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ARTICLE

Conformational change of influenza virus hemagglutinin is sensitive to ionic concentration

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Abstract The homotrimeric spike glycoprotein hemagglutinin (HA) of influenza virus undergoes a low pH-mediated conformational change which mediates the fusion of the viral envelope with the target membrane. Previous approaches predict that the interplay of electrostatic interactions between and within HA subunits, HA 1 and HA2, are essential for the metastability of the HA ectodomain. Here, we show that suspension media of low ionic concentration promote fusion of fluorescent labelled influenza virus X31 with erythrocyte ghosts and with ganglioside containing liposomes. By measuring the low pH mediated inactivation of the fusion competence of HA and the Proteinase K sensitivity of low pH incubated HA we show that the conformational change is promoted by low ionic concentration. We surmise that electrostatic attraction within the HA ectodomain is weakened by lowering the ionic concentration facilitating the conformational change at low pH.

Keywords Influenza virus · Hemagglutinin · Fusion · Ionic concentration · Fluorescence

Abbreviations

HA	Hemagglutinin
PBS	Phosphate buffered saline
R18	Octadecylrhodamine B chloride
FDQ	Fluorescence dequenching

Introduction

The influenza virion is an enveloped virus. Viruses bind to sialic acid containing receptors of the host cell via their spike glycoprotein hemagglutinin (HA). HA is the key protein for cell entry and the release of the viral genome into the cytoplasm of the host cell.

The HA is organized as a noncovalent associated homotrimer formed by the precursor monomers HA0 in the Endoplasmic Reticulum. To activate its fusogenic properties, each HA0 monomer has to be proteolytically processed into the two subunits: HA1 and HA2. Both subunits remain linked by a disulfide bond. The three-dimensional crystal structure of the bromelain-cleaved ectodomain of HA from influenza strain X31 (subtype H3) at neutral pH has been obtained at a resolution of 3 Å (Wilson et al. 1981). Recently, the crystal structures of the HA ectodomain from H1, H5,

Dedicated to Prof. K. Arnold on the occasion of his 65th birthday.

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H9 and H7 subtypes have been reported (Gamblin et al. 2004; Stevens et al. 2004, 2006; Ha et al. 2002; Russell et al. 2004). Overall, the ectodomain consists of a globular head region and of an elongated fibrous stem region largely formed by a triple stranded coiled coil.

The processed HA is supposed to be metastable (Carr and Kim 1993). Upon endocytosis of virions, the acidic pH of the endosomal lumen triggers an irreversible conformational change of the HA ectodomain. Thereby an extended trimeric coiled-coil structure is formed (Bullough et al. 1994; Chen et al. 1998), and the 20 amino acid long hydrophobic N-terminus of HA2 (the so-called fusion peptide) is moved to the distal end of HA and exposed towards the target membrane. The conformational change mediates the fusion between the viral and the endosomal membrane releasing the viral genome from the envelope. Destabilisation of the target membrane by the fusion peptide is believed to initiate fusion (Lüneberg et al. 1995; Durrer et al. 1996).

Recently, based on the crystal structure of HA from X31, we have shown that electrostatic interactions between HA1 and HA2 monomers are a determinant of the stability of the HA ectodomain at neutral pH (Huang et al. 2002, 2003). At low pH electrostatic attraction is weakened by the enhanced protonation of amino acid residues of the ectodomain. However, this conclusion warranted further justification. We hypothesized that if electrostatic interaction is essential for the tight association of the HA monomers, the stability of the ectodomain should be sensitive to the ionic concentration of the suspension medium and, thus, may affect the fusion properties of HA. To test this hypothesis, we have investigated here the influence of physiological and low ionic concentrations on the conformational change of HA and on fusion of influenza virus X31 with erythrocyte membranes and with liposomes containing the ganglioside GD_{1a}. We used influenza virus X31 (H3 subtype) because its fusion behaviour at low pH has been well described previously.

