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Review

Early steps of the conformational change of influenza virus hemagglutinin to a fusion active state
Stability and energetics of the hemagglutinin

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Abstract

A conformational change of the homotrimeric glycoprotein hemagglutinin (HA) of influenza virus mediates fusion between the viral envelope and the endosome membrane. The conformational change of the HA ectodomain is triggered by the acidic pH of the endosome lumen. An essential step of the conformational change is the formation of an extended coiled-coil motif exposing the hydrophobic fusion peptide toward the target membrane. The structures of the neutral-pH, non-fusion active conformation of the HA ectodomain and of a fragment of the ectodomain containing the coiled-coil motif are known. However, it is not known by which mechanism protonation triggers the conformational change of the stable neutral-pH conformation of the ectodomain. Here, recent studies on the stability of the HA ectodomain at neutral pH, the energetics of the conformational change toward the fusion-active state and of the unfolding of the HA ectodomain are summarised. A model for the early steps of the conformational change of the HA ectodomain is presented. The model implicates that protonation leads to a partial dissociation of the distal domains of the HA monomers that is driven by electrostatic repulsion. The opening of the ectodomain enables water to enter the ectodomain. The interaction of water with respective sequences originally shielded from contact with water drives the formation of the coiled-coil structure.

Keywords: Fusion; Virus; Influenza; Hemagglutinin; Conformation; Stability

1. Introduction

Membrane fusion between enveloped viruses and host cell membranes is an obligatory process of viral infection mediated by viral glycoproteins, e.g. by influenza virus hemagglutinin (HA) [1] and by gp41 of human immunodeficiency virus 1 (HIV-1) [2,3]. Enveloped viruses fuse either with the plasma membrane (e.g. HIV) or after endocytic uptake with the endosome membrane.

Influenza virus is taken up by endocytosis. Acidification of the endosome lumen triggers a conformational change of HA [4–6] mediating the fusion of the viral envelope with the endosome membrane. HA is one of the best-studied viral proteins mediating fusion. Upon enzymatic cleavage of the precursor HA0, each monomer of the homotrimeric organised HA consists of two subunits, HA1 and HA2, linked by a single disulfide bond (Fig. 1). The three-dimensional structure of the bromelain-cleaved ectodomain of HA from influenza strain X31 (subtype H3) at neutral pH is known from X-ray crystallography at a resolution of 3 Å [1] (monomer: Fig. 1A; trimer: Fig. 1B and C, left). The first 20 amino acids of the N terminus of HA2—the so-called fusion peptide—are located ~ 100 Å away from the distal part of the ectodomain and ~ 35 Å away from the viral membrane, oriented towards the trimer interface (Fig. 1A). At acidic pH the HA ectodomain converts into a fusogenic conformation, thereby expos-

Abbreviations: HIV, human immunodeficiency virus; HA, hemagglutinin; BHA, bromelain-cleaved hemagglutinin; TBHA2, thermolysin treated BHA2; DSC, differential scanning calorimetry; CD, circular dichroism; EM, electron microscopy

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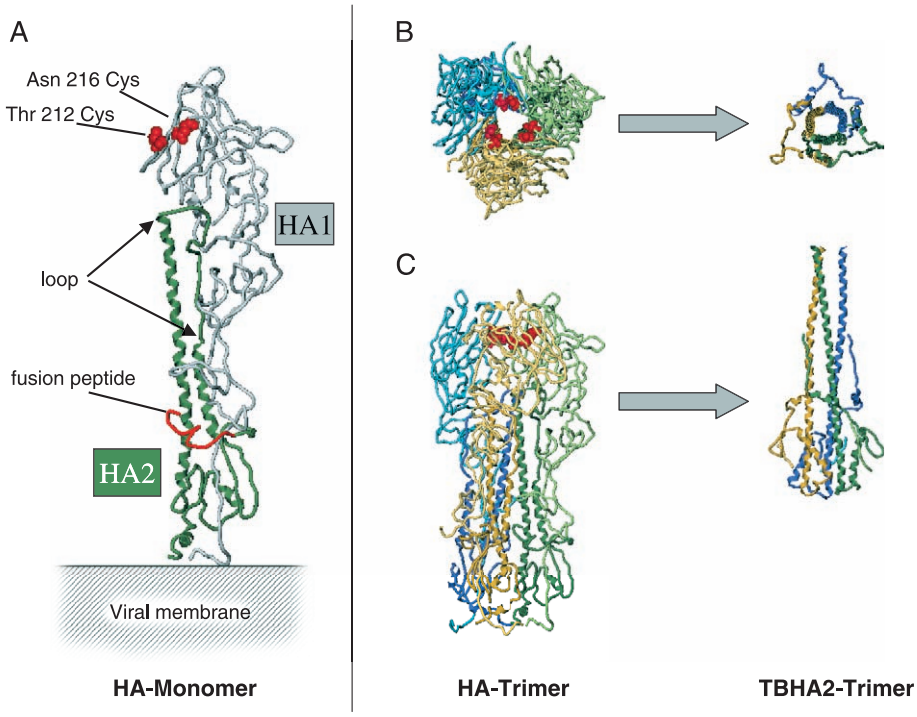

Fig. 1. Crystal structure of the HA ectodomain of influenza virus X31 in the neutral-pH form [1]. (A) A monomer with the HA1 (grey) and the HA2 (green) subunits. (B and C) Left: the homotrimeric ectodomain with the monomers in blue, green and yellow colour (B—top view; C—side view). The loop region connecting the short and long α helices of the HA2 subunits is indicated by two arrows (A). The viral membrane is indicated (A). The low-pH form of the soluble fragment of HA (TBHA2) with the extended coiled-coil motif is shown in B and C (right) (B—top view; C—side view). By substituting residues Asn 216 and Thr212 for Cys (red) by site directed mutagenesis (B and C, left), the HA1 subunits become locked by intermolecular disulfide bonds in the distal [26,27]. This cross-linking has been shown to inhibit the conformational change of HA and the formation of the extended coiled-coil structure (B and C, right). The structures were obtained by the protein explorer. The PDB ID codes of the neutral form and of TBHA2 are 1 hgf and 1 htm, respectively.

ing the fusion peptide. X-ray crystallographic studies of a fragment of the ectodomain suggest that the loop of the HA2 subunit connecting two α-helical segments in the neutral-pH form (Fig. 1A) transforms into an α-helix at low pH. As a result an extended trimeric coiled-coil structure is formed [7–11] (Fig. 1B and C, right). By this so-called spring-loaded mechanism [7] the fusion peptide is moved to the tip of HA and exposed towards the target membrane. Destabilisation of the target membrane by the fusion peptide is believed to initiate membrane fusion [12,13]; for reviews see Ref. [14] and in this issue, R.M. Epand: *Fusion peptides and the mechanism of viral fusion*. For a detailed discussion of the conformational change of HA see also reviews in Refs. [15,16]. Recently, a site-directed mutagenesis approach in the region undergoing the spring-loaded conformational change revealed another role of the spring-loaded mechanism beyond the presentation of the fusion peptide to the target membrane [17]. Even after a reduction of the length of the extended coiled-coil structure by about 50% as revealed by electron microscopy (EM), the fusion peptide still inserted into the target membrane but no fusion was observed. It was hypothesised that binding of the C terminus of HA2 to the N terminus of the full-length extended coiled-coil structure is essential to pull membranes together.

