of the receptor-binding chain (HA_1) is enough to allow HA_2 to adopt the stable state. Further, they provide direct evidence that low pH is not required to form the membrane-fusion conformation but acts to make this state kinetically accessible in viral HA.**

Infection by enveloped viruses proceeds by fusion of viral and cellular membranes that transfers the genetic material of the virus into the cell. The influenza virus hemagglutinin (HA), a homotrimer of the receptor-binding (HA_1) and the membrane-anchoring (HA_2) polypeptide chains, first binds the virus to a sialylated cell-surface receptor after which it is internalized by endocytosis. At the low pH of endosomes, between pH 5 and pH 6, the membrane fusion potential of the HA is activated (1–3). This activation exposes the hydrophobic N terminus of HA_2, called the fusion peptide (4, 5). *In vitro* the conformational change has been induced both by low pH and by high temperature (4, 6). Digestion of native HA with bromelain produces a soluble molecule, BHA, by removing the HA_2 C-terminal membrane anchor (7, 8). Tryptic digestion of BHA in the fusion pH conformation to remove the HA_1 domains (4, 9, 10) and thermolysin digestion to remove the fusion peptide (9, 11) produced a soluble trimeric fragment of HA in the low-pH-induced conformation called $TBHA_2$ (9, 11). The three-dimensional structure of $TBHA_2$, which is composed of HA_2 residues 38–175 [HA_2-(38–175)] disulfide bonded to HA_1 residues 1–27 [HA_1-(1–27)], was determined by x-ray crystallography and has a triple-stranded α-helical coiled coil from residues 38 to 105, a turn around residue 107, and a small domain composed of a short α-helix and three strands of β-sheet (including HA_1 residues 12–16) packed back against the lower part of the coiled coil (12).

The observed irreversibility of the fusion-pH-induced conformational change (4) and the increased thermostability of $TBHA_2$ compared to HA even at neutral pH (9) suggested that the low-pH-induced structure is more thermodynamically stable than the neutral structure [i.e., that the neutral pH structure of HA is metastable (6, 12, 13)]. The simplicity of the long α-helical coiled-coil fold of $TBHA_2$ (12), the finding that synthetic segments of the coiled-coil region form α-helical trimers in solution (13), and the observation that $TBHA_2$ was more thermodynamically stable than HA suggested that a soluble fragment of HA_2 would fold in *Escherichia coli* or could be refolded from denaturants, although neither have been accomplished for the HA molecule.

In this paper, we describe that the recombinant HA_2-(38–175), which corresponds to the HA_2 portion of $TBHA_2$, folds *in vivo* into the low-pH-induced conformation of viral HA_2. In contrast to short synthetic trimeric coiled-coil peptides (14), this triple-stranded coiled-coil-containing structure, like $TBHA_2$, does not bind to lipid vesicles. These results provide evidence that the structure of the low-pH-induced fold of HA_2 ($TBHA_2$) observed crystallographically is the lowest-energy-state fold of the HA_2 polypeptide in the absence of HA_1.

METHODS

Expression and Purification. DNA encoding HA_2-(38–175) was cloned into a plasmid with a T7 promoter and expressed in *E. coli* [BL21 (DE3)]. Dramatic improvement in expression was observed when three rare Arg codons (Arg-123, -124, and -127) were changed from AGG to CGT. DNA sequence was confirmed on both strands. Cys-137, which forms a disulfide bond with HA_1 residue 14 (15, 16), was changed to Ser for expression, but the two cysteines (Cys-144 and Cys-148) that form a disulfide bond within HA_2 were retained. The 100,000 × *g* supernatant from lysed *E. coli* was heated (68°C, 45 min) and cooled to 20°C. A massive precipitant was removed by centrifugation (100,000 × *g*, 60 min), and then 0.5% polyethylenimine (Sigma) was added to precipitate nucleic acids. The protein was precipitated with 50% saturated ammonium sulfate (30 min, 20°C), collected by centrifugation (10,000 × *g*, 15 min), and resuspended (50 mM Hepes, pH 7.5/1 M NaCl). *E. coli*-expressed HA_2 residues 38–175 ($EBHA_2$) was eluted as a single peak on size exclusion chromatography (Superdex 200). Crystals of $EBHA_2$ have been observed from pH 4.6 to 7.5 by using 2-propanol and 2-methyl-2,4-propanediol as precipitants.

