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# Modulation of the pH Stability of Influenza Virus Hemagglutinin: A Host Cell Adaptation Strategy

Santiago Di Lella,[1,2] Andreas Herrmann,[1] and Caroline M. Mair[1,*]
[1]Institute of Biology, Humboldt Universität zu Berlin, Berlin, Germany; and [2]Departamento de Química Biológica e IQUIBICEN-CONICET, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad de Buenos Aires, Argentina

ABSTRACT Proteins undergo dynamic structural changes to function within the range of physical and chemical conditions of their microenvironments. Changes in these environments affect their activity unless the respective mutations preserve their proper function. Here, we examine the influenza A virus spike protein hemagglutinin (HA), which undergoes a dynamic conformational change that is essential to the viral life cycle and is dependent on endosomal pH. Since the cells of different potential hosts exhibit different levels of pH, the virus can only cross species barriers if HA undergoes mutations that still permit the structural change to occur. This key event occurs after influenza A enters the host cell via the endocytic route, during its intracellular transport inside endosomes. The acidic pH inside these vesicles triggers a major structural transition of HA that induces fusion of the viral envelope and the endosomal membrane, and permits the release of the viral genome. HA experiences specific mutations that alter its pH stability and allow the conformational changes required for fusion in different hosts, despite the differences in the degree of acidification of their endosomes. Experimental and theoretical studies over the past few years have provided detailed insights into the structural aspects of the mutational changes that alter its susceptibility to different pH thresholds. We will illustrate how such mutations modify the protein's structure and consequently its pH stability. These changes make HA an excellent model of the way subtle structural modifications affect a protein's stability and enable it to function in diverse environments.

Hemagglutinin (HA) is the major spike protein of influenza A viruses and is the most abundant antigen on the viral surface. The natural reservoir of influenza is aquatic wild birds, in which 16 serotypes of HA have been identified, and two additional serotypes (H17 and H18) have been discovered in viruses isolated from bats (1,2). Only three of these 18 serotypes (H1, H2, and H3) have managed to adapt to human hosts, with devastating consequences. They are responsible for the global pandemics in 1918, 1957, and 1968, and are also the cause of seasonal flu outbreaks. Their continuous circulation in the human population has raised major concerns, particularly in light of repeated human infections with avian influenza A viruses of other subtypes, including H5, H7, and H9. These factors and specific aspects of influenza virus biology suggest that another pandemic strain may well lie on the not-too-distant horizon.

A number of barriers normally prevent viruses from jumping between species. However, influenza has evolved efficient strategies to overcome these obstacles. One occurs when different viral strains coinfect the same host. The genomes of each strain consist of eight RNA segments that can mix with each other in a process called antigenic shift, producing entirely new influenza virus strains with unique compositions of segments. Another mechanism, antigenic drift, is the result of mutations that viral proteins undergo during the course of replication. This can alter their properties in ways that permit them to function despite differences in the chemical environments of the cells of a new host. Often antigenic shift and drift combine to produce new, highly potent forms of influenza. This was the case in the pandemic of 2009, for example, when a new form of H1N1 arose from a mixing of the genomes of several strains and mutations in viral proteins (3). The highly pathogenic H5N1 strain, on the other hand, was largely the product of mutational adaptations that took place in wild birds and poultry, creating a form that could be directly transmitted from birds to humans. Antigenic drift was also responsible for the H1N1 virus pandemic in 1918 (4).

We still lack a full understanding of the properties of viral proteins that support the processes of infection, replication, and successful establishment of a given virus in a specific host, as well as the types of changes they must undergo to permit their spread to new species. For influenza A, the spike protein HA has two pivotal functions in the viral life cycle. First, it attaches the virus to the cell it will infect. After the uptake of the virus via the endocytic pathway, HA mediates the fusion of viral and endosomal membranes,

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*Correspondence: carolinemair001@gmail.com
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which permits the release of viral genome segments (5,6). At the cell surface, the protein binds to sialic acid (SA) receptors of the host cell, preferably to either the avian $\alpha$-2,3-conjugated SA ($\alpha$-2,3-SA) or human $\alpha$-2,6-conjugated SA ($\alpha$-2,6-SA) form (7). Which form it prefers depends mostly on the amino acid composition of the site within HA that binds to the receptor. In several subtypes, even one mutation has proven to be sufficient to switch the receptor specificity from $\alpha$-2,3-SA to $\alpha$-2,6-SA (8–12). After binding and endocytosis have occurred, the virus is transported inside endosomal vesicles toward the nucleus. The environment inside late endosomes has a mildly acidic pH, causing protonation and inducing a major structural rearrangement of HA (Fig. 1). This triggers the merging of the viral and endosomal membranes and causes the release of the viral genome into the cytoplasm.