Materials and methods

Materials

Octadecylrhodamine B chloride (R18) was purchased from Molecular Probes (Eugene, OR, USA). Fresh blood from healthy donors was obtained from the local Blood Bank. Phosphatidylcholine (PC), phosphatidylserine (PS), disialoganglioside GD_{1a} and Proteinase K were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Virus preparation

Influenza virus was grown for 48 h in the allantoic cavity of 11-day-old embryonated hen eggs. The allantoic fluid was collected, and cell debris was removed by a low speed spin (880×*g*, 30 min). The virus was pelleted by spinning the allantoic fluid at 95,000×*g* for 90 min. The pellet was resuspended in PBS and homogenized with a Teflon-coated homogenizer.

Buffers

The following buffers were used: phosphate-buffered saline (5.8 mmol of phosphate per liter, pH 7.4 or pH 5.0) (1) with 150 mmol/l NaCl (PBS 150) or (2) without NaCl (PBS 0), and (3) sodium acetate buffer (20 mmol/l of sodium acetate, 150 mmol/l of NaCl; pH , 5.0) (NaAc).

Proteinase K assay

After incubation of influenza virus at the desired conditions (pH, ionic concentration, temperature), 50 µg of virus were resuspended in 200 µl PBS 150, pH 7.4, mixed with 30 µl Proteinase K (1 mg/ml in PBS) and incubated for 30 min at 37°C. Subsequently, the mixture was vortexed with 0.5 ml trichlor acetic acid (diluted by 1:5) for 5 min at room temperature and centrifuged at 20.000×*g* (4°C) for 5 min. The supernatant was discarded and 1 ml of ice-cold ethanol was added to the pellet. After vortexing and centrifugation, the pellet was washed twice in PBS 150 and resuspended in 50 µl PBS 150. 12.5 µl of sample buffer was added and 30 µl of this sample was transferred to 12.5% polyacrylamide gel. After staining with coomassie blue gels were imaged using a DESAGA ProviDoc documentation system (SARSTEDT AG, Nümbrecht, Germany) and bands were analysed with AIDA Image Analysis Software (Raytest GmbH, Straubenhardt, Germany).

Erythrocyte and ghost preparations

After removal of buffy coat and plasma, erythrocytes were washed three times in PBS 150 (pH 7.4). Unsealed erythrocyte ghosts were prepared by the method of Dodge et al. (1963).

Liposome preparation

Lipids and gangliosides were dissolved in chloroform and mixed in a glass tube with a molar ratio of PC:PS:GD_{1a} 50:45:5. Solvent was evaporated under nitrogen stream. Lipids were dissolved in a small volume of ethanol (final concentration below 1% v/v), 1 ml PBS buffer was added and vortexed. Afterwards five freeze–thaw cycles were performed by freezing for 10 min at –70°C and thawing for 5 min at 50°C (waterbath). To prepare large unilamellar vesicles (LUV) suspension was passed 10 times through a 0.1 µm-polycarbonate filter using an extruder (Lipex Biomembranes, Vancouver, BC, Canada). LUV were stored at 4°C for further use.

Labelling of virus for fusion

A 1.25 µl volume of a 2 mM stock solution of R18 in ethanol was added by rapid vortexing to 0.25 ml of influenza virus (1 mg of virus protein/ml). After incubation for 30 min at room temperature (in the dark), the virus was washed by high-speed centrifugation (45,000×*g*) with ice-cold PBS 150 to remove unbound R18 and resuspended to a concentration of 1 mg of virus protein/ml (Arbuzova et al. 1994, Herrmann et al. 1993a). The final concentration of added probe corresponds to approximately 2 mol% of total viral lipid.

The protein concentration of viruses as well as of ghosts was determined by the method of Lowry et al. (1951).

Inactivation of influenza virus

R18 labelled influenza virus (1 mg/ml) in PBS 150 or in PBS 0 was preincubated at low pH (see the figure legends) in the absence of the target membrane. After different periods, samples of the virus suspension were neutralized (pH 7.4) and kept on ice in PBS 150. Subsequently, virus was bound to ghosts and fusion was measured in sodium acetate buffer at pH 5.0, 37°C, as described subsequently.