Crosslinking. $EBHA_2$ (0.54 mg/ml in Hepes) was crosslinked with bis(sulfosuccinimidyl)suberate (BS^3) (Pierce) at room temperature for 30 min and quenched by adding

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Abbreviations: HA, hemagglutinin; HA_1, the receptor-binding chain of HA; HA_2, the membrane anchoring chain of HA; BHA, bromelain-produced soluble HA fragment; $TBHA_2$, a proteolytic fragment of the low-pH-induced conformation of HA; $EBHA_2$, *E. coli*-expressed HA_2 residues 38–175; EM, electron micrograph.

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Tris·HCl (pH 6.8) to 20 mM. BS^3 concentrations used ranged from 0 to 50 mM.

Ellman's Assay. $EBHA_2$ (19.5 μM in 6 M guanidium hydrochloride/0.1 M sodium phosphate, pH 7.3) was reduced with 20 mM dithiothreitol for 20 min at 20°C followed by dialysis against 0.1 M sodium phosphate (pH 7.3) containing 6 M guanidium hydrochloride, 1 mM EDTA, and 0.1 M acetic acid for 24 h. Ellman's reagent [3 mM in 0.1 M sodium phosphate (pH 7.3)] was added in excess (50 equivalents) to reduced $EBHA_2$ (9.7 μM in dialyzed buffer) or nonreduced $EBHA_2$ [6.3 μM in 6 M guanidium hydrochloride/0.1 M sodium phosphate (pH 7.3)]. The number of free thiol groups was calculated from the absorbance at 412 nm and a calibration curve, which was calculated from standard glutathione solutions in the same buffer.

CD Spectra. CD spectrum of $EBHA_2$ (107 μg/ml) in 250 mM KF (pH 7.5), 25°C, 1-mm cell, was obtained with an Aviv Associates (Lakewood, NJ) 62DS spectropolarimeter by averaging three measurements. Protein concentration was calculated from absorbance at 280 and 288 nm in 6 M guanidine hydrochloride (17).

Lipid Binding. Lipid binding was assessed by mixing 20 μg of $TBHA_2$ or 200 μg of $EBHA_2$ with 850 μg of liposomes (as in ref. 18, plus 0.1 mol % gangliosides) at pH 5.0 or 7.0. Incubation for 30 min at 37°C was followed by addition of sucrose to make 30% (wt/vol). A discontinuous gradient was formed with the 30% sucrose sample at the bottom, overlaid by 10% sucrose, overlaid by PBS, and centrifuged at 250,000 $\times$ *g* for 1 h at 20°C. Samples shown in Fig. 3*A* were taken from the bottom soluble fraction and the top lipid vesicle fraction and examined by SDS/PAGE and immunoblot analysis with a polyclonal anti-BHA serum (R186) detected by ECL (Amersham).

Liposome Association. BHA associated with liposomes after low pH treatment was digested first with trypsin and then at pH 6.0 or pH 5.0 for 2 h at 37°C with thermolysin (figure 4 of ref. 18). Samples were incubated further at pH 6.0, 5.0, and 4.0 for 1 h then centrifuged (100,000 $\times$ *g*, 30 min, 4°C) to pellet the lipid vesicles.