HA is a typical class I fusion protein in which acid-induced refolding is irreversible (6,13,14). This means that after it is exposed to an acid, it undergoes a conformational change even in the absence of a target membrane, but this leads to viral inactivation. The role of the acid stability of HA in viral replication, pathogenesis, and transmissibility has only recently been appreciated, and it is now seen as a key determinant of host adaptation and a major potential therapeutic target (15).

Here, we focus specifically on the membrane fusion function of HA during adaptation to a specific new host. We review recent ideas regarding two major issues: the sequence of structural rearrangements that the protein undergoes as it induces fusion, and the amino acids that are involved in pH sensing. The stability of HA under different acid conditions is determined by ionizable residues and their interactions. We show how single adaptive mutations in HA affect these interactions. As a specific example, we examine the relevance of these mutations with regard to the genesis of the human pathogenicity of H5 and 2009 pandemic H1 subtypes. Gaining a precise understanding of the mechanisms that permit HA to adapt to different hosts may be an important step toward developing inhibitors of the viral life cycle and finally eradicating pandemics caused by influenza.

## Structure of HA at neutral and low pH

Crystal structures of HA have been solved at neutral and low pH, yielding pre- and postfusion structures of the protein (16,17). At neutral pH, HA is a 135-Å-long, homotrimeric, integral membrane glycoprotein (6,16). Each monomer consists of two subunits linked by two disulfide bonds. Subunit HA1, the globular receptor binding domain, is responsible for recognizing and docking to the host cell through interactions with SA-containing glycans. HA2, which connects the HA protein to the viral membrane via its transmembrane domain (TMD), is the fusion-mediating domain (Fig. 1). HA1 is mainly composed of antiparallel $\beta$-sheets and harbors the receptor binding site (RBS) and major epitopes for neutralizing antibodies (18). The dominant feature of the stalk-like HA2 domain is three long, parallel $\alpha$-helices (helices C, 76 Å), one from each monomer, which associate to form a triple-stranded coiled-coil. Each of these helices is connected by a flexible region at position 59–76, the so-called B-loop, to a shorter $\alpha$-helix (helix A) that extends distally from the membrane (16,19).

The two subunits HA1 and HA2 are structurally distinct and are only generated upon cleavage of HA monomers that are initially synthesized as single polypeptide precursors (HA0) in infected cells. After trimerization, glycosylation, and processing of the monomers occur, cleavage into HA1 and HA2 unleashes the highly conserved N-terminus of HA2, the so-called fusion peptide, which is buried in a negatively charged cavity at the oligomeric interface of HA2 (the fusion peptide region) (19,20).

As is typical of class I fusion proteins, the postfusion form of HA2 is a coiled-coil composed of six $\alpha$-helices (a six-helix bundle) with the fusion peptide and the TMD positioned at the same end of the trimer (17). Although the HA1 globular head domains were removed through proteolysis and

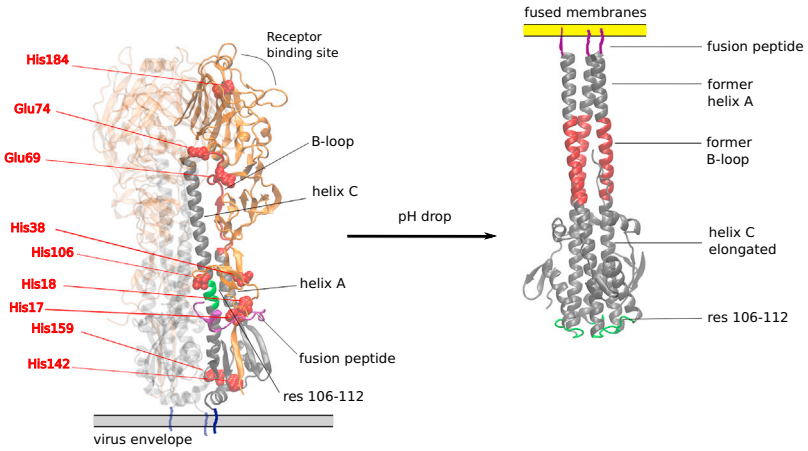


FIGURE 1 Three-dimensional structure of the influenza A HA ectodomain in its prefusion (PDB: 2YPG) and postfusion (PDB: 1HTM) states (H3 subtype). The trimeric quaternary structure of HA is presented in a cartoon representation with the HA1 subunit in orange and the HA2 subunit in gray. The TMD is shown in blue (not part of the crystal structures). The B-loop region (shown in *dark red*) and residues 106–112 of HA2 (displayed in *green*) undergo secondary-structure changes upon a drop in pH, and the fusion peptide (*purple*) is anchored in the fused membrane.

thus were not present in the low-pH crystal structure, they seemed to detach from each other and from the stalk while the overall fold remained intact (20). In the absence of HA1, the postfusion conformation of HA2 is spontaneously adopted even at neutral pH (21). Therefore, in the native prefusion structure, HA1 is thought to clamp onto HA2 and prevent the protein from undergoing a conformational change at neutral pH before the stage of infection (14).