While both, the initial conformation—the 3D conformation of the neutral-pH, non-fusion-active form of HA—and an important motif of the putative final conformation of HA—the extended coiled-coil structure—are known, the identity and structure of possible intermediates during the fusion process despite many efforts are not well characterised. Even the relation between formation of the extended coiled-coil structure and the fusion event is not clearly established. Indeed, so far it is not unambiguously shown whether the formation of extended coiled-coil structure occurs before, during or after the fusion of the membranes. Likewise, the structures of well-documented intermediates between the neutral-pH and the extended coiled-coil form and their relation to steps of the membrane fusion process are not known, for example, the structure of the fusion-committed state of HA. This intermediate that has been described for HA of influenza X31 [18] is adopted at low pH and low temperature before triggering of the merger of viral and target membrane. It has the capacity to trigger fusion even at pH 7.4 upon elevation of the temperature. The fusion-committed state can be stabilised over a longer period at neutral pH by keeping viruses at low temperature [18]. This shows that only the initial phase of the conformational change requires a low pH, later stages towards the fusion-active conformation may not. The fusion capacity of the committed state requires only HA2, but not HA1 since fusion was not affected upon enzymatic removal of the HA1 subunit [18].

To understand the molecular details of the HA mediated fusion process, intermediate structures and the requirements to proceed from one intermediate to the other have to be identified. In particular, an essential question is which step(s) of the conformational change is (are) dependent on acidic pH. Here we will focus on the early steps of the conformational change and will address by which mechanism acidification of the suspension medium can initiate the conformational change of HA. Of course, this question is strongly related to the stability of the neutral-pH form and the energy associated with the conformational change. Therefore, we will first review the energetics of the conformational change of HA; subsequently, we will address how low pH could destabilise the stable neutral-pH structure and what are the likely steps of the conformational change after destabilisation by low pH. Several lines of independent evidence suggest that the initial step of the conformational change is an opening of the distal HA1 domains. However, in particular, studies employing EM, circular dichroism (CD), and differential scanning calorimetry (DSC) indicate that activation of the fusion activity of HA does not require a dissociation of the HA1 domains and a disruption of the spike morphology with tight association of the HA. A partial dissociation of the HA1 domains preserving essentially the compact spike morphology is sufficient to trigger the fusion-mediating properties of HA. The preservation of this morphology does not interfere with the formation of the extended coiled-coil structure and might be an important structural feature to orientate the extended coiled-coil structure with the fusion peptide on its tip toward the target membrane. We will suggest a mechanism explaining how protonation of the HA ectodomain causes an opening of the HA1 domain, and that this step might be the essential or even the only step of the conformational change requiring low pH.

2. Energetics of the conformational change

The cleaved, neutral-pH, non-fusion active conformation of HA has been suggested to be a metastable intermediate in which the ectodomain is kinetically trapped behind an energy barrier due to extensive noncovalent interactions between HA subunits [7,19]. This concept suggests that the fusogenic state of HA is thermodynamically more stable than the neutral-pH state. This conclusion was based on the experimental fact that the conformational change of HA at neutral pH, triggered with either heat or the denaturant urea, is indistinguishable from the change triggered by low pH [19]. In each case, the conformational change leads to induction of the membrane fusion activity. The concept implicates also that during biosynthesis of HA, folding into the thermodynamically more stable structure achieved at low pH is efficiently prevented. In particular, the fusion-incompetent precursor, HA0, may not be able to adopt this stable structure. Only subsequently to the generation of the mature, two-subunit HA1/HA2 state by proteolytic cleavage, the thermodynamically most stable structure of HA can be achieved.

To address whether the native, neutral-pH form is a metastable conformation, DSC is the method of choice to elucidate whether the conformational transition of the cleaved HA ectodomain to a fusion-active state is associated with exothermic processes. Only recently, two studies investigated in a systematic way the energetics of the unfolding of the HA ectodomain as a function of pH and temperature.

Remeta et al. [20] undertook a combined approach utilising DSC, CD and fluorescence spectroscopy, and analytical centrifugation to characterise the unfolding pathway of the HA ectodomain of X31 and its energetics including intermediate states. The study was undertaken on isolated, full-length HA in its cleaved (HA1/HA2), pre-fusion conformation. These authors found that the neutral-pH, non-fusion active conformation of the HA ectodomain exhibits a significant conformational and thermal stability. The DSC profile was characterised by a single co-operative endotherm at 66 °C with an enthalpy change of 800 kcal/mol trimer and a co-operative ratio of 3. A deconvolution analysis of the endotherm revealed a best fit to a model of three independent two-state unfolding transitions in agreement with the homotrimeric organisation of HA. These results are consistent with the previous observations that the HA globular domains unfold independently [21]. Remarkably, the transition temperature is significantly decreased upon lowering the pH. It is shifted to 45 and 42 °C at pH 5.4 and 5.0, respectively. The enthalpy was 260 and 76 kcal/mol trimer at pH 5.4 and 5.0, respectively. This significant decrease of the transition temperature is a strong indication that acidification triggers structural changes in the HA ectodomain, which lower the endotherm transition but are different from those alterations associated with the thermal unfolding detected by DSC. Nevertheless, the structural changes caused by acidification are irreversible. As suggested by Remeta et al. [20], the decrease of the transition temperature most likely results from a loss of HA1–HA1 contacts within the trimer and HA1–HA2 contacts in the stem region. Presumably, only a part of these contacts is disrupted. Indeed, from the correlation of the temperature dependence of the intrinsic tryptophan fluorescence of the HA and the DSC endotherms, it was concluded that the temperature-induced unfolding of HA is accompanied by an abolishment of the residual domain contacts. Thermal unfolding at low pH was characterised by a loss of co-operativity, suggesting that exposed hydrophobic residues in the thermally labile HA1 domains interact during thermal unfolding and the collapsed structure unfolds as a single unit [20].

Similar results were obtained when monitoring the unfolding of the HA ectodomain by DSC for intact viruses [22]. However, the difference between neutral and low pH was much lower compared to the isolated, full-length HA [20]. While at pH 7.4 the endotherm transition was similar to that of the isolated full-length HA, the endotherm transition temperature decreased only to 60 °C by shifting the pH to

5.0 without significant changes of the enthalpy. This was taken as an indication that the HA is stabilised by interaction with other molecular components in the intact virus in comparison to isolated HA being organised as rosettes. However, one has to keep in mind that the HA protein comprises only about 30% of the total virus protein [23] and, therefore, those studies are complicated by the presence of the remaining viral proteins. Nevertheless, the endothermic DSC peaks of HA unfolding could be well resolved as confirmed by their disappearance after enzymatic removal of the ectodomain. Epand and Epand [22] did not find any evidence for an exothermic transition. From that they concluded that the neutral-pH, non-fusion active conformation does not correspond to a metastable conformation.