RESULTS

Recombinant HA_2 Portion of $TBHA_2$ Is a Stable Trimer. We expressed HA_2-(38–175) plus an N-terminal Met in *E. coli* (named $EBHA_2$) and found that it folded into a soluble trimer (Fig. 1). The $EBHA_2$ polypeptide (Fig. 1*A*, lane 1) was purified from the soluble fraction of lysates of transfected bacteria by heat treatment at 68°C (pH 7.5), taking advantage of the expected thermostability of the low-pH-induced conformation (6). Evidence that the one disulfide bond, between residues 144 and 148, had formed was provided by a difference in mobility between reduced and unreduced $EBHA_2$ on SDS/PAGE and by measuring the number of free Cys residues in denatured $EBHA_2$ with Ellman's reagent. $EBHA_2$ has the apparent molecular mass of a trimer, 48 kDa, measured by gel filtration (data not shown), and shows the three bands expected for a trimer when crosslinked chemically (Fig. 1*A*, lanes 1–9), like viral $TBHA_2$ (Fig. 1*A*, lane 10).

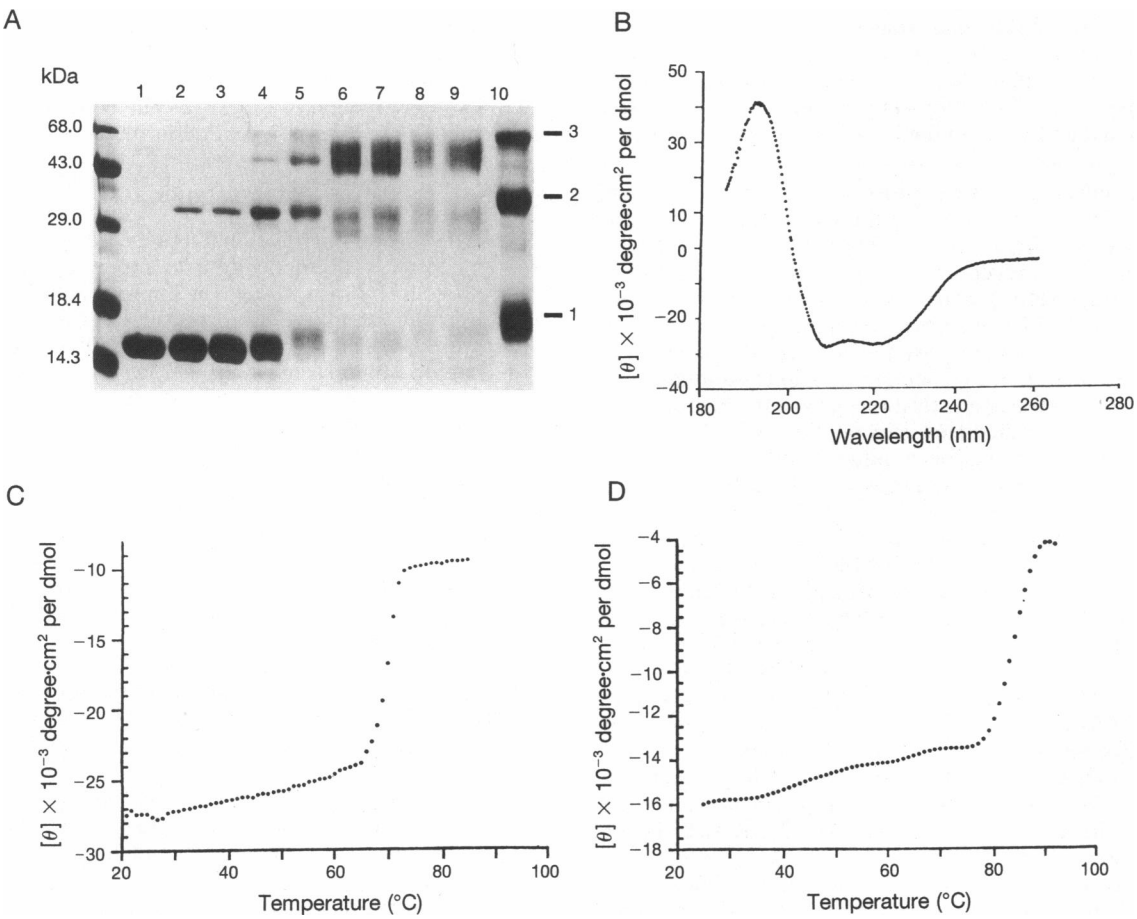

FIG. 1. (*A*) SDS/PAGE (12% gel) of chemically crosslinked $EBHA_2$. Lanes: 1–9, $EBHA_2$ with crosslinking reagent 0, 0.01, 0.02, 0.05, 0.2, 10, 20, 40, and 50 mM, respectively; 10, $TBHA_2$ crosslinked with 5 mM bis(sulfosuccinimidyl) suberate; bands 1–3 correspond to the monomer, dimer, and trimer, respectively. (*B*) CD spectrum of $EBHA_2$. Minima at 208 and 222 nm are characteristics of α-helices. (*C*) Thermal denaturation curve of $EBHA_2$ at pH 7.2. (*D*) Thermal denaturation curve of $EBHA_2$ at pH 5.2. The curves were obtained by monitoring the CD signal at 222 nm with a scan rate of 0.7°C per min.

CD and thermal stability measurements indicate that $EBHA_2$ has the high α-helical content and high thermal stability characteristic of the α-helical coiled coil in $TBHA_2$ (6, 9, 13) (Fig. 1*B*). Thermal denaturation of $EBHA_2$ was observed by monitoring the changes in CD at 222 nm. At pH 7.2, a single unfolding transition was observed at melting temperature (T_m) $\approx$ 70°C (Fig. 1*C*). This is comparable to the unfolding transition of $TBHA_2$ (T_m = 76°C), which contains in addition to HA_2-(38–175), one oligosaccharide