## Low-pH-induced transition from a pre- to postfusion structure

The crystal structures reported for HA, obtained under different conditions, have provided insights into the structural rearrangements of HA1 and HA2 that are involved in the fusogenic activity of the protein, including the 1) HA1-HA1 head dissociation, 2), loop-to-helix transition of the B-loop of HA2, 3) movement of the N-terminal fusion peptide by 100 Å toward the target membrane, 4) collapse of the extended helix at the so-called hinge region (residues 106–111 of HA2), and 5) packing of the C-terminal domain of HA2 as three extended loops against the central coiled-coil (17). In addition, a number of mutational and computational studies have permitted the identification of possible intermediate structures that arise during the course of refolding at low pH. The proposed models are summarized in Fig. 2. Whereas they all describe similar structural rearrangements, the order of these events and the existence of associated intermediate structures are still matters of debate.

Many studies have concluded that the initial event of the HA conformational change that is triggered by low pH is the partial dissociation of HA1 monomers from each other (22,23), as shown in Fig. 2, *A* and *B*. This can be easily rationalized from the high isoelectric point of the HA1 subunit (pI~8) (24). It has been suggested that the protonation of HA1 leads to a significant increase of positive net charge and thus the repulsion of HA1 monomers (25,26). Accordingly, the cross-linking of HA1 monomers by the introduction of three intermonomer disulfide bonds abolishes any conformational change and fusion activity of the protein (23,27). Although Bizebard et al. (28) found that the crystal structure of the majority of HA1 (residues 43–309) was not affected by acidic pH, others detected subtle conformational changes in some portions of HA1 (22,29). This invariance of crystal structures strongly suggests that at least a partial detachment of HA1 monomers is required for the fusion-inducing refolding of HA: first, it enables the formation of a cavity that allows water to interact with the previously shielded HA2 domain (30,31), and second, it provides this domain with a certain flexibility. However, although reconfigurations in HA1 seem to be essential for the conformational change of the protein, they may not directly participate in membrane fusion.

Indeed, electron cryo-microscopy has indicated that a central cavity forms through the entire ectodomain upon acid exposure of the bromelain-cleaved HA (22). It was further suggested that the dissociation of HA1 monomers unleashes HA2, permitting the spring-loaded transition of the unstructured B-loop to an extended $\alpha$-helix, which drives the movement of the fusion peptide toward the target membrane (the spring-loaded model) (14,32). Huang et al. (30,31) proposed that water molecules enter the newly formed cavity and interact with the loop residues, providing the driving force for this transition (the water-driven model). These structural transitions result in the