One may wonder whether an exothermic process—perhaps the formation of the extended coiled-coil structure (see below)—is buried under the endothermic peak. However, one has to reconcile that the endotherm DSC peak observed in both studies occurs at temperatures well above the temperature at which fusion is observed at acidic pH. Thus, the formation of the extended coiled-coil motif that is an essential step of fusion takes place at temperatures below the observed endothermic peak. One may speculate that alterations of the HA ectodomain, namely the loss of HA1–HA1 and HA1–HA2 contacts (see above) occurring well below the transition temperature and causing a lower endothermic transition peak, lead to the formation of the extended coiled-coil structure. Thus, an exothermic formation of the extended coiled-coil structure should be in principle visible by DSC. However, in this respect, one has to consider that the extended coiled-coil could has been formed already prior to DSC measurement as already acknowledged by Epand and Epand [22] even at low temperature. Indeed, exposure of the fusion peptide of HA from X31 at pH 5.0 has been observed for a temperature of 0 °C while the spike morphology remained unchanged as probed by antibodies [24]. Likewise, Böttcher et al. [25] found by cryoEM on HA of A/Japan (subtype H2), at pH 5.0 and 4 °C, a rearrangement of the distal HA1 domains while the overall shape of the ectodomain was preserved (see below and Fig. 3). Under those conditions, fusion of both X31 and A/Japan has been observed, suggesting that the extended coiled-coil motif has been formed. According to the experimental procedure of both DSC studies, full-length HA [20] and intact virus [22] have been mixed with buffer of the desired pH before starting scans. Thus, at this time point the extended coiled-coil structure may have already been formed.

The situation might be different at pH 7.4. Here, the formation of the extended coiled-coil structure may not be achieved below the endothermic transition as at acidic conditions, and occurs only in the temperature range of the endothermic transition.[1] Thus, an exothermic peak caused by the formation of the coiled-coil structure could be well buried under the endothermic conditions.

What is the relation between the exothermic energy of the loop to coiled-coil transformation and the energy of the endothermic transition? By employing molecular modelling methods, the energy of the loop to coiled-coil transformation of HA2 can be estimated. It is in the order of about −25 kcal/mol HA2 trimer assuming a pH value of 5.0 and a temperature of 30 °C (Huang and Herrmann, unpublished results). Thus, in comparison to the endothermic peak, the exothermic peak of loop to coiled-coil formation is rather low and might be difficult to resolve.

An important result of both DSC studies is that fusion occurs significantly below the unfolding transition. Two interpretations of this result have to be considered: (i) either the ectodomain does not require a major unfolding for triggering fusion or (ii) only a small, undetectable number of HAs needs to unfold to induce fusion occurring already below the unfolding transition detected by DSC. However, the fact that fusion requires the dissociation of the globular head domains [26,27] and the formation of the extended coiled-coil may not necessarily argue for the latter interpretation, which was favoured by Remeta et al. [20].

3. Preservation of spike morphology under fusion conditions

DSC studies of Remeta et al. [20] show that acidification induces changes in the conformation of HA as disruption of HA1–HA1 and HA1–HA2 contacts which do not necessarily lead to an unfolding of the ectodomain (see above). This is supported by the study of Bizebard et al. [28] who found that HA1 domains dissociate but remain globular with a rearranged tertiary structure. This suggests that even upon acidification, the typical spike morphology of the ectodomain can be essentially preserved, which is confirmed by several studies.

White and Wilson [29] have probed the extent and kinetics of the conformational change of HA from X31 by various antibodies having a significantly higher affinity to the low-pH form in comparison to the neutral-pH, non-fusion active conformation of HA. They found a rapid binding of respective antibodies to the fusion peptide after acidification. Subsequently, antigenic sites of the interface between the trimers in the HA1 top domain became exposed, indicating a dissociation of the globular heads of the ectodomain. However, while the half-time of the exposure of the fusion peptide at pH 5.0 and 37 °C was about 15 s and only slightly enhanced upon lowering the temperature to 25 °C, the dissociation of the globular heads was much slower. The half-time was about 4 min at 37 °C while about 50 min at 25 °C preceded by a lag time of 5 min. Conformational alterations of similar kinetics were found by measurement of the intrinsic fluorescence of tryptophan residues of the X31 HA ectodomain [30]. Importantly, fusion proceeds

[1] As pointed out by Remeta et al. [20], above 55 °C, fusion measurements are not possible because complexes of the membranes to be fused are unstable.

much faster in comparison to the dissociation of the head domain under those conditions [20,31–33]. This suggests that dissociation of the head domain is a rather slow process, and that the spike morphology of the ectodomain is not lost after acidification. Furthermore, these studies provide evidence that the exposure of the fusion peptide as well as fusion do not require the dissociation of the globular heads of the ectodomain. However, these data do not contradict the occurrence of alterations of the head domain necessary for achieving fusogenic activity. For example, rearrangement of the head domain accompanied by a partial dissociation (opening) may occur (see below), not sufficient for respective antibodies to have access to antigenic sites in the interface region [29].

Preservation of the spike morphology of fusion active HA after acidification was observed by various studies employing EM. Kanaseki et al. [34] studied fusion between influenza X31 and liposomes by quick-freeze EM. They did not observe any alteration of the morphology of the HA ectodomain at pH 5.0, 23 °C, with respect to the neutral-pH form. Under the conditions chosen, fusion of influenza virus with liposomes has already occurred. Similar observations of a preserved, compact spike morphology of fusion active HA were reported from other studies [33,35–37]. The morphology of the HA ectodomain of influenza virus X31 even after 1 h at pH 5.4, 37 °C, was similar to that observed at neutral pH [33]. Under those conditions, rapid fusion with erythrocyte membrane (half time about 4 min) was observed [20,32,33]. In contrast, spike morphology was rapidly lost and showed a fuzzy appearance when X31 viruses were incubated in the absence of target membranes at pH 5.0, 37 °C. Very likely, the loss of spike morphology is related to inactivation of the fusion mediating properties of HA, but not related to the fusion active state. This is supported by CD measurement in the near UV sensitive to alterations of the tertiary and quaternary structure of the HA ectodomain [20,32]. At pH 5.4, 37 °C, neither a loss of the fusion activity nor a decline of the CD signal was observed for HA of X31 even after 1 h of incubation [32]. This strongly argues that the fusion active HA ectodomain can preserve a spike morphology comparable to that of the neutral-pH form. In contrast, the rapid inactivation of X31 at pH 5.0, 37 °C, was paralleled by a rapid loss of the CD signal which, presumably, coincides with the fuzzy appearance of the ectodomain observed by cryoEM (see above). Comparing the kinetics of inactivation and the loss of the CD signal with that of the accessibility of antibodies to the trimer interface in the HA1 domain [29] (see above), the dissociation of the globular domains is rather associated with the inactivation process, also causing the fuzzy appearance of the HA ectodomains. Notably, these data do not point to a disappearance of the higher order structure and a rather disordered arrangement of the fusion-inactivated HA because no alteration of the secondary structure was observed consistent with other studies [20,38]. Furthermore, these changes of the near-UV CD signal occur well below the respective temperature of thermal unfolding detected by DSC.