on HA_2 and 27 residues with two oligosaccharides of HA_1 (9, 12, 16). $TBHA_2$ (9) and low-pH-treated BHA (6) are more stable to thermal denaturation at pH 5.0 than pH 7.0 by at least 19°C. Similarly, $EBHA_2$ denatures at a temperature 14°C higher at pH 5.2 (T_m = 84°C) than at pH 7.2 (Fig. 1*D*). Thus the secondary structure as measured by CD, the thermal stability, and the pH dependence of the unfolding transitions of $EBHA_2$ folded in *E. coli* are similar to those of $TBHA_2$ produced proteolytically from low-pH-treated viral HA.

Structure Features of $EBHA_2$ Are the Same as $TBHA_2$. Two other specific features of the $TBHA_2$ structure (12) can be probed biochemically, one proteolytically and the other with specific antibodies (18). A number of proteases when incubated with $TBHA_2$ at pH 7.0 yield a stable 10-kDa fragment. Thermolysin incubated with $TBHA_2$ yields a 10-kDa fragment that N-terminal sequencing and mass spectrometry identify as residues 38–125; trypsin yields fragments containing residues 40–124 and 40–127 (18) (Fig. 2*A*). Tryptic digestion of $EBHA_2$ under similar conditions produces the identical $\approx$10-kDa fragments (Fig. 2*A*), which N-terminal sequencing and mass spectrometry also identify as residues 40–124 and 40–127. The production of similar 10-kDa fragments after digestion of both $TBHA_2$ and $EBHA_2$ with a variety of proteases (trypsin, thermolysin, or bromelain; data not shown) argues that cleavage at these positions is not simply a function of the amino acid sequence but represents digestion of an exposed region on both structures, the end of the short α-helix (residues 113–128), that yields a stable substructure, the helical hairpin: helix (residues 40–105), turn (residues 106–112), and helix (residues 113–128). An intermediate fragment of $\approx$12 kDa observed as a trypsin digestion product of $EBHA_2$ (Fig. 2*A*) was identified as residues 40–139 by mass spectrometry and N-terminal sequencing. Residue 139 is at the end of the last β-strand in $TBHA_2$, so that the observed cleavage would remove an unusual extended and partially disordered segment of $TBHA_2$ (12).

