Structure *14*, 1481–1487, October 2006 DOI 10.1016/j.str.2006.07.011

The Role of Histidine Residues in Low-pH-Mediated Viral Membrane Fusion Hypothesis

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Summary

A central event in the invasion of a host cell by an enveloped virus is the fusion of viral and cell membranes. For many viruses, membrane fusion is driven by specific viral surface proteins that undergo large-scale conformational rearrangements, triggered by exposure to low pH in the endosome upon internalization. Here, we present evidence suggesting that in both class I (helical hairpin proteins) and class II (β-structure-rich proteins) pH-dependent fusion proteins the protonation of specific histidine residues triggers fusion via an analogous molecular mechanism. These histidines are located in the vicinity of positively charged residues in the prefusion conformation, and they subsequently form salt bridges with negatively charged residues in the postfusion conformation. The molecular surfaces involved in the corresponding structural rearrangements leading to fusion are highly conserved and thus might provide a suitable common target for the design of antivirals, which could be active against a diverse range of pathogenic viruses.

Introduction

Membrane fusion is an essential step during the entry of enveloped viruses into their host cell. Depending on the type of virus, membrane fusion is initiated by various mechanisms, including receptor binding (e.g., HIV-1), changes in pH (e.g., influenza and dengue viruses), or a combination of both (e.g., avian sarcoma and leukosis virus), with the mechanism of fusion being related to the individual viral life cycle. In viruses that enter cells via endocytosis, fusion is initiated upon the acidification of the endosomal vesicle (Helenius et al., 1980), leading to the release of the viral genome into the cytoplasm and infection of the cell. Membrane fusion is a fundamental biological process that occurs in a range of organisms and processes, and both viral and eukaryotic fusion proteins show a high degree of structural similarity (Sollner, 2004). This finding raises the questions: (1) What are the underlying triggering mechanisms? (2) Are these common to all viral fusion proteins?

Before the fusion event, viral surface proteins that drive fusion (fusion proteins) adopt a "meta-stable" conformation (referred to here as "prefusion" conformation). After a specific regulatory event such as receptor binding or a change in pH, they undergo a series of structural transitions, ultimately leading to a more stable "postfusion" conformation. All viral fusion proteins contain a hydrophobic segment referred to as the "fusion peptide," which, in most cases, is initially buried within the prefusion form; however, once fusion is triggered, it is exposed and can associate with the membrane of the host cell. In this transition phase, the protein is anchored in the viral envelope and the host cell membrane simultaneously, and further conformational changes drive the two membranes to fuse (Harrison, 2005; Schibli and Weissenhorn, 2004).

Two major classes of viral membrane fusion proteins have been characterized. Class I fusion proteins are found in the envelopes of viruses belonging to the *Coronaviridae*, *Filoviridae*, *Arenaviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, and *Retroviridae* families (Colman and Lawrence, 2003; Earp et al., 2005; Poumbourios et al., 1999). The influenza virus hemagglutinin (HA) is the best studied and the archetypical class I fusion protein. In the prefusion conformation, HA forms trimeric spikes on the virion surface (Wilson et al., 1981). During fusion, the proteins rearrange, forming hairpin postfusion structures, in which the C-terminal membrane anchor and the fusion peptide are juxtaposed at the same end of a rod-like structure (Bullough et al., 1994).

The class II fusion proteins, such as those encoded by the viruses of the *Togaviridae* and *Flaviviridae* families, have a very different molecular architecture (Allison et al., 2001; Kielian, 2006; Lee et al., 1997; Lescar et al., 2001; Modis et al., 2003; Rey et al., 1995). They have three domains that are rich in β structure, and their fusion peptide is located within an internal loop. While the fusion proteins from different viruses in this class share a remarkably similar tertiary structure, the processing of the fusion proteins differs. The flavivirus (E) and alphavirus (E1) fusion proteins initially assemble as a heterodimer, and this assembly involves a companion viral surface protein that acts as a folding chaperone (prM in flaviviruses, PE2 in alphaviruses). Maturation of the viral particle involves proteolytic cleavage of the chaperone by furin, leading to the rearrangement of the fusion protein as an E homodimer in flaviviruses, and as an E1/E2 heterodimer in alphaviruses (where E2 is PE2 derived). Once activated, the dimer rearranges further into fusion protein homotrimers, which expose the fusion peptide at the tip of a rod-like structure (Bressanelli et al., 2004; Gibbons et al., 2004; Modis et al., 2004). Although the details of the conformational transition in class I and class II proteins are different, they nevertheless share common mechanistic features. Notably, in both cases, the protein folds back onto itself, causing the two membrane attachment points to be located close together in the postfusion conformation, which,