Virus binding to ghost membranes or liposomes

Labelled virus (0.1 mg of protein) was incubated for 15 min on ice with 0.1 ml of erythrocyte ghost suspension (6–7 mg of protein/ml) or 0.8 ml of liposomes (1.2 µmol lipid/ml).

Fusion assay

Fusion was measured by monitoring the fluorescence dequenching (FDQ) of the lipid-like fluorophore R18 upon fusion of R18-labelled viruses with ghost mem-

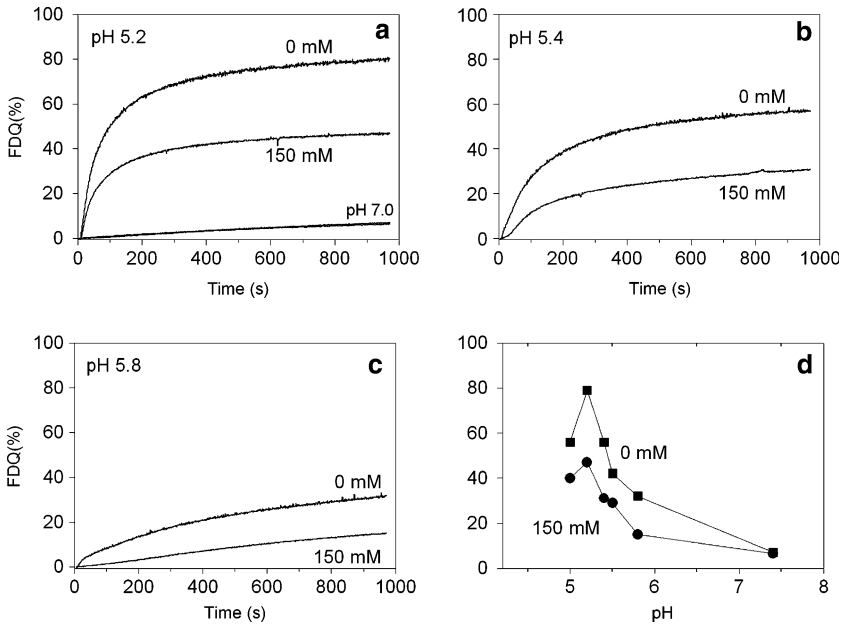

Fig. 1 Fusion of influenza virus with erythrocyte ghost membranes as a function of pH and ionic concentration at 37°C. **a–c** Kinetics of fusion were measured by fluorescence dequencing (FDQ) of the lipid-like fluorophore R18 incorporated initially into the viral membrane at self-quenching concentrations (see "Materials and methods") at pH 5.2 (**a**), 5.4 (**b**), and 5.8 (**c**). Fusion was measured either at physiological NaCl concentration (PBS 150, 150 mM) or at low ionic concentration (PBS 0, 0 mM). At neutral pH only a very slow and low fluorescence dequenching was observed (see **a**, pH 7.0). **d** Extent of FDQ measured 960 s after triggering fusion. Typical experiments out of three are shown