Two monoclonal antibodies have been described (18) that recognize HA in the low-pH-induced form but not in the native fusion-inactive conformation. One, LC89, which exhibits binding that is sensitive to an amino acid substitution at HA_2 residue 107 recognizes the turn (residues 106–112) between the long and short α-helices of $TBHA_2$ (18). The other, IIF4, recognizes $TBHA_2$ (residues 38–175) but not the 10-kDa tryptic (residues 40–124/127) or thermolytic products (18). Both of these antisera were shown by ELISA to bind equally to $EBHA_2$ and $TBHA_2$ (Fig. 2*B*). Thus, $EBHA_2$ appears to have the low-pH-induced conformation of both $TBHA_2$ and low-pH-treated HA including the turn at residue 107.

In EMs (Fig. 2 *C* and *D*), $EBHA_2$ and $TBHA_2$ are indistinguishable long rods (the coiled coil) with one terminal knob (the β-sheet and short α-helix domain) (see also refs. 9 and 18). EMs of $EBHA_2$ complexed with antibody IIF4 show $EBHA_2$ rods with antibodies bound to one end as observed with $TBHA_2$ (18).

Neither $EBHA_2$ nor $TBHA_2$ Binds Lipid Vesicles. It has been suggested that the coiled-coil portion of the low-pH-induced conformation of the HA may dissociate in the presence of lipid vesicles and insert into the lipids (14), from electron paramagnetic resonance studies of a synthetic peptide of 40 residues