Similar relations between fusion activity, inactivation and the CD signal at near UV were established for two other subtypes of influenza virus: A/PR 8/34 (H1) and A/Japan 305/57 (H2) [32]. Again, these results are consistent with cryoEM studies on the morphology of the HA ectodomain. Shangguan et al. [37] found that influenza virus A/PR 8/34 retained its fusion activity after preincubation in the absence of target membranes at pH 4.9 and 30 °C. During this period no morphological changes of HA occurred. The spikes on the surface of the virions are distinct and well ordered. However, longer incubation at these conditions leads to inactivation of A/PR 8/34 HA paralleled by a loss of spike morphology.[2] Finally, incubation of influenza virus A/Japan 305/57 at 37 °C for 15 min in the absence of target membrane did affect neither the spike morphology [36] nor the fusion activity [32,36]. However, pH 5 and 37 °C-treated HA of A/Japan had undergone conformational changes as indicated by an increased hydrophobicity, exposure of antibody epitopes, and susceptibility to protease digestion. For A/Japan 305/57 prebound to erythrocyte membranes, fusion under those conditions was rapid with a half time of less than 1 min [32,36].

Recently, the three-dimensional structures of the *complete* HA of influenza virus A/Japan/305/57 have been determined by cryoEM and image reconstruction in its native, neutral-pH form and membrane fusion-competent conformation at low pH at a resolution of 10 and 14 Å, respectively, [25] (see also Fig. 3). In agreement with other studies, in the fusion-competent form the subunits remain closely associated, preserving typical overall features of the trimeric ectodomain at neutral pH. However, various significant differences between the two structures were observed. For the fusion-competent, low-pH forms a flattening of the top of the distal domains, a refolding of the stem region of the trimer and a torsion of the ectodomain mainly in the part located above the viral membrane were found. The latter causes a widening of the trimer interior. The N terminus of the HA2 may also be turned outwards from the subunit interface, giving rise to an enhanced flexibility of the HA2 subunit. Most striking was the formation of a continuous central cavity through the whole ectodomain of the trimer at low pH.

In conclusion, the conformational change of the ectodomain leading to fusion is not necessarily accompanied with a loss of the compact trimeric spike morphology of the neutral-pH, non-fusion active conformation. However, in this respect, one has to note that typically cryoEM studies showing the preservation of the spike morphology were done in the absence of the target membrane. These studies cannot preclude that later steps of the fusion process require a complete dissociation of the HA1 domains. For example, refolding of the C terminus of the HA2 ectodomain leading

[2] It should be noted that Korte et al. [32] observed a much faster inactivation of A/PR 8/34 as reported by Shangguan et al. [37].

to the formation of the antiparallel helix bundle may require the complete dissociation of the HA1 domains.

4. Opening of the HA1 distal domain is an essential step of the conformational transition

Different approaches have suggested that a rearrangement of the HA1 globular (distal) domains of HA occurs at low pH and is an essential step of the conformational change of the HA ectodomain toward a fusion active state [25–27]. This is supported by studies employing chemical cross-linking, antibody binding, proteolysis, EM and site-specific mutation [25–27,29,39–45]. Locking the HA1 subunits by intermolecular disulfide bonds in the distal part (see Fig. 1B and C, left) prevented the conformational change of HA and abolished its fusion activity [26,27]. Recently, it has been shown that an antibody that binds simultaneously to and, by that, cross-links the membrane distal HA1 domains of two HA monomers prevents the low-pH-mediated conformational change of HA [45]. Thus, a sideward reorientation of the originally tightly associated monomers of the HA1 globular domain, at least of their distal parts, seems to be essential for formation of the fusion active conformation of HA. Furthermore, the exposure of the fusion peptide requires an opening of the distal domains. These studies suggest that the opening of the distal HA1 domain is an early, perhaps the first, step of the conformational change of the HA ectodomain toward a fusion active state. Remarkably, as outlined above, an opening of the HA1 distal domain and a formation of a cavity spanning the whole ectodomain were observed by cryoEM and image reconstruction for a fusion-competent state of the ectodomain of HA from A/Japan [25].

5. Protonation of the ectodomain destabilises the neutral-pH form

A partial dissociation of the HA1 subunits of influenza virus HA is considered to be the initial step of conformational changes of the HA ectodomain leading to a membrane fusion active conformation (see above). An attractive hypothesis is that at low pH the enhanced protonation of the solvent-exposed HA1 domain destabilises this domain because the largest contribution to the electrostatic potential within a protein arises from the protonable amino acids that can carry a net charge.

According to the X-ray structure of HA from strain X31 [1], at neutral pH and room temperature, the net charge in the HA1 domain is basically positive (Fig. 2). The HA1 domains by itself would not be able to form a stable trimer. The HA2 domain, in particular the distal part, is locally enriched with negative charges, while the corresponding contact position of the HA1 domain is enriched with positive charges. This indicates that in the HA ectodomain at neutral pH, the electrostatic force between either three subunits of HA1 or of HA2 is repulsive, however, that between HA1 and HA2 domains is attractive. The relevance of electrostatic interaction between HA1 and HA2 for the stability of the HA ectodomain and the tight association of the monomers is confirmed by screening of fusion mutants of influenza virus [46] and site-directed mutagenesis at the interface of HA2–HA1 subunits (Sivaramakrishna and Herrmann, unpublished results). For example, destroying the electrostatic interaction between Glu 67 in the loop region of HA2 and Arg 269 of the HA1 domain by substitution of the latter residue for Gly leads to a significant reduction of the stability of the HA ectodomain of X31. Conversely, substitution with amino acids aimed at introducing new attractive electrostatic interactions increased the stability of the HA ectodomain (Sivaramakrishna and Herrmann, unpublished results).