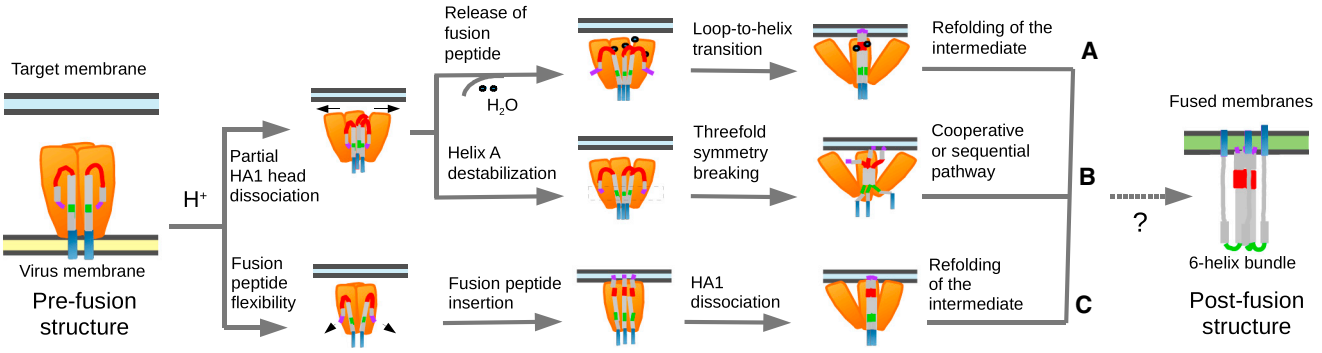


FIGURE 2 Models of multistep HA1 and HA2 refolding upon endosomal acidification. (*A* and *B*) A decrease in pH could lead to a partial dissociation of the HA1 subunit, followed by HA2 refolding via one pathway or a combination of at least two pathways. In all of these models, the first step in the HA2 transition is the release of the fusion peptide. In model (*A*), the protonation of a few residues (25,26) and the specific interaction of incoming water molecules with the B-loop region (30,31) facilitate its transition into a helix (spring-loaded loop transition), which results in the generation of the extended intermediate structure. The subsequent refolding of residues 106–111 into a loop is thought to draw the membranes together, driving membrane fusion and the formation of the postfusion structure. (*B*) A recently proposed two-step mechanism first involves a fast destabilization of the kink at position 106–111, resulting in the release of the fusion peptide (24). The originating quasi-stable asymmetric intermediate then transits into a slow pathway of sequential or cooperative phenomena to reach the postfusion conformation. (*C*) Alternatively, as recently observed by Garcia et al. (34), acidification could lead to an initial increase in the flexibility of the fusion peptide, with no concomitant immediate dissociation of the HA1 subunit, an event that occurs after the spring of the loop into the target membrane.

formation of a needle-shaped structure in which the peptide is inserted into the target membrane, whereas the C-terminus of HA2 remains anchored in the viral envelope (the extended intermediate structure). The collapse of the intermediate structure at the hinge region is thought to subsequently draw viral and endosomal membranes together and induce membrane fusion. In support of these models, Gruenke et al. (33) provided evidence that headgroup separation and fusion peptide exposure precede the formation of a stable coiled-coil, which is required for fusion to progress from target membrane binding to a merging of the membranes.

Results obtained by applying the principles of energy landscape theory to HA2 (24) have recently provided support for an alternative pathway of HA2 refolding through which local disorder in the region around residues 106–111 can drive the release of fusion peptides. Subsequent competition between the formation of the extended coiled-coil and C-terminal melting results in a quasi-stable intermediate that allows either a sequential or cooperative route for the HA2 conformational transition (for details, see (24)). However, further experimental evidence for such a model is necessary.

Another recent study employed hydrogen-deuterium exchange coupled with mass spectrometry (HDX-MS) to observe dynamic changes of HA in response to pH (34). As presented in Fig. 2 *C*, structural changes were mostly observed for the fusion peptide and adjacent regions, whereas the HA1 head domains remained stably associated. These findings imply that fusion peptide exposure and target-membrane binding precede HA1 unclamping and large-scale HA2 refolding (the fusion peptide release model). Support for this scenario comes from previous studies in which fusion was detected before headgroup separation occurred under acidic pH conditions at different temperatures (0°C and 30°C) (35,36). Furthermore, cryo-electron tomography of acid-activated virus particles revealed an outward rotation of the fusion peptides while the HA1 domain remained unchanged (29). A stabilized H2 HA mutant ($R106_2H$) at low pH revealed only subtle changes in the HA1 domain, whereas the B-loops adopted an open-like conformation (20). Biochemical methods have also detected such reversible structural changes before HA1 dissociation (37).

However, as mentioned by Fontana et al. (29), a small reorganization in HA1 might be too subtle to be detected by cryo-electron tomography. In studies employing crystallographic methods, a modest reorganization of HA1 could be observed at low pH, though not in the membrane-distal part as described in the HDX-MS study (20,29,34). Nevertheless, the insertion of the fusion peptides into the target membrane and the formation of a central coiled-coil release a significant amount of energy, indicating that this process is rather irreversible and occurs at a later stage of conformational change, either simultaneously with HA1 detachment or in its aftermath (37).

## Residues that determine the pH-dependent stability of HA

In the biological context of an influenza virus infection, the fusion-inducing conformational change of HA is triggered only upon exposure to the mildly acidic pH inside endosomal vesicles. Key residues at critical interface regions are protonated, weakening the interactions and triggering structural rearrangements that lead to membrane fusion. However, as demonstrated by in vitro assays, the kinetic barrier of this transition can also be overcome by exposure to high temperatures or denaturants such as urea (14). As the transition from the pre- to postfusion form is irreversible and inactivates protein fusion, it provides a readout for acid stability, which can be measured over a range of different temperatures or pH values (38).