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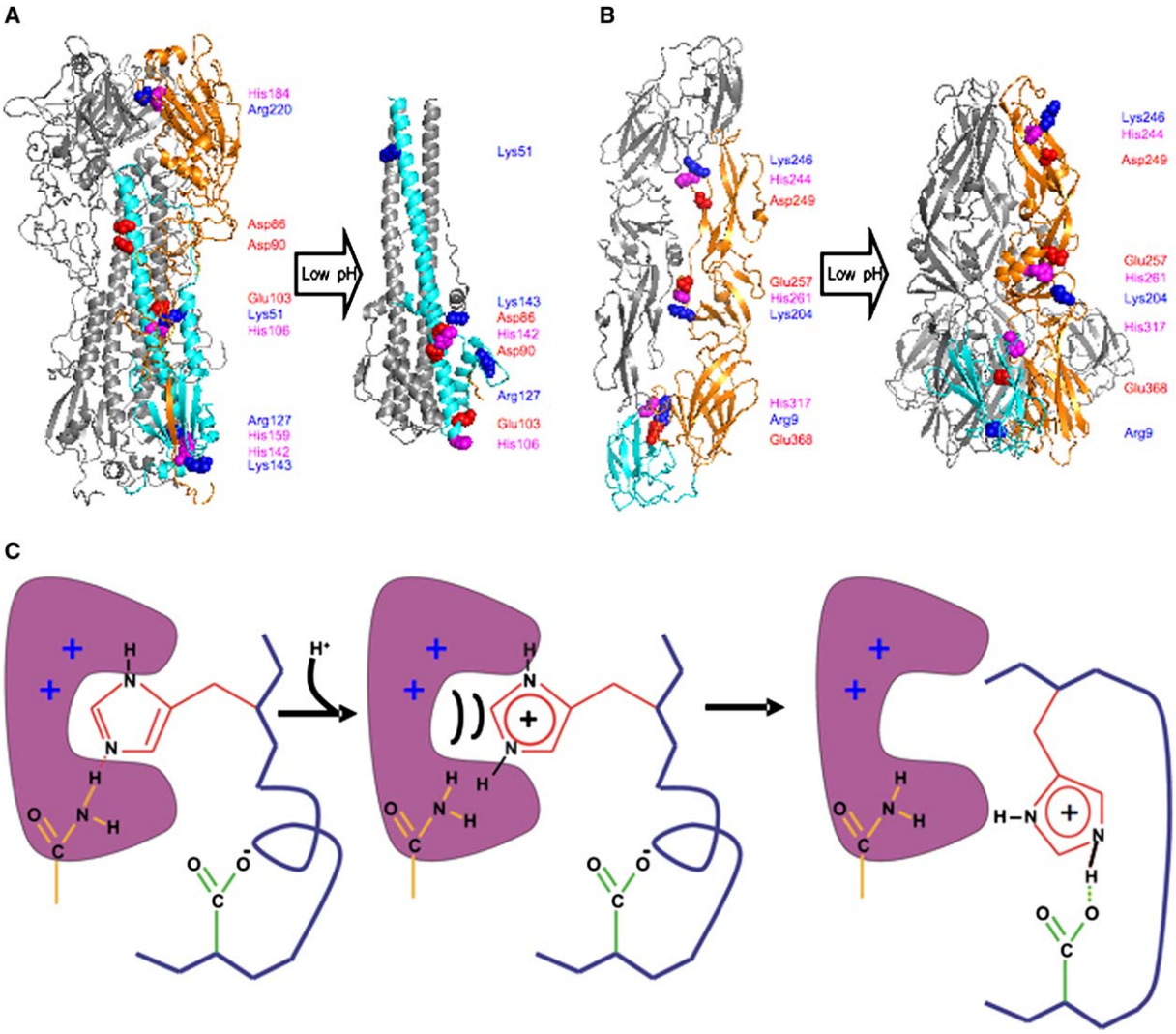