Since the net charge in the HA1 domain is positive, it was suggested recently that an enhanced protonation of this domain will lead to an even more positive charge that will give rise to an additional electrostatic repulsion between the three subunits of the HA1 domain lowering the stability of the trimeric monomer association (Fig. 2) [47]. To model this quantitatively, the electrostatics state of the HA1 domain was characterised recently by a single parameter—the number of associated protons with this domain [47]. Based on the X-ray structure of HA, the average numbers of associated protons with HA1 domain at different pH values and temperatures were calculated based on a continuum solvent model and Monte Carlo sampling techniques [48–50]. The calculations showed that the protonation of the HA1 domain is enhanced at the conditions (pH and temperature) known to trigger conformational changes of the HA ectodomain (see also Ref. [19]). It was reported that the dependence of the calculated relative degree of protonation of the HA1 domain on temperature and pH was similar to that observed experimentally for the conformational change of HA assessed by proteinase K sensitivity [47]. A close quantitative correlation between the pH thresholds of the conformational change at a given temperature and the number of associated protons with the HA1 domain at similar conditions was established. This result indicated that at various temperature-dependent pH thresholds of the conformational change, the electrostatic interaction between HA1 subunits is similar. Since protonation enhances the positive net charge of each HA1 subunit and, by that, the repulsive component, the stability of the ectodomain may become reduced. In particular, the tight contact of HA1 subunits could be affected, which may lead to a sideward relocation of the HA1 subunits releasing constraints of other conformational alterations. This modelling approach provides a theoretical basis and explanation to the puzzling observations on conformational changes of HA occurring at higher temperature and neutral pH as reported by Carr et al. [19]. Furthermore, a weakened attraction between the HA subunits may facilitate not only a relocation of the HA1 subunits, but also enable the subsequent steps of conforma-

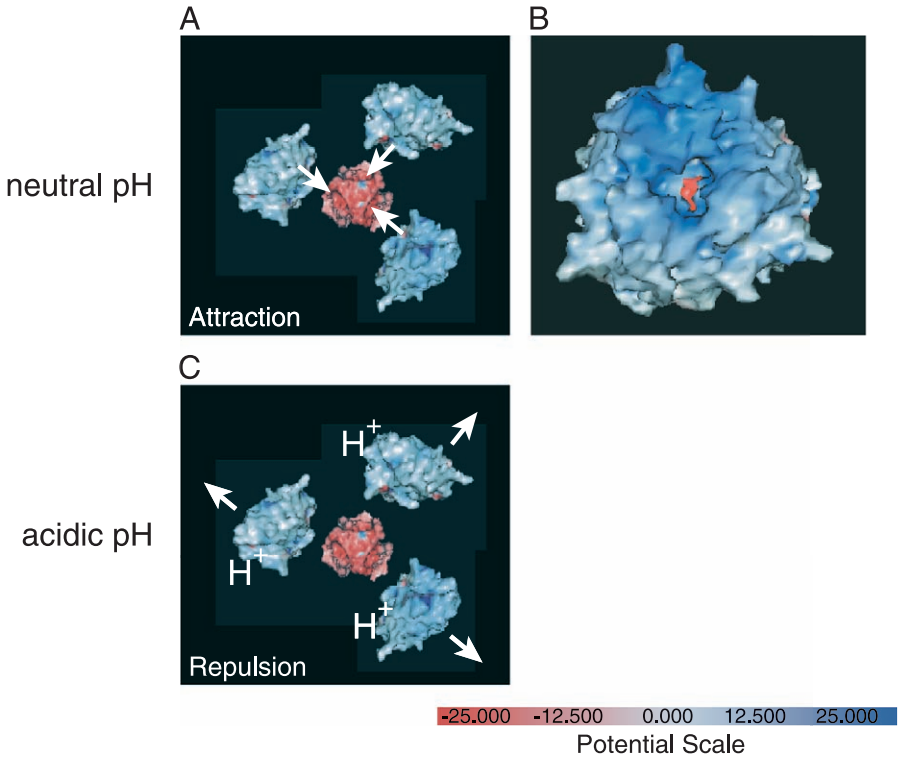

Fig. 2. Electrostatic interactions between the HA1 and the HA2 subunits (top view). The HA2 domain, in particular the distal part, is locally enriched by negative charges, while the corresponding contact position of the HA1 domain is enriched of positive charges. The results [47] indicate that in the HA ectodomain at neutral pH (A), the electrostatic force between either three subunits of HA1 or of HA2 is repulsive; however, that between HA1 and HA2 domains is attractive. The opponent surface potentials give rise to an attractive interaction between both subunits essential for its stability at neutral pH (B). Since the net charge in the HA1 domain is positive, enhanced protonation of this domain will lead to an even more positive charge that will give rise to an additional electrostatic repulsion between the three subunits of the HA1 domain reducing the stability of the ectodomain. The enhanced repulsion may cause a (partial) dissociation of the HA1 subunits as indicated in C. For details see text and Huang et al. [47]. Surface electrostatic potential of HA1 domain and HA2 domain with GRASP [60]. Electrostatic potential is colour-coded using a sliding scale indicated in the lower part of the figure (unit in k_BT/e). Red represents negative electrostatic potential, blue represents positive electrostatic potential, and white is neutral.

tional change of HA into a fusogenic state by the spring-loaded mechanism [7].

6. Model for early steps of the conformational change of HA

The initial step of the conformational change of HA at low pH (Fig. 3) is a partial dissociation or opening of the HA1 domain. We surmise that this step is driven by electrostatic forces. The strong electrostatic attraction between the positively charged HA1 subunits and the negatively charged HA2 subunits in the neutral-pH form is perturbed at low pH. Protonation enhances the positive charge of the HA1 domain and thus causes an enhancement of the electrostatic repulsive forces. Structural evidence for a partial opening of the HA1 domain is given by the fusion-competent, low-pH conformation of the HA ectodomain from A/Japan 305/57 detected by cryoEM [25] (see above). Furthermore, this study revealed a central cavity along the central axes. Through this opening and the formation of the cavity, water can enter the interior of the HA ectodomain and interact with the loop region of HA2 connecting the short and the long alpha helix in the neutral-pH form. In the latter conformation, the loop region is essentially shielded from water contact. Interaction with water triggers the transformation of the loop region into an alpha helical structure. The loop-to-helix transformation is accompanied by the formation of the extended coiled-coil structure found by Bullough et al. [8]. This structural change and the formation of the extended coiled-coil structure are energetically favoured and resemble an exothermic process. Notably, this step does not depend on low pH. As revealed by molecular modelling (Huang and Herrmann, unpublished results), the associated free energy of the loop to helix transformation is about -75 and -120 kcal/mol trimer at pH 5.0 and pH 7.4, respectively.

This model suggests that acidification is essentially necessary for an opening of the HA1 head domains, allowing water to enter the interface regions of the subunits and to get in contact with the loop domain of HA2 connecting the short and the long alpha helix. Once this contact is established, the conformational change of the loop region is driven leading eventually to the extended coiled-coil structure. The spontaneous formation of the coiled-coil structure is indicated by the studies of HA2 of Carr and Kim [7] on a long peptide comprising the short alpha helix as well the loop region of