Acid stability has been found to vary among HA subtypes (38–40) as well as between viruses of the same subtype (41–43). Based on their primary sequences, HA subtypes have been classified into two groups (group 1: H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18; and group 2: H3, H4, H7, H10, H14, and H15), which have been further divided into four different clades (44). Variations in the presence and location of ionizable residues have been found among the different groups and clades. These might account for differences shown by the subtypes in terms of electrostatic interactions that are relevant to protein stability and the order in which structural rearrangements occur (18,45). Generally, pH sensing has been attributed to histidine residues in class I and class II viral fusion proteins. Histidine is the only amino acid whose protonation state changes in the same range of pH values ($pk_a$~6.5, in solution) in which HA refolding has been found to occur (46). Sensing histidines have been described as being 1) uncharged at neutral pH and 2) colocalized with other histidines or basic residues (Arg or Lys) in the prefusion state. Upon protonation at low pH, the His side chain becomes positively charged and repels other positively charged neighbor residues, leading to a destabilization of the prefusion structure. In the HA protein, the highly conserved histidine residues $His184_1$, $His106_2$, $His142_2$, and $His159_2$ (H3 numbering, where the subindex indicates HA1 or HA2 residues) have been proposed as potential sensors (Fig. 1) (46). Whereas $His184_1$ and $His142_2$ are conserved among all subtypes, $His106_2$ is conserved only within group 2. In group 1, on the other hand, the residue at position 106 is a positively charged amino acid (Arg/Lys). The histidine residue at position 17 in proximity to the fusion peptide is only conserved within group 2. In group 1, a tyrosine is located at that position; this establishes two stabilizing interactions with residues of the

fusion peptide and thus eliminates its potential use as a pH sensor. Instead, to account for the missing histidines at HA1 position 17 and HA2 position 106, histidines at positions 18, 38 (HA1), and 111 (HA2) in group 1 were suggested (18,26,47,48). It is thus not surprising that site-directed mutagenesis of residues at these critical positions or of their interaction partners affects the acid stability of HA differently depending on the subtype and the amino acid replacement, as demonstrated by a number of mutational studies (20,47,49,50). For example, Arg/Lys$106_2$ has been found to co-occur only with Glu$105_2$ or Asp$105_2$ in group 1 HAs, whereas the less basic His$106_2$ in group 2 HAs is always matched with a glutamine at position 105. The mutation of any of these residues shifts the threshold of fusion activation away from the normal fusogenic pH, indicating that a mismatch of the two residues disrupts the precise balance of charged residues in the fusion peptide region (20).

A recent comprehensive experimental and computational study (51) demonstrated that not all amino acid substitutions for the highly conserved His$184_1$ at the membrane-distal HA1-HA1 interface abrogate the conformational change of H5 HA and its ability to induce fusion (Fig. 3). The results suggest that His$184_1$ participates in triggering the partial dissociation of HA1 head domains at low pH. A possible pH-regulatory role has been ascribed to other charged residues in the vicinity, including Arg$220_1$ and Arg$229_1$ (36,51). Additionally, a recent computational study suggested that the head must partially dissociate to permit subsequent structural rearrangements, including the release of the fusion peptide (26). All of these studies support the idea of an initial HA1 head opening as precondition for HA conformational change.

However, Zhou et al. (26) suggested that, instead of His$184_1$, the His$18_1$ and Glu$103_2$ pH sensor groups may act as pH-dependent regulators of the HA conformational change by controlling the release of fusion peptides at a lower critical pH than that required for HA1 head dissociation. Although this notion has not been experimentally confirmed, their study suggested that ionizable residues other than histidines might serve as molecular triggers. Also, Harrison et al. (52) pointed out that the low $pK_a$ values of aspartate and glutamate (3.9 and 4.2 in solution, respectively) can be substantially increased depending on the local environment, which positions them as interesting candidates in pH-dependent fusion proteins. Indeed, specific aspartate residues were experimentally demonstrated to serve as molecular switches for the reversible fusion that mediates the conformational change of glycoprotein G of vesicular stomatitis virus (53).

It was suggested that in the prefusion structure of HA, the anionic residue pair Glu$69_2$ and Glu$74_2$ (2.6 Å) at the HA1-HA2 interface promote the dynamic transition of the unstructured B-loop to a highly stable $\alpha$-helix (52). For a mutated H2 HA with an enhanced acidic pH stability, the protonation of highly conserved Glu$69_2$ (and of partly conserved Glu$64_2$) at low pH was shown to facilitate outward rotation of the B-loop by contributing to the stability of the central coiled-coil (20). As a consequence, complete HA1 dissociation and HA2 spring unloading were suggested