Figure 1. Low-pH-Induced Refolding of Class I and Class II Viral Fusion Proteins during Membrane Fusion

(A and B) The structures of pre- (left) and postfusion (right) viral fusion proteins are shown for (A) influenza virus HA and (B) dengue virus E protein. The protein backbone is shown in ribbon representation. Selected residues are shown in CPK representation (histidines, magenta; positively charged residues, blue; negatively charged residues, red). In HA, HA1 is colored orange and HA2 is colored cyan. In E protein, domains I and II are colored orange and domain III is colored cyan. Only one subunit from each polypeptide is shown in color; the other subunits are gray. The figure was prepared with PyMOL (DeLano, 2002). Additional histidine residues may contribute to the fusion process but are not specifically mentioned here because their role is not clear; they are not highly conserved or have less optimal interactions in the available structures. Note that the available structures correspond only to fragments of viral fusion proteins.

(C) Schematic diagram of the interactions of histidine residues in viral fusion proteins in pre- and postfusion states. In the prefusion state, the histidine residues (magenta) are located in the vicinity of positively charged residues (blue "+"). At low pH, these histidine residues become doubly protonated and positively charged. This will favor electrostatic interactions with negatively charged side chains, which may lead to the formation of new salt bridges (red) and thereby facilitate refolding into a more stable configuration. Hydrogen bonds involving a histidine residue as an acceptor will also be perturbed upon protonation.

in turn, facilitates membrane fusion (Bressanelli et al., 2004; Bullough et al., 1994; Fass et al., 1996; Gibbons et al., 2004; Jardetzky and Lamb, 2004; Kielian and Rey, 2006; Kobe et al., 1999; Modis et al., 2004; Schibli and Weissenhorn, 2004; Roche et al., 2006) (Figure 1).

pH-activated fusion commonly occurs at pH values around 6. Histidine is the only amino acid whose protonation state changes near this pH value ($pK_a \approx 6–7$). Generally, histidine is considered uncharged at neutral pH, and it becomes doubly protonated and positively charged at pH $\approx$ 6 and below, although the effective pK_a of a specific histidine depends on its local environment. There are many examples of histidine protonation triggering structural changes at low pH (Nordlund et al., 2003), including changes induced within the endosome (Lazar et al., 2003). It is therefore expected that histidine residues play a critical role in the process of viral fusion (Bressanelli et al., 2004; Chen et al., 1998; Da Poian et al., 2005; Roussel et al., 2006; Stevens et al., 2004). The question regarding the nature of this role remains. Specifically, is fusion triggered by the protonation of one or more critical histidine residues, or are the initial steps in fusion triggered by the general effects of an increase in surface charge? Here, we extend previous analyses

(Bressanelli et al., 2004; Chen et al., 1998; Roussel et al., 2006; Stevens et al., 2004) to consider whether specific histidines play similar roles in viral fusion proteins from both class I and class II.

Our sequence analyses identified a small number of highly conserved histidine residues that lie in key structural locations. We propose that these specific histidines and their interaction partners play a central role in initiating the structural transition leading to viral fusion, and that they use a similar triggering mechanism involving (1) colocation of histidines and positively charged residues in the prefusion form and (2) the protonation of these histidines, which promotes their rearrangement to form salt bridges with specific negatively charged residues in the postfusion form. We review the experimental evidence for this hypothesis, and we provide supporting theoretical calculations.

Activation of Class I Fusion Proteins

The prototypical class I fusion protein is the influenza virus protein HA. The crystal structures of the three different forms of the protein, the precursor form, the prefusion form, and a proteolytic fragment of the postfusion form, have been determined (Bullough et al., 1994; Chen et al., 1998; Skehel and Wiley, 2000; Wilson et al., 1981). HA1 residue His184 and HA2 residues His106, His142, and His159 are located in the vicinity of positively charged residues in the prefusion structure (Figure 1A). While HA1 residue His184 and HA2 residue His159 are not resolved in any structure of the postfusion form of HA, His142 forms salt bridges with negatively charged side chains (Asp86 and Asp90), and both side chain nitrogens of His106 act as hydrogen-bond donors near a glutamic acid in the postfusion structure (Figure 1A). Not only are the histidine residues themselves highly conserved among the many different influenza serotypes, but the neighboring residues in the prefusion structure are as well (Figure 2A).