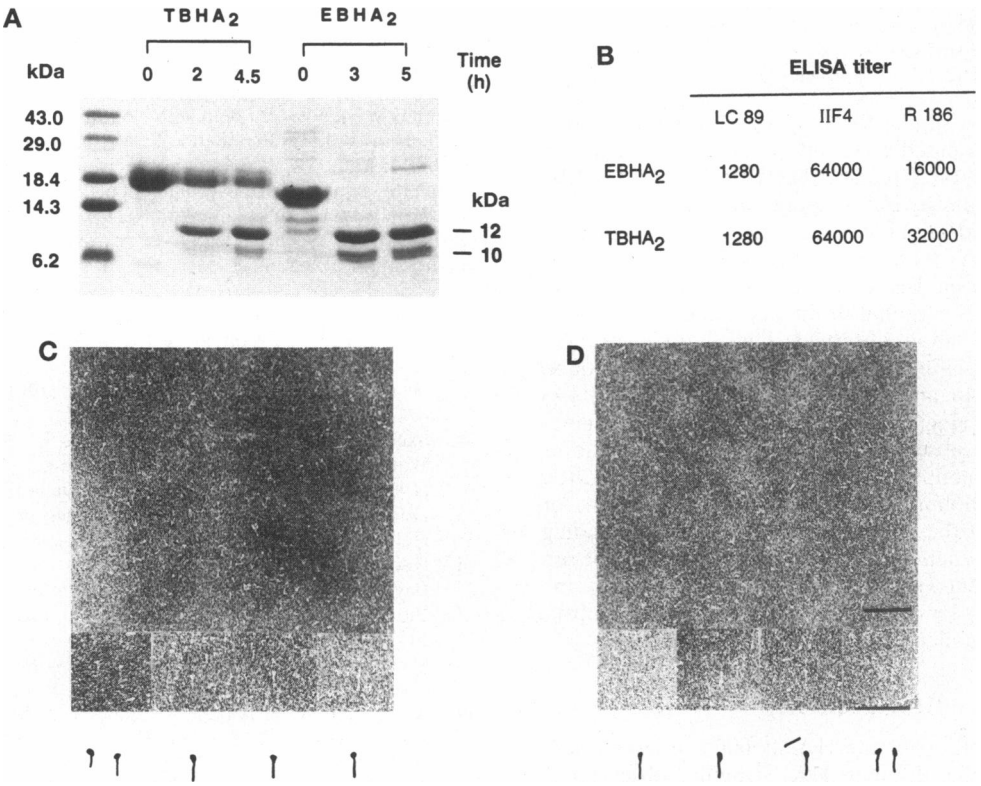

FIG. 2. Proteolysis products, antibody binding, and EM images are similar for $EBHA_2$ and $TBHA_2$. (*A*) Trypsin treatment of $TBHA_2$ and $EBHA_2$ at pH 7.0 showing three products, one at $\approx$12 kDa and two near 10 kDa. (*B*) ELISA titers of $EBHA_2$ and $TBHA_2$ with monoclonal antibodies (LC89 and IIF4) raised against low-pH-treated HA and a polyclonal rabbit antisera (R186) that binds equally well to native and low-pH-induced HA. R186 is used here as a control to be sure $EBHA_2$ and $TBHA_2$ bound to ELISA plates comparably. Electron micrographs (EMs) of $EBHA_2$ (*C*) and $TBHA_2$ (*D*) showing long rods with a knob visible at one end on some molecules. Samples were prepared for EMs as in ref. 18.

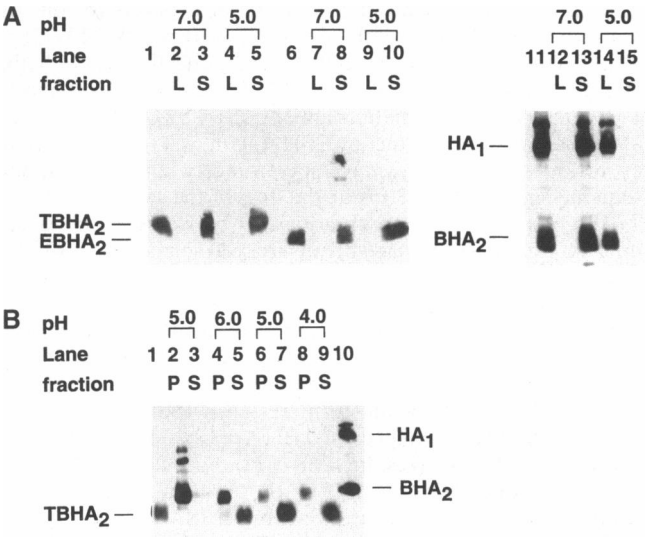

FIG. 3. Neither $EBHA_2$ nor $TBHA_2$ bind lipid vesicles. (*A*) SDS/PAGE of $TBHA_2$ (lanes 2–5), $EBHA_2$ (lanes 7–10), and BHA (lanes 12–15) after incubation with lipid vesicles (23) at the pH shown and separated from the lipid vesicles fraction (L) by flotation in a sucrose gradient from the soluble (not bound to lipid) fraction (S). $TBHA_2$ and $EBHA_2$ are found exclusively unbound (S) to lipid (lanes 3, 5, 8, and 10) at pH 7.0 and pH 5.0. BHA is unbound to lipid at pH 7.0 (lane 13) but binds to lipid vesicles as expected at pH 5.0 (lane 14). Lanes 1, 6, and 11 are untreated protein controls. (*B*) SDS/PAGE of the supernatants (S) and lipid vesicle pellets (P) after incubation of BHA_2 with liposomes at low pH and proteolysis to remove the HA_2 N terminus. Lanes: 1, $TBHA_2$ marker; 2 and 3, low-pH-treated BHA pelleted bound to the lipid vesicles; 4–9, results of thermolysin digests at pH 6 (lanes 4 and 5) and pH 5 (lanes 6–9) of vesicles prepared as in lane 2 [and subsequently incubated at pH 6 (lanes 4 and 5), pH 5 (lanes 6 and 7), and pH 4 (lanes 8 and 9)]. In each case, $TBHA_2$ resulting from thermolytic digestion is found in the supernatant (lanes 5, 7, and 9) and not bound to the lipid vesicles (lanes 4, 6, and 8). [Some undigested BHA_2 remains associated with the vesicles (lanes 4, 6, and 8).]