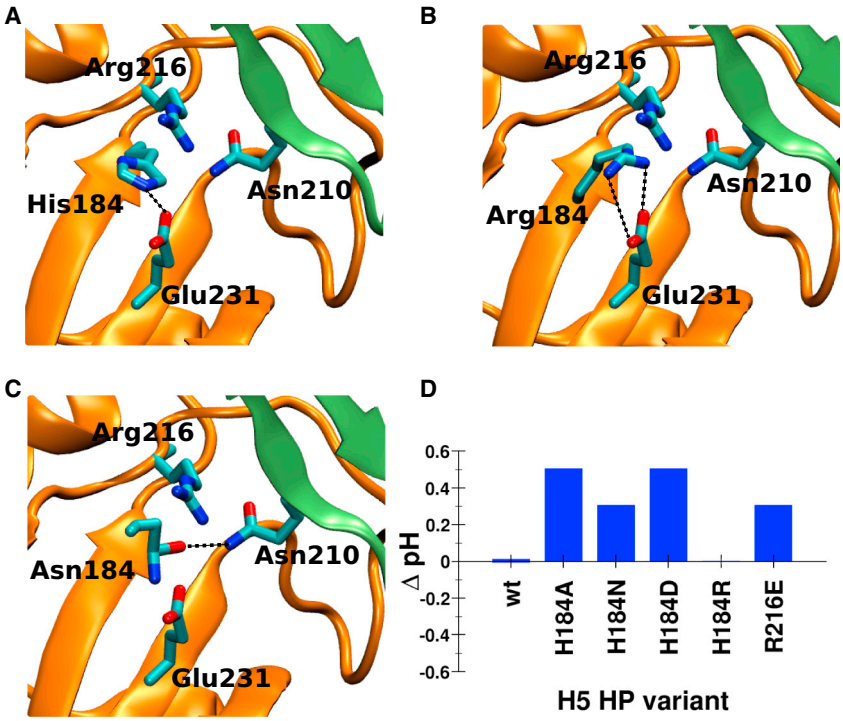


FIGURE 3 Structural representation of the HA1-HA1 interface of wild-type H5 HP (PDB: 2IBX) and of some in silico mutations at position 184. (*A–C*) In the crystal structure of wild-type H5 HP, His184 exhibits a hydrogen-bond interaction with Glu231 (*A*), which is strengthened by mutation $H184_1R$ (*B*) and abrogated by mutation $H184_1N$ (*C*). (*D*) Subtle changes in the fusion pH are observed upon these and other (not represented) mutations. A higher fusion pH threshold than that observed for the wild-type indicates a destabilization of the HA1-HA1 interface. The $H184_1R$ mutation abolishes any conformational change and fusion of H5 HA at low pH (51).

as concurrent, synchronized events during fusion as proposed for pathway A of HA refolding. However, as mentioned above, since the presence and location of ionizable residues vary among HA subtypes, the sequence of structural rearrangements might differ as well.

## Relevance of HA acid stability for host adaptation

Whereas ionic interactions and residues are not always conserved among all subtypes, the relative structural location and positioning of crucial domains have been found to be highly preserved (with root mean-square deviations of ~1 Å within domains), emphasizing their importance for the functionality of the protein (18,45). This has also been described for other viral fusion proteins, such as the glycoprotein G of the rhabdovirus family (54). However, in response to different environmental conditions, adaptive mutations have emerged that affect the acid stability of HA (55,56) and have been mapped to seven different regions (15).

The basis for viral adaptation relies on the ability of HA to preserve its functions in receptor binding and membrane fusion activity in spite of mutations. Whereas mutations in the RBS are essential to mediate attachment of the virus to host-specific receptors, they have also been shown to alter the fusion pH, sometimes reducing viral fitness and thus viability (12,57–59). In ferrets, it was additionally shown that the transmissibility of the virus via the airborne route—the primary mode of transmission between humans—requires a certain pH stability of the viral HA (60,61). Thus, the acid stability of HA seems to be highly relevant for human host adaptation, an idea that is supported by the finding that the fusion pH of human-adapted subtypes is in general lower than that of their avian counterparts (42).

Indeed, the degree of pathogenicity in avian and mammalian hosts could be related to the acid stability of HA (43,62), with a decreased stability (fusion pH 5.6–6.0) facilitating the infection of ducks (63) and an increased stability (pH 5.4–5.6) being favored for replication in lower mammals (64). A pH of fusion lower than 5.4 or higher than 5.9 did not support viral replication in any of these hosts. As a consequence, an optimal threshold pH of fusion was suggested to be required—a precondition that was related to host-specific conditions such as the extent and kinetics of endosomal acidification during infection (43,65–70). In summary, for successful adaptation of avian viruses to the human host, adaptive mutations in addition to those required for human-type receptor binding seem to be indispensable.