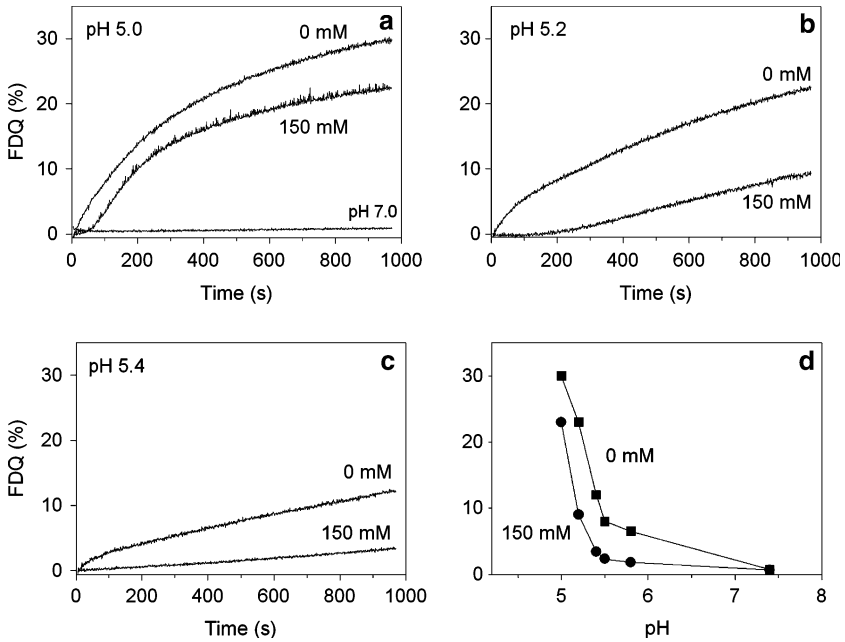

Fig. 2 Fusion of influenza virus with erythrocyte ghost membranes as a function of pH and ionic concentration at 20°C. **a–c** Kinetics of fusion were measured by fluorescence dequenching (FDQ) of the lipid-like fluorophore R18 incorporated initially into the viral membrane at self-quenching concentrations (see "Materials and methods") at pH 5.0 (**a**), 5.2 (**b**), and 5.4 (**c**). Fusion was measured either at physiological NaCl concentration (PBS 150, 150 mM) or at low ionic concentration (PBS 0, 0 mM). At neutral pH fluorescence dequenching was negligible (see **a**, pH 7.0). **d** Extent of FDQ measured 960 s after triggering fusion. Typical experiments out of three are shown

branes (Hoekstra et al. 1984). Subsequently 30 µl of ice-cold virus-ghost suspension was transferred to a quartz cuvette containing 1.8 ml of sodium acetate buffer or PBS buffer (PBS 150 or PBS 0) at the pre-adjusted temperature and the respective pH (pH 5.0–7.4). The suspension was stirred with a Teflon-coated magnetic stir bar. Fusion was monitored continuously by measuring FDQ (λ_{ex} = 560 nm; λ_{em} = 590 nm; cut-off filter, 570 nm), with a time resolution of 1 s, with an AMINCO Bowman II fluorescence spectrometer. At the end of each experiment, Triton X-100 (final concentration, 0.5%) was added to obtain maximum R18 fluorescence, $F(\max)$. The percentage of FDQ was calculated as described previously (Blumenthal et al. 1987):

$$\mathrm{FDQ} = \frac{F(t) - F(0)}{F(\max) - F(0)} \times 100\%$$

where $F(0)$ and $F(t)$ are the fluorescence intensity before starting fusion and the fluorescence intensity at a given time, t, respectively.

For fusion of virus with ganglioside containing LUV, 90 µl of virus-liposome suspension were used and the experiment was performed as described earlier.

Results

Low pH-triggered fusion of R18-labelled influenza virus X31 with erythrocyte ghost membranes was measured in buffered suspension at physiological NaCl concentration (PBS 150) and at low ionic concentration in the absence of NaCl (PBS 0) at 37°C (Fig. 1) and at 20°C (Fig. 2). Fusion was assessed by a lipid mixing assay utilizing the fluorescence dequenching of the lipid-like fluorophore R18 initially incorporated at selfquenching concentration in the virus membrane. As shown previously for X31, a lag phase occurs between the low pH trigger and the onset of lipid mixing (Clague et al. 1991). This phase may provide clues for the various processes affecting HA-mediated fusion. It has been shown to depend on the surface density of HA (Clague et al. 1991; Danieli et al. 1996), pH (Clague et al. 1991), temperature (Pak et al. 1994; Schoch et al. 1992), packing of the membrane (Herrmann et al. 1993b) and nonabsorbing polymers (Herrmann et al. 1993a). The lag phase can hardly be detected at optimal conditions of fusion (pH ~5.0, 37°C) as evident from Fig. 1a. However, suboptimal pH and temperature allow resolving the lag time. Therefore, we have studied virus fusion also at those conditions.