The role of histidines and their protonation may be viewed in two contexts. First, in their neutral, singly protonated form (pH $\sim$7), they will interact strongly with the positively charged residues (usually as hydrogen-bond acceptors) found in their vicinity in the prefusion form. This will effectively lock the structure into the prefusion form until it is doubly protonated. The proximity to positively charged residues will make protonation more difficult by lowering the effective pK_a value of the histidine and increasing the initial barrier to activation. Second, once doubly protonated, their newly acquired charge will destabilize these prefusion interactions, leading to the formation of stable salt bridges with specific negatively charged residues. The formation of such salt bridges will stabilize the doubly protonated form, resulting in an increase in the pK_a of the histidine, and may make the change effectively irreversible (Figure 1C) (Tanford, 1970; Warwicker, 1989, 1992).

Histidine residues have been previously proposed to play important roles in the structural transitions of HA (Chen et al., 1998; Stevens et al., 2004), and a computational analysis of the HA structure identified His142 in HA2, as well as regions surrounding several other histidines, as energetically critical for the overall stability of the protein (Isin et al., 2002). The mutation of His17 in HA1 to glutamine or arginine resulted in an increase in the pH of fusion (Daniels et al., 1985); note that while this histidine is only partially conserved, it is located in the vicinity of several other histidines at positions 18 and 37 in HA1 and 106 and 111 in HA2, suggesting that these residues may cooperatively regulate the structural transitions.

Activation of Class II Fusion Proteins

A prototypical class II fusion protein is the dengue virus E protein. In the structure of the prefusion dimer (Modis et al., 2003), the highly conserved histidine residues His244, His261, and His317 are located in the vicinity of positively charged residues (Figure 1B). His261 and His317 form conserved salt bridges in the postfusion structure. His244 does not form a salt bridge in the postfusion structure of dengue virus E protein, but the equivalent residue in tick-borne encephalitis (TBE) E protein (His248) does form a salt bridge with Asp253. These histidine residues are located at the interface between domains I and III (His317) and at the dimer interface (His244 and His261), both of which undergo extensive reorientation during conversion to the postfusion structure. The residues that make up the local environment of these three histidines are also conserved in a wide range of flaviviruses (Figure 2B).

In the alphavirus Semliki Forest virus (SFV) envelope protein E1, residues His3 and His125 also fit the pattern observed for the proposed key histidines in fusion proteins from influenza viruses and flaviviruses. It is also possible that other histidines play similar roles in fusion activation, but their identification must await the availability of more complete structures (e.g., in SFV, the prefusion form of the E1-E2 complex). In SFV, the substitution His230Ala abrogates membrane fusion (Chanel-Vos and Kielian, 2004); while this residue is structurally analogous to His244 in the dengue virus E protein, its role in fusion is not clear from the available SFV E1 protein structures. The TBE virus residues (residues 146 and 323) equivalent to dengue virus E protein residues His144 and His317, respectively, have been proposed to allow the breakage of domain I/domain III contacts at the initiation of the conformational changes leading to fusion (Bressanelli et al., 2004). The environment of His144 in dengue virus E protein is consistent with that of the other presumably important histidines discussed above; however, this residue forms a hydrogen bond with a negatively charged residue already in the prefusion structure.

Environment of the Histidine Residues

The propensity of specific histidines to be doubly protonated will depend strongly on the local environment, including the proximity of alternative proton-donating groups and the degree of hydrophobic shielding from the polar solvent (Tanford, 1970; Warwicker, 1989, 1992). This provides a means by which the protonation of critical histidines (and hence the triggering process) could be modulated. While making protonation more difficult, the presence of positive charges will provide a repulsive force driving conformational changes once the side chain is doubly protonated. An analogous mechanism has been proposed for the process of viral uncoating (Warwicker, 1989, 1992). The degree of accessibility to solvent will also modulate the effect on