## How adaptive mutations affect HA acid stability

As described above, the stability, flexibility, and function of HA largely depend on electrostatic interactions at domain and subunit interfaces that are important for keeping the meta-stable conformation at neutral pH. Adaptive mutations that emerge as a consequence of different environmental conditions modulate the interaction network within HA and hence its stability. As shown in the example of avian H5 and 2009 pandemic H1 HA proteins, such stability-modulating mutations were found to be predominantly located in or near the RBS (HA1 residues 226, 228, 158, and 160) and at the HA1-HA1 interface (HA1 residues 94 and 216), the HA1-HA2 interface (HA1 residues 104, 110, 115, and 318; HA2 residues 47, 51, and 58), the fusion peptide region (HA1 residues 23 and 24; HA2 residue 114), and the central axis of the HA2 trimer (HA2 residue 111), indicating that these regions are the most sensitive to structural alterations, possibly due to their significant involvement in the structural reorganization of the protein at low pH (Fig. 4 *A*) (49).

Mutations that occur at the HA1-HA1 interface are located in or near the loop between positions 210 and 220, and are often involved in the exchange of an ionic residue. One example is the naturally evolved mutation $E216_1R/K$ in avian H5 HA, which has been suggested to increase HA acid stability by modulating the $pK_a$ of $His184_1$ and facilitating fine-tuning of the fusion pH (51). The exchange of charge at position 216 by mutagenesis indeed affects the pH dependence of the conformational change that is induced, providing indirect evidence for such an adaptive mechanism. A similar example of a mutation fine-tuning the histidine environment is the stabilizing mutation $T318_1I$ at the HA1-HA2 interface, which emerged in an airborne-transmissible H5N1 strain as a result of an experimental adaptation of the virus to ferrets (61). The residue is located close to $His18_1$ in the fusion domain of HA1, a group in the H5 subtype that was proposed by Zhou et al. (26) to serve as a sensor. The exchange of Thr318 for isoleucine was shown to alter the conformation of $His38_1$ in proximity to $His18_1$ (71). This may in turn interfere with the putative pH sensor function of $His18_1$ at low pH (Fig. 4 *B*).

An adaptive mutation at the HA1-HA2 interface might inhibit the movement of HA1 with respect to HA2. An example is the adaptive mutation $E47_2K$, which evolved in the pandemic H1 HA in 2010, causing higher thermal stability and fusion activation at lower pH than was found in strains isolated in 2009 (72). HA2 residues Lys47 and Glu21 established a salt bridge between two monomers in the 2010 isolate, a stabilizing interaction that was not present in H1 HA of 2009 (Fig. 4 *C*). The latter carries Glu at position 47, which cannot form an intermonomeric salt bridge, and this suggests a structural basis for the different thermal stabilities of the two viruses and the different pH values at which fusion activation occurs (72). Viral evolution of the 2009 pandemic H1 HA toward increased acid stability in the human host was also reported by Castelán-Vega et al. (73). In addition to the $E47_2K$ mutation, the $D104_1N$ mutation was detected, which is particularly interesting