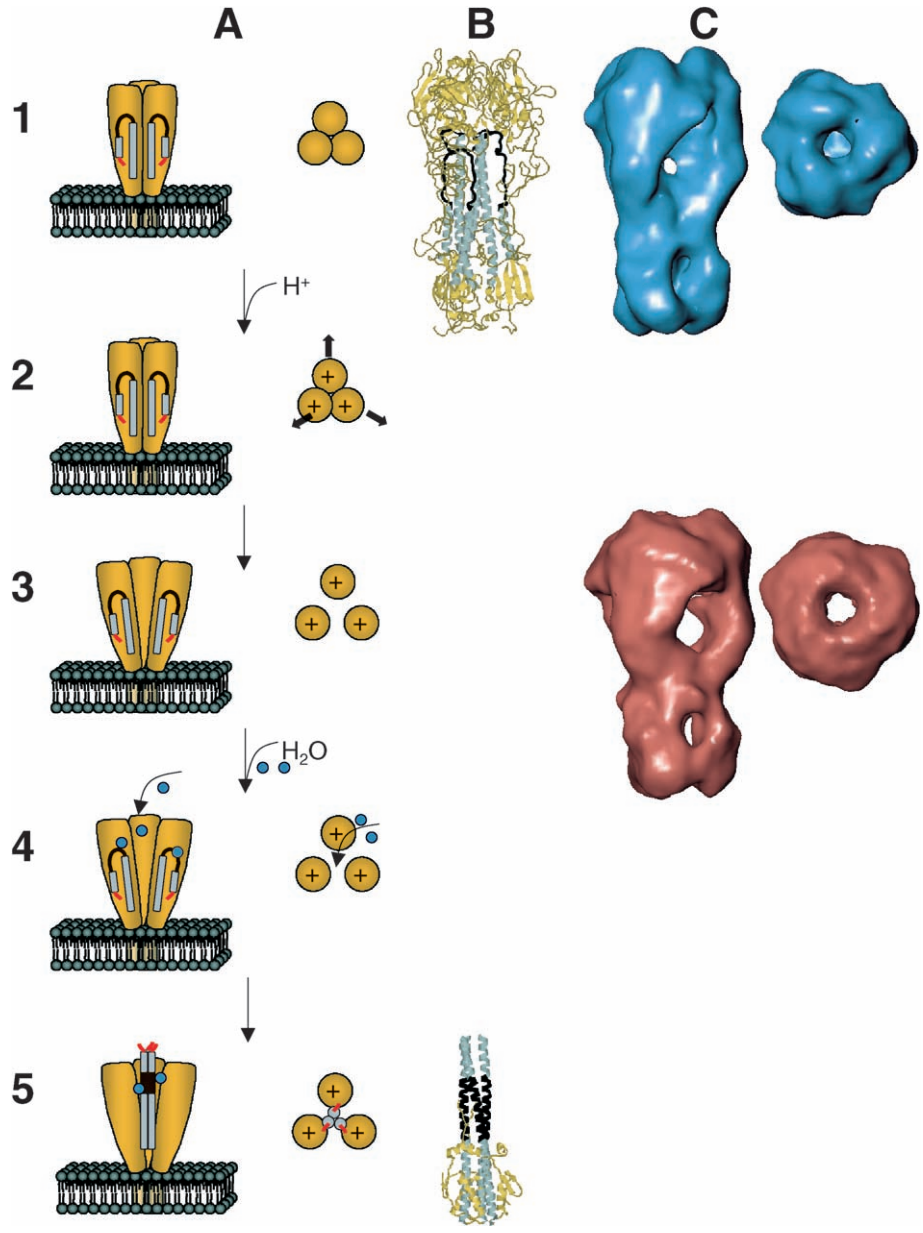

Fig. 3. Model of the initial stages of the conformational change of the HA ectodomain (A). In (B) structures of the HA ectodomain known from X-ray crystallography are shown: the neutral-pH conformation of the HA ectodomain of influenza X31 (B, top) [1] and the low-pH form of the soluble fragment of HA (TBHA2) (B bottom) [8]. The PDB ID codes are 1 hgf and 1 htm, respectively. On the right, the neutral-pH conformation (C, top) and the low-pH conformation (C, middle) of the intact HA of influenza virus A/Japan 305/57 are presented obtained from 3D reconstruction of cryoEM images [25]. In the model the long and the short α-helix of HA2 at neutral are indicated by grey colour, the connecting loop domain in the neutral-pH conformation of HA (1A) by black, and the fusion peptide by red colour (A and B). Lowering the pH leads to an enhanced protonation of the HA1 domain (2A). This gives rise to a repulsive interaction between the HA1 subunits (2.A) which causes a partial (partial) dissociation of the top domain of HA (3A). A partial dissociation is in accordance with the 3D conformation of HA from A/Japan at fusion conditions obtained by reconstruction of cryoEM images (C, middle) [25]. Different to the neutral-pH conformation, a cavity has been formed at low pH (see arrow). The partial dissociation of the top domain allows to enter water into the ectodomain (4A). The aqueous environment triggers the conformational change of the loop domain into an α helical structure, forming an extended trimeric coiled-coil structure (5A) of the HA2 domain. The extended coiled-coil structure has been identified by X-ray crystallography (B, bottom). The only partial dissociated HA1 subunits and the cavity may guide the formation of the coiled-coil and, thereby, the fusion peptide distal to the target membrane.

HA2. Furthermore, the observation that the bacterial expressed ectodomain of HA2 comprising amino acids 23–185 folds spontaneously at neutral pH into the coiled-coil conformation [10] is also consistent with this notion. Therefore, we suggest that the formation of the extended coiled-coil structure of HA2 per se does not require a low pH and forms also at neutral pH similar to respective domains of fusion proteins of enveloped viruses fusing with their target at neutral pH. Thus, the extended coiled-coil formation is essentially a water, but not protonation driven process. As

reviewed above, the stability of the loop region of HA2 in the native, neutral-pH structure of HA has been ascribed to a metastable, kinetically controlled state of the ectodomain [19]. The tight association of the HA1 domain may act as a clamp preventing the formation of the extended coiled-coil. However, the limited access of water molecules to the HA2 loop region in the native HA structure may resemble another reason for the suppression of the HA2 loop-to-helix transition. Thus, differently from the kinetically controlled model mainly based on the irreversibility of the formation of the extended coiled-coil structure with respect to pH [51], the conformational changes of the HA ectodomain may also be under thermodynamic control.

It was proposed [25] that the HA1 domain does not only prevent the conformational change of the HA2 subunit at neutral pH [7], but it also controls the conformational change of HA2 at low pH to enable fusion. This supports a recent study of Gray and Tamm [52] that suggested a key role of HA1 at acidic pH for correct orientation of HA2 with respect to the target membrane enabling fusion. Presumably, the transient stability of the association of the HA1 membrane-distal domains is an important factor retarding the inactivation of influenza virus at low pH, too. Dissociation of the domains exposes the fusion sequence with its strong hydrophobic properties [14]. Coiled-coil structures with an exposed hydrophobic fusion sequence at the tip immediately tend to aggregate and/or insert into the viral membrane in the absence of a target membrane [53]. This would drive the ectodomain into a fusion-inactivated conformation. Thus, membrane fusion requires a distinct sequence of conformational changes of viral fusion proteins. This sequence is determined by the structure of the fusion protein as well as the presence of the target membrane.