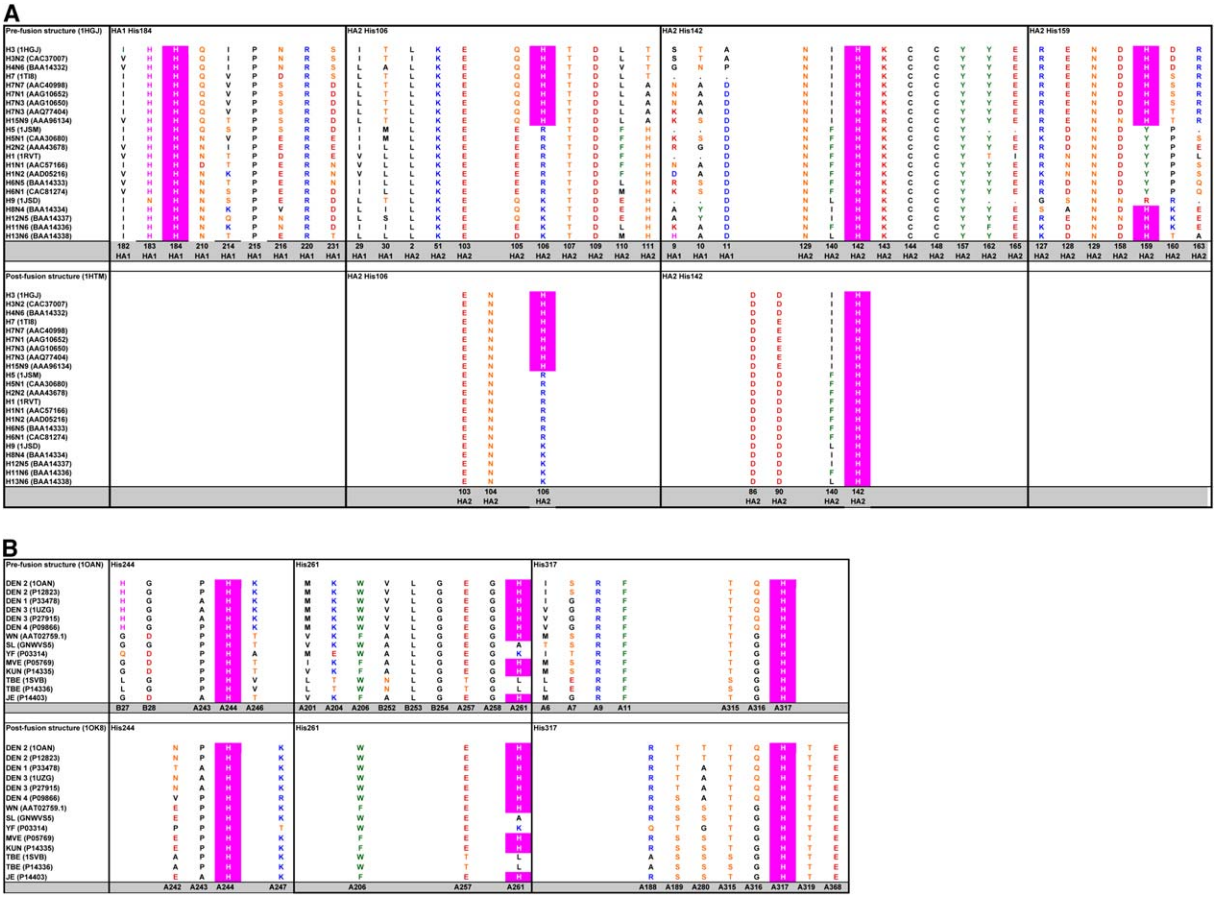

Figure 2. Sequence Conservation of Key Histidines and Residues in Their Vicinity

(A) Sequence alignment of influenza virus HA proteins from 16 different influenza virus serotypes (Phipps et al., 2004); 1HGJ, hemagglutinin from A/Aichi/2/1968 (H3N2) isolate (Protein Data Bank ID 1HJG) (Sauter et al., 1992); 1TI8, H7 hemagglutinin (Russell et al., 2004); 1RVT, 1930 H1 hemagglutinin (Gamblin et al., 2004); 1JSM, avian H5 hemagglutinin (Ha et al., 2002); 1JSD, swine H9 hemagglutinin (Ha et al., 2002). All residues within 5 Å of the side chain nitrogens of selected histidines are shown. Colors: histidines, magenta; negatively charged residues, red; positively charged residues, blue; polar residues, orange; nonpolar residues, black; aromatic residues, green. The alignments show that HA2 residue His111 may substitute for the role of His106 in some serotypes.