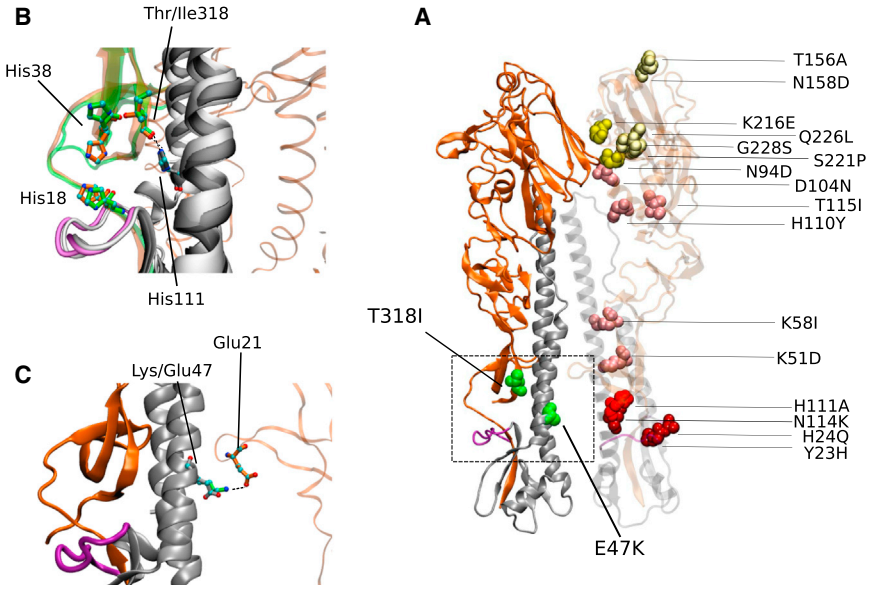


FIGURE 4 Adaptive mutations that modulate the acid stability and thus the pH of fusion of the 2009 pandemic H1 and avian H5 subtype (PDB: 4BH1). Two monomers are shown, with HA1 and HA2 displayed in orange and gray, respectively. The fusion peptide is colored in purple. (*A*) Mutations of residues that lead to a pH shift are categorized in the following regions: the region in and near the RBS (residues in *light yellow*), the HA1-HA1 interface (residues in *dark yellow*), the HA1-HA2 interface (residues in *pink*), the central axis of the HA2 trimer (residues in *red*), and the fusion peptide region (residues in *dark red*). Residues in the figure were numbered according to the literature. (*B* and *C*) As examples of the effects of certain adaptive mutations on the acid stability of HA, the changes induced by $T318_1I$ and $E47_2K$ (*green spheres*) in the intramonomeric (*B*) and intermonomeric (*C*) interactions in the prefusion structure are shown in detail. HA1 residue 318, which is observed differentially in the natural (PDB: 4BH1, *orange*) and ferret-transmissible (PDB: 4BH3, *green*, superimposed) mutants, alters the conformation of His38 in the structure, possibly abrogating the triggering function of the His18 pH sensor group at low pH (*B*). (*C*) The intermonomeric interaction between residues HA1-21 and HA2-47 determines the threshold pH of fusion. Lys47 in H1 HA of the Bris/10 strain forms a stabilizing hydrogen bond with Glu21, lowering the pH of fusion to 5.0, whereas in the Cal/09 strain the presence of Glu47 inhibits this kind of interaction and the observed pH of fusion is 5.4 (72).

since it was also found in avian H5N1 along with the $I115_1T$ mutation (43). The latter mutations stabilize the HA1 globular head atop the central stalk due to hydrogen bonds formed with Leu73 of HA2 and Leu111 of HA1. Similarly, the mutation $H110_1Y$, which as $T318_1I$ enables the airborne transmissibility of avian H5N1 between ferrets, stabilizes the protein due to a new hydrogen bond that forms between Tyr110 of the HA1 head and HA2 residue Asn413 of the stalk domain (60,74). In summary, these stabilizing mutations at the HA1-HA2 interface seem to inhibit movement of the HA1 domain with respect to HA2 (Fig. 4 *A*), indicating that HA1 must undergo at least a subtle deformation to allow the structural changes in HA2 observed by Xu and Wilson (20).

Whereas most of the mutations that have been described exemplify a host adaptation strategy of the avian H5 HA and 2009 pandemic H1 HA toward increased stability, a number of other mutations in which HA stability is reduced have been detected in laboratory strains, naturally adapted virus strains, and even in viruses isolated from human patients, such as the $N94_1D$ mutation in H5 HA (75) (Fig. 4 *A*). Although Asp94 at the HA1-HA1 interface initially strengthens the interaction within one monomer by hydrogen bonding with Glu227, the electrostatic repulsion of Asp94 from Thr204 of the neighboring HA1 monomer leads to a destabilization of the trimeric structure.

To conclude, for human-adapted influenza viruses, a general trend of HA evolution toward increased acid stability can be observed. However, it has not yet been clarified whether such stabilizing mutations in the highly pathogenic H5 subtype will be sufficient over the long run to support the successful adaptation of avian H5N1 viruses to human hosts and allow them to follow the pattern seen in H1, H2, and H3 viruses in the past.

## Conclusions

Recent studies combining experimental and computational methods have offered a submicroscopic view of the molecular determinants of HA's stability under different pH conditions. These studies have not only revealed possible intermediate structures in the refolding pathway of HA but also highlighted the contributions of specific residues and interactions to the acid stability of the protein. A number of studies have emphasized that histidine residues play prominent roles as pH sensors. However, their absence often fails to abolish the conformational change of HA that induces fusion, and other residues may also affect the pH threshold at which the protein can function. Therefore, although this sensor function may not be a global property of histidine residues in general, we suggest that specific histidine residues are probably most relevant, and indeed required, for establishing the pH threshold of HA.

HA has several possible means of adapting to host-specific conditions, and mutations of such histidines might well modulate their sensor functions. Adaptive mutations were preferentially found in interdomain and intersubunit regions of the HA ectodomain. A specific association between the regional preference of these mutations and specific host cell conditions has not yet been established. In any case, subtle changes in the structure of HA modulate

its acid stability and thus enable the virus to replicate under different host cell conditions, ensuring viral infectivity and transmissibility to other species. It remains to be clarified whether such changes support the natural selection of highly pathogenic avian influenza viruses over the long run and enable their successful adaption to human hosts.

## AUTHOR CONTRIBUTIONS

S.D.L., A.H., and C.M.M. wrote the manuscript. S.D.L. and C.M.M. designed the figures.

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