[HA_2-(54–93)] that is similar to a 36-residue synthetic peptide that forms triple-stranded α-helical coiled coils in solution (13). Fig. 3 demonstrates that neither $EBHA_2$ nor $TBHA_2$, both of which contain the HA_2 segment of residues 54–93 of the coiled-coil, binds lipid vesicles. Neither by incubating $EBHA_2$ and $TBHA_2$ with lipid vesicles (Fig. 3*A*) nor by creating $TBHA_2$ from BHA already associated with lipid vesicles through its N-terminal fusion peptide (Fig. 3*B*) (4, 9, 11) was lipid association observed. Under both of these conditions the low-pH-induced conformation of BHA bound lipid (Fig. 3 *A*, lane 14, and *B*, lanes 2, 4, 6, and 8) but $EBHA_2$ and $TBHA_2$ did not (Fig. 3 *A*, lanes 2, 4, 7, and 9, and *B*, lanes 5, 7, and 9). These observations are consistent with earlier indications that the nonpolar N-terminal of HA_2 was required for interaction with lipids (4, 9, 11). The implication for membrane fusion of the pH dependence of the lipid binding reported for the synthetic model coiled coils (figure 4 of ref. 14) is also inconsistent with earlier observations of membrane fusion at higher pHs by mutant HAs with amino acid substitutions outside the coiled-coil region (5, 19–22).

DISCUSSION

The spontaneous folding of the HA_2 domain observed here demonstrates that the alternate HA_2 structure observed in viral HA (16) is metastable. Viral HA (HA_1 + HA_2) is synthesized as a single-chain precursor HA_0, whose three-dimensional structure must predispose HA_2 to adopt the metastable HA_2 conformation after proteolytic cleavage to HA_1 and HA_2 chains. Comparing the three-dimensional structures of BHA and $TBHA_2$ suggests that the HA_1 polypeptide has kinetically trapped the metastable HA_2 fold through extensive contacts with parts of HA_2 that must refold to achieve the stable state (12). Formation of the stable HA_2 structure directly in *E. coli* in the absence of the HA_1 sequence, as we have observed, suggests that removal of HA_1 from viral HA would permit folding to the stable HA_2 state. This conclusion is unlikely to be affected by the presence or absence of the N-terminal fusion peptide of HA_2 because EM observations (9, 18) and thermodynamic measurements (6, 9) indicate that the same rod-like structure forms and is more stable than the metastable HA_2 structure in the presence and absence of the fusion peptide. A shorter HA_2 fragment, residues 38–125 ($EBHA_2$ is residues 38–175), produced by proteolysis of viral HA (18) or expressed in *E. coli* (C. Carr and P. Kim, personal communication) also forms a stable triple-stranded oligomer.

Because the folding observed here occurs at neutral pH, it is likely that the biological requirement of low endosomal pH for the HA function in influenza virus infection (1–3) is not to stabilize a fusion-active HA conformation or to promote a pH-dependent step in bilayer fusion but is only required to remove the kinetic constraints preventing the formation of the fusion-pH structure. This suggests the possibility that if the membrane anchor and fusion peptides normally associated with the ends of HA_2 could be restored to $EBHA_2$, then it would be fusogenic at neutral pH. Expression of a soluble domain, devoid of the nonpolar fusion peptide and membrane anchor peptides, may be a general method for studying the structure of membrane fusion proteins from other viruses (and cells), especially where the mechanism by which the fusion active conformation is achieved is less well characterized (as in paramyxoviruses and retroviruses) or when the molecule is not amenable to proteolytic dissection.

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