(B) Sequence alignment of the flavivirus E proteins, including the four dengue virus serotypes (DEN1, DEN2, DEN3, and DEN4: dengue virus types 1, 2, 3, and 4, respectively) and closely related flaviviruses (WN, West Nile virus; SLE, St. Louis encephalitis virus; YF, yellow fever virus; MVE, Murray Valley encephalitis virus; KUN, Kunjin virus; TBE, tick-borne encephalitis virus; JE, Japanese encephalitis virus). 1OAN, dengue virus type 2 E protein (Protein Data Bank ID 1OAN) (Modis et al., 2003); 1UZG, dengue virus type 3 E protein (Modis et al., 2005); 1SVB, TBE E protein (Rey et al., 1995). A and B denote monomers 1 and 2, respectively. The residue selection and colors are as in (A).

pK_a due to the neighboring positive charges. In general, the less the histidine side chain is accessible to solvent, the stronger the effect will be.

Molecular-Modeling Studies

To further examine whether the protonation of histidines could be the critical step in the structural transition from the pre- to postfusion conformation, molecular dynamics (MD) simulations were performed on the prefusion dimer of dengue virus E protein (Modis et al., 2003) (Figure 3; see the legend for simulation conditions). After 10 ns of simulation, little change in the overall structure of the dimer was observed at pH 7 (Figure 3B). By contrast, at pH 6 (Figure 3C), a separation in the dimer of the central region near domain I of one monomer could be observed. Distinct local differences were also evident; at pH 6, the interdomain salt bridges Arg9–Glu368 were disrupted in both subunits. No changes in this region were observed at pH 7. This separation is consistent with the dissociation of the dimer and the formation of the postfusion trimer.

The simulations support the suggestion that the exchange of interaction partners is the critical first step in the events leading to acid-activated fusion. These critical interactions could be experimentally probed in model systems through site-directed mutagenesis of the proposed residue partners, followed by examination of the fusion phenotype.

As noted above, the prefusion conformation is considered to be meta-stable, and certain steps of the transition from the pre- to postfusion structure are irreversible. The simulations suggest that in dengue virus E protein the Arg9–Glu368 salt bridge may act as a "linchpin" maintaining the structure in the prefusion state. We propose that once this interaction is lost, the conformational transition from the pre- to postfusion state occurs

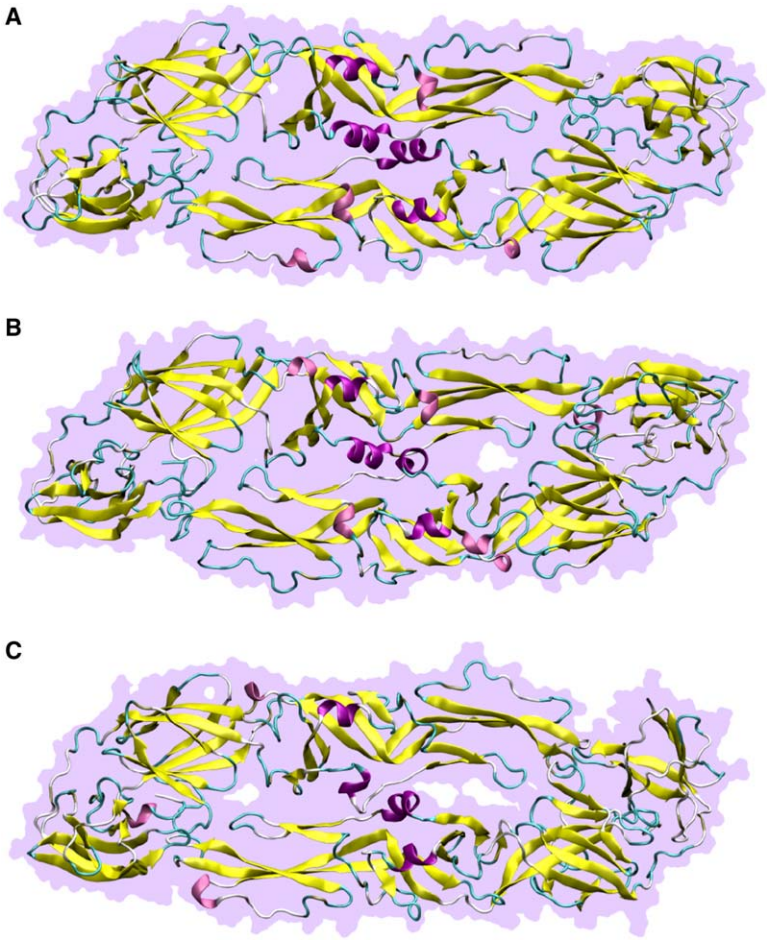

Figure 3. Molecular Dynamics Simulations Show Separation of Monomers in the Prefusion Dimer Structure of Dengue Virus E Protein at Low pH, but Not at Neutral pH