The ionic concentration has a significant effect on the kinetics of fusion. At low ionic concentration fusion was much faster in comparison to physiological ionic concentration. At physiological ionic concentration, we observed a lag phase at 37°C for pH ≥ 5.2 (Fig. 1a–c) and at 20°C for all acidic pH values studied (Fig. 2a–c). In particular, at suboptimal conditions we found rather long lag phases. At 10°C the delay time of the onset of fusion was about 80 and 360 s at pH 5.0 and pH 5.2. In contrast, no lag phase was observed at low ionic concentration neither at 37°C (Fig. 1a–c), 20°C (Fig. 2a–c) nor at 10°C (data not shown).

Furthermore, the fusion extent was enhanced at low ionic concentration (Figs. 1d, 2d). However, the pH dependence of the extent was not affected.

To address whether the low ionic concentration affects the properties of the viral fusion machinery or makes the surface of ghosts more fusogenic, we studied the fusion of influenza viruses with liposomes containing the ganglioside GD_{1a} as a receptor at 37°C and 20°C (Fig. 3). We found similar effects of low ionic concentration on fusion as observed for ghosts. Fusion was faster, in particular at 20°C, and the extent was enhanced in the absence of NaCl. Although we could not detect a lag phase at 150 mM NaCl (PBS 150) at 37°C (Fig. 3a, b), we found a significant delay at 20°C (Fig. 2c, d). Again, at low ionic concentration we could not detect any delay time even at 20°C.

These results indicate that the ionic concentration affects directly the fusion properties of hemagglutinin. To explore this further, we preincubated influenza virus at low pH. Several studies have shown that the fusiogenic properties of HA rapidly inactivate at low pH in the absence of target membranes, in particular at 37°C (White et al. 1982; Sato et al. 1983; Korte et al. 1997). R18-labelled influenza virus was preincubated at pH 5.2, 37°C, for 10 min at physiological (PBS 150) and low (PBS 0) ionic concentrations. Subsequently, preincubated viruses were bound to ghosts on ice at neutral pH. Fusion was measured at pH 5.0, 37°C. As can be seen fusion activity was decreased by about 50% for viruses preincubated at physiological ionic concentration. In contrast, fusion was almost completely abolished when viruses were preincubated at low ionic concentration (Fig. 4a). The control sample was treated in a similar way except that preincubation was done at neutral pH. No difference was observed between control samples preincubated either at high (shown in Fig. 4a) or low ionic concentration (not shown).

Low pH mediated inactivation becomes very slow when viruses were incubated at 20°C at physiological ionic concentration (Fig. 4b), confirming our previous results (Korte et al. 1997). However, when viruses were preincubated at low ionic concentration, 20°C, again, fusion activity was abolished.

To further support the influence of ionic concentration on the conformational change, we treated low pH preincubated influenza virus with Proteinase K. It has been shown that the ectodomain becomes sensitive to Proteinase K upon low pH triggered conformational change of HA (Doms et al. 1985). We measured digestion of HA by Proteinase K after preincubation of