A continuous cavity within the trimer as found for the fusion-competent ectodomain of HA from A/Japan 305/57 might facilitate the relocation of the 'fusion peptide'. It has been shown that the loop region between the two α-helices of the neutral-pH form remains highly flexible at low pH [54]. Both the flexibility of the loop region and the cavity may give rise to conformational alterations as formation of the extended coiled-coil structure and facilitate the relocation of the fusion peptide towards the tip of the ectodomain, the site of the target membrane. In this way, the transient association of the HA1 globular domains and the cavity represent essential features of the fusion-competent structure directing the fusion sequence to the target membrane and preventing the interaction of hydrophobic sequences that leads to inactivation (see above). Eventually, in the presence of the target membrane, the formation of the extended trimeric extended coiled-coil structure enables the insertion of the fusion peptide into, and destabilisation of, the target membrane.

It is not known whether the partial dissociation of HA1 domains is reversible or whether it is accompanied by irreversible rearrangements preventing a reassociation of the HA1 domains. Of course, low pH may also destabilise other domains of the ectodomain. From the X-ray structure of the neutral form of HA X31 [1], it was concluded that the localisation of the N terminus of HA2 is stabilised by four intra-chain hydrogen bonds. At acidic conditions, these bonds may become destabilised facilitating the release of the fusion peptide. Indeed, studies on mutants of HA X31 suggested that a loss of the intra-chain hydrogen bonds results in a local destabilisation and facilitated the extrusion of the N terminus at higher pH with respect to the wild type [46,55,56]. Furthermore, rearrangement of other domains paralleling the opening of the HA1 domains is evident from the cryoEM structure of the HA ectodomain of A/Japan 305/57 [25]. Korte et al. [32,33] found, after preincubation of A/Japan 305/57 and X31 at suboptimal acidic pH in the absence of target membranes, an enhanced fusion activity after binding to target membranes and triggering fusion. This enhanced activity was not abolished by re-neutralisation, arguing for irreversible alterations of the HA ectodomain conformation differently from that leading to inactivation. Taken together, very likely, the partial dissociation or opening of HA1 head domains is irreversible. Presumably, as soon as water gets into contact to the HA2 loop region, the spontaneous transformation of this domain to an α-helix and the formation of the extended coiled-coil structure will prevent any reassociation of the HA1 head domains.

The fact that that formation of the extended coiled-coil structure occurs spontaneously as soon as water has access to the loop region of the HA2 domain may shed some new insights in the structure of the HA ectodomain of the commitment intermediate. It has been shown that after adopting the commitment state, for later steps leading to fusion, only the HA2 subunit is required. The HA1 domain could be removed enzymatically without affecting the fusion capacity of the commitment state [18]. From this observation, one can conclude that in the commitment state the loop region of HA2 is accessible to water and that the extended coiled-coil motif has been formed. The question is why fusion is still arrested and requires further incubations at low pH or higher temperatures at neutral pH although the extended coiled-coil structure has already been formed. One may speculate that the formation of the extended coiled-coil structure is not sufficient to mediate fusion and that other conformational changes are necessary which occur only after longer incubation at low pH or at higher temperatures at neutral pH. For example, the formation of the antiparallel helix bundle and the binding of the C terminus to the grooves of the N terminal coiled-coil may resemble such a step, which might be necessary for fusion giving rise to the tension to pull the membranes together [17]. Furthermore, the nature of the interaction of the fusion peptide with a membrane may change as a function of pH [57].

The presented model suggests that only the first step of the conformational change—the opening (partial dissociation) of the HA1 domain—requires a low pH. Further steps, leading eventually to fusion, also proceed at neutral pH. However, this does not preclude that those later steps reveal a

pH dependence, facilitating fusion at acidic pH. It has been observed recently that rapid membrane fusion at lower temperature mediated by the extended coiled-coil structure of the HA2 ectodomain (amino acids 1–127) requires protonation [58,59]. However, one has to be aware that fusion between two membranes mediated by an already preformed extended coiled-coil structure might be not directly comparable to a situation where the process of coiled-coil formation leads to fusion. Indeed, although the 1–127 construct shows similarities as the pH dependence of membrane fusion, significant differences exist with respect to intact HA. For example, the 1–127 construct is not able to mediate full fusion, which means mixing of content subsequently to pore formation [59].

7. Implication for fusion of influenza virus with the endosome membrane

Very likely, preservation of the spike morphology (i.e., a rather close association of the HA subunits) and its suggested function of guiding the coiled-coil and the fusion sequence toward the target membrane will be also characteristic for fusion of influenza virus at the native, biologically relevant situation, namely fusion of virus with the endosome membrane subsequent to its endocytic uptake. The endosome lumen is acidified by the increase of H^+-concentration due to the H^+-ATPase pump activity. However, the pH of the endosome lumen is decreased gradually, but does not drop instantaneously to about pH 5 where maximum fusion has been observed for various influenza viruses. Thus, the conformational change of HA and fusion will be triggered in the endosome already at pH values well above pH 5. At those pH values, the conformational change is not associated with a dissociation of the head domains and a fuzzy appearance in the time course of fusion (see above). Furthermore, at those pH values inactivation does not interfere significantly with the fusion activity. Thus, the structure of HA is optimised with respect to its fusion-mediating function to the specific acidic conditions of the endosome.