(A–C) Two systems were simulated in which the protonation states of pH-sensitive residues (His, Asp, and Glu) were assigned to those expected for slightly acidic and neutral conditions (pH 6 and 7), respectively. At pH 6, all histidines were protonated at both nitrogen positions. At pH 7, all histidines were singly protonated. The structures of dengue virus E protein are shown at (A) pH 7, t = 0; (B) pH 7, t = 10 ns; and (C) pH 6, t = 10 ns. The secondary structures are shown in cartoon representation; β structure is colored yellow, helices are purple and mauve, turns are cyan, and the coil is white. The violet shading indicates the contours of the protein solvent-accessible surface. The prefusion structures (the starting structure was Protein Data Bank ID 1OKE with the molecule of β-octylglucoside omitted [Modis et al., 2003]) were simulated in explicit water by using the SPC-water model (Berendsen et al., 1981) for 20 ns. The systems were simulated in a rectangular box under periodic conditions and standard parameters. All simulations were performed with the GROMACS 3.2.1 software (Van Der Spoel et al., 2005) in conjunction with the GROMOS96 43a1 force field (van Gunsteren et al., 1996). The temperature (T = 300 K) and pressure (p = 1 bar) were held constant by weak coupling to an external bath. A twin-range cutoff of 0.8 and 1.4 nm was used in conjunction with a reaction field (εRF = 80) to correct for the truncation of electrostatic interactions beyond the long-range cutoff. Within the short-range cutoff, the interaction pair list was updated at every time step. Longer-range interactions, together with the pair list, were updated every 10 steps. Bonds were constrained by applying the LINCS algorithm (Hess et al., 1997). The integration time step was 2 fs. Initial atom velocities were assigned from a Maxwell distribution at T = 300 K. Images were produced with the VMD program (Humphrey et al., 1996).

spontaneously. Clearly, to confirm the existence of such a mechanism, the long-term stability of the native state, as well as the full extent of the structural changes, would need to be shown after the Arg9–Glu368 salt bridge has been disrupted. It also has to be noted that the process is complex and is aided by additional factors; for example, liposomes are required in addition to low pH for the transition of dengue and TBE E protein ectodomains to the trimeric state (Modis et al., 2004; Stiasny et al., 2002).

Concluding Remarks

In this work, we present evidence that in a wide range of pH-activated viral fusion proteins for which structural information is available, the initial conformational changes associated with the transition from the pre- to postfusion forms, such as the separation of the prefusion dimeric form of the dengue virus E protein, are triggered specifically by the protonation of a small number of conserved histidine residues, as opposed to a more general effect of increasing surface charge. The protonation of these histidines, located adjacent to positively charged residues in the prefusion form, leads to their expulsion and subsequent formation of specific salt bridges and could make the transformation essentially irreversible. The essential role of histidine residues may extend to proteins such as glycoprotein G from vesicular stomatitis virus, a member of the *Rhabdoviridae* family, although the structural transition in this protein is reversible (Carneiro et al., 2003); the structure of the low-pH form determined very recently suggests that a number of residues including histidines would have a destabilizing effect upon deprotonation (Roche et al., 2006).

As many of the viruses for which membrane fusion is induced at low pH are significant pathogens, the surfaces associated with the histidine-triggered structural rearrangements might represent important potential target sites for antiviral compound design (Hoffman et al., 1997; Luo et al., 1997). Indeed, the conserved triggering mechanism of viral membrane fusion may open the possibility for the design of antiviral compounds with a broad spectrum of efficacy.

Acknowledgments

This work was funded by a grant from the National Health and Medical Research Council (NHMRC) (to P.R.Y. and B.K.). B.K. is an

Australian Research Council (ARC) Federation Fellow and an NHMRC Honorary Research Fellow, and A.E.M. is an ARC Federation Fellow.

Received: March 10, 2006
Revised: July 18, 2006
Accepted: July 23, 2006
Published: October 10, 2006

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