References

[1] I.A. Wilson, J.J. Skehel, D.C. Wiley, Nature 289 (1981) 366–373.
[2] E.O. Freed, M.A. Martin, J. Biol. Chem. 270 (1995) 23883–23886.
[3] D.C. Chan, D. Fass, J.M. Berger, P.S. Kim, Cell 89 (1997) 263–273.
[4] T. Maeda, S. Ohnishi, FEBS Lett. 122 (1980) 283–287.
[5] R.T. Huang, R. Rott, H.-D. Klenk, Virology 110 (1981) 243–247.
[6] J. White, K. Martin, A. Helenius, J. Cell Biol. 89 (1982) 674–679.
[7] C.M. Carr, P.S. Kim, Cell 73 (1993) 823–832.
[8] P. Bullough, F.M. Hughson, J.J. Skehel, D.C. Wiley, Nature 371 (1994) 37–43.
[9] H. Qiao, S.L. Pelletier, L. Hoffman, J. Hacker, R.T. Armstrong, J.M. White, J. Cell Biol. 141 (1998) 1335–1347.
[10] J. Chen, S.A. Wharton, W. Weissenhorn, L.J. Calder, F.M. Hughson, J.J. Skehel, D.C. Wiley, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 12205–12209.
[11] J. Chen, J.J. Skehel, D.C. Wiley, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 8967–8972.
[12] J. Lüneberg, I. Martin, F. Nüssler, J.M. Ruysschaert, A. Herrmann, J. Biol. Chem. 270 (1995) 27606–27614.
[13] P. Durrer, C. Galli, S. Hoenke, C. Corti, R. Gluck, T. Vorherr, J. Brunner, J. Biol. Chem. 271 (1996) 13417–13421.
[14] S.R. Durell, I. Martin, J.M. Ruysschaert, Y. Shai, R. Blumenthal, Mol. Membr. Biol. 14 (1997) 97–112.
[15] J.J. Skehel, D.C. Wiley, Ann. Rev. Biochem. 69 (2000) 531–569.
[16] D.M. Eckert, P.S. Kim, Ann. Rev. Biochem. 70 (2001) 777–810.
[17] J.A. Grunke, R.T. Armstrong, W.N. Newcomb, J.C. Brown, J.M. White, J. Virol. 76 (2002) 4456–4466.
[18] C. Schoch, R. Blumenthal, M.J. Clague, FEBS Lett. 311 (1992) 221–225.
[19] C.M. Carr, C. Chaudhry, P.S. Kim, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 14306–14313.
[20] D.P. Remeta, M. Krumbiegel, C.A.S.A. Minetti, A. Puri, A. Ginsburg, R. Blumenthal, Biochemistry 41 (2002) 2044–2054.
[21] D.C. Wiley, I.A. Wilson, J.J. Skehel, Nature 289 (1981) 373–378.
[22] R.M. Epand, R.F. Epand, Biochem. J. 365 (2002) 841–848.
[23] J.S. Oxford, T. Corcoran, A.L. Hugentobler, J. Biol. Stand. 9 (1981) 483–491.
[24] T. Stegmann, J.M. White, A. Helenius, EMBO J. 9 (1990) 4231–4241.
[25] C. Böttcher, K. Ludwig, A. Herrmann, M. van Heel, K. Stark, FEBS Lett. 463 (1999) 255–259.
[26] L.J. Godley, J. Pfeifer, D. Steinhauer, B. Ely, G. Shaw, R. Kaufmann, E. Suchanek, C. Pabo, J.J. Skehel, D.C. Wiley, S. Wharton, Cell 68 (1992) 635–645.
[27] G.W. Kemble, D.L. Bodian, J. Rose, I.A. Wilson, J.M. White, J. Virol. 66 (1992) 4940–4950.
[28] T. Bizebard, B. Gigant, P. Rigolet, B. Rasmussen, O. Diat, P. Bosecke, P.S.A. Wharton, J.J. Skehel, M. Knossow, Nature 376 (1995) 92–94.
[29] J.M. White, I.A. Wilson, J. Cell Biol. 105 (1987) 2887–2896.
[30] M. Krumbiegel, A. Herrmann, R. Blumenthal, Biophys. J. 67 (1994) 2355–2360.
[31] T. Stegmann, D. Hoekstra, G. Scherphof, J. Wilschut, J. Biol. Chem. 261 (1986) 10966–10969.
[32] T. Korte, K. Ludwig, M. Krumbiegel, D. Zirwer, G. Damaschun, A. Herrmann, J. Biol. Chem. 272 (1997) 9764–9770.
[33] T. Korte, K. Ludwig, F.P. Booy, R. Blumenthal, A. Herrmann, J. Virol. 73 (1999) 4567–4574.
[34] T. Kanaseki, K. Kawasaki, M. Murata, Y. Ikeuchi, S. Ohnishi, J. Cell Biol. 137 (1997) 1041–1056.
[35] F.P. Booy, in: J. Bentz (Ed.), Viral Fusion Mechanisms, CRC Press, Boca Raton, FL, 1993, pp. 67–88.
[36] A. Puri, F. Booy, R.W. Doms, J.M. White, R. Blumenthal, J. Virol. 64 (1990) 3824–3832.
[37] T. Shangguan, D.P. Siegel, J.D. Lear, P.H. Axelsen, D. Alford, J. Bentz, Biophys. J. 74 (1998) 54–62.
[38] R.W.H. Ruigrok, S.R. Martin, S.A. Wharton, J.J. Skehel, P.M. Bayley, D.C. Wiley, Virology 155 (1986) 484–497.
[39] P.N. Graves, J.L. Schulman, J.F. Young, P. Palese, Virology 126 (1983) 106–116.
[40] R.G. Webster, L.E. Brown, D.C. Jackson, Virology 126 (1983) 587–599.
[41] R.W. Doms, A. Helenius, J. White, J. Biol. Chem. 260 (1985) 2973–2981.
[42] R.W.H. Ruigrok, N.G. Wrigley, L.J. Calder, S. Cusack, S.A. Wharton, E.B. Brown, J.J. Skehel, EMBO J. 5 (1986) 41–49.
[43] R.W.H. Ruigrok, A. Aitken, L.J. Calder, S.R. Martin, J.J. Skehel, S.A. Wharton, W. Weis, D.C. Wiley, J. Gen. Virol. 69 (1988) 2785–2795.
[44] R.S. Daniels, S. Jeffries, P. Yates, G.C. Schild, G.N. Rogers, J.C. Paulson, S.A. Wharton, A.R. Douglas, J.J. Skehel, D.C. Wiley, EMBO J. 6 (1987) 1459–1465.
[45] C. Barbey-Martin, B. Gigant, T. Bizebard, L.J. Calder, S.A. Wharton, J.J. Skehel, M. Knossow, Virology 294 (2002) 70–74.

[46] R.S. Daniels, J.C. Downie, A.J. Hay, M. Knossow, J.J Skehel, M.L. Wang, D.C. Wiley, Cell 40 (1985) 431–439.
[47] Q. Huang, R. Opitz, E.-W. Knapp, A. Herrmann, Biophys. J. 82 (2002) 1050–1058.
[48] P. Beroza, D.R. Fredkin, M.Y. Okamura, G. Feher, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 5804–5808.
[49] D. Bashford, M. Karplus, J. Phys. Chem. 95 (1991) 9556–9561.
[50] G.M. Ullmann, E.W. Knapp, Eur. Biophys. J. 28 (1999) 533–551.
[51] D. Baker, D.A. Agard, Structure 2 (1994) 907–910.
[52] C. Gray, L. Tamm, Protein Sci. 6 (1997) 1993–2006.
[53] S.A. Wharton, L.J. Calder, R.W.H. Ruigrok, J.J. Skehel, D.A. Steinhauer, D.C. Wiley, EMBO J. 14 (1995) 240–246.
[54] C.-H. Kim, J.C. Macosko, Y.G. Yu, Y.-K. Shin, Biochemistry 35 (1996) 5339–5365.
[55] W.I. Weis, S.C. Cusack, J.H. Brown, R.S. Daniels, J.J. Skehel, D.C. Wiley, EMBO J. 9 (1990) 17–24.
[56] D. Shental-Bechor, T. Danieli, Y.I. Henis, N. Ben-Tal, Biochim. Biophys. Acta 1565 (2002) 81–90.
[57] X. Han, J.N. Bushweller, D.S. Cafiso, L.K. Tamm, Nat. Struct. Biol. 8 (2001) 715–720.
[58] R.F. Epand, J.C. Macosko, C.J. Russell, Y.K. Shin, R.M. Epand, J. Mol. Biol. 286 (1999) 489–503.
[59] E. Leikina, D.L. LeDuc, J.C. Macosko, R. Epand, R. Epand, Y.K. Shin, L.V. Chernomordik, Biochemistry 40 (2001) 8378–8386.
[60] A. Nicholls, GRASP: Graphical Representation, Analysis of Surface Properties, Columbia Univ., New York, 1992.