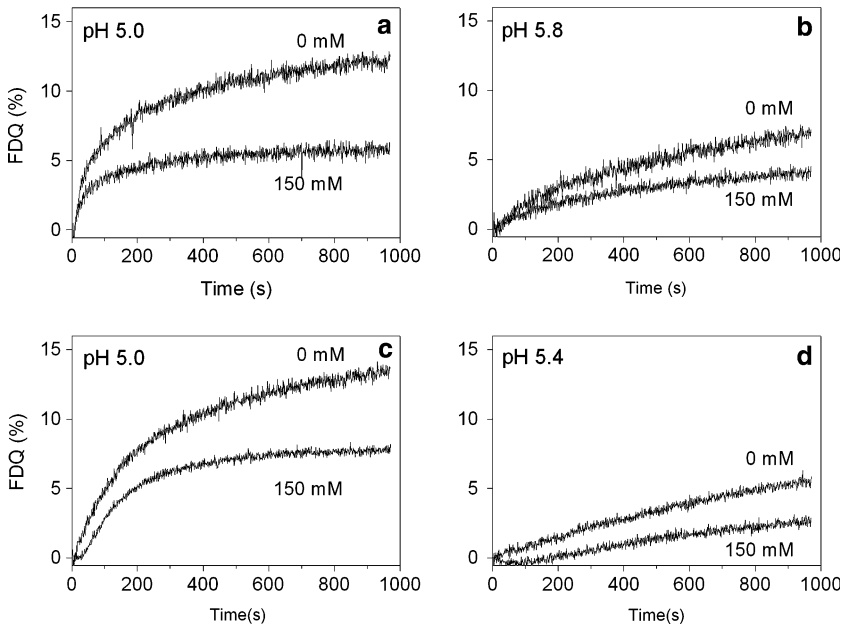

Fig. 3 Fusion of influenza virus with liposomes containing the ganglioside GD_{1a}. Liposomes were prepared from PC, PS and GD_{1A} (5 mol%) and incubated with R18 labelled influenza virus as described in "Materials and methods". Kinetics of FDQ were measured at 37°C (**a**, **b**) or at 20°C (**c**, **d**) at physiological (PBS 150, 150 mM) or at low ionic concentration (PBS 0, 0 mM) at various pH. No FDQ was observed at neutral pH (not shown). Typical experiments out of three are shown

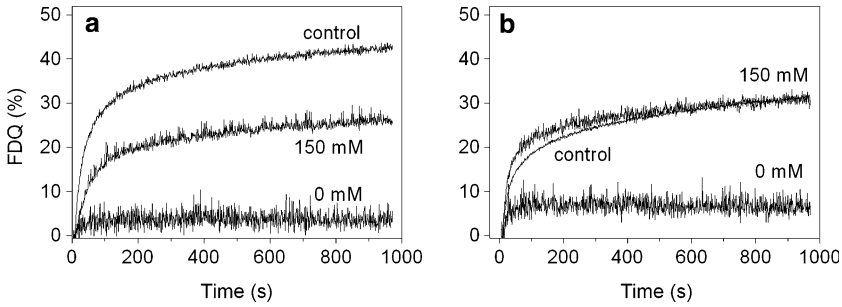

Fig. 4 Inactivation of influenza virus by preincubation at acidic pH. R18-labelled influenza virus was preincubated at physiological NaCl concentration (PBS 150, 150 mM) or at low ionic concentration (PBS 0, 0 mM) at low pH for 10 min. After preincubation virus was bound to ghost membranes at neutral pH on ice (see "Materials and methods"). Subsequently fusion was measured in sodium acetate buffer at pH 5.0, 37°C. Preincubation was done at pH 5.2, 37°C (**a**) and at pH 5.0, 20°C (**b**). Control–preincubation for 10 min at pH 7.4 in PBS 150

influenza virus at low pH (pH 5.2) and 37°C. In Fig. 5, the results of three independent experiments are shown. In each experiment we found a higher sensitivity to Proteinase K treatment after preincubation at low ionic concentration. The relatively large standard error is due to the high variability of the data between independent experiments.

Discussion

In the present study we observed that membrane fusion of influenza virus X31 is faster, and the fusion extent becomes enhanced in suspension media of low ionic concentration with respect to physiological ionic concentration. When influenza virus X31 was preincubated at acidic pH in the absence of target membranes, we found that the inactivation of the HA fusion competence was much higher at low ionic concentration. Furthermore, HA became more sensitive to Proteinase K digestion when acidic pH incubation was done at low ionic concentration. Since both the inactivation and the Proteinase K sensitivity are related to the conformational change of HA, the results indicate that low ionic concentration supports destabilization and the structural transition of the HA ectodomain at low pH.

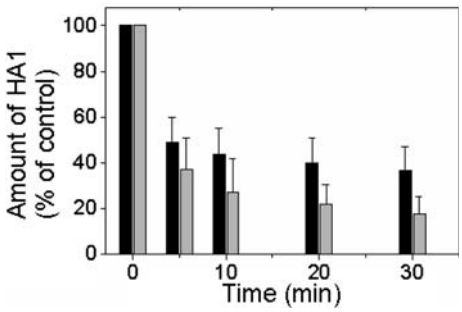

Fig. 5 Proteolytical digestion of the HA ectdomain. Influenza virus was preincubated at pH 5.2, 37°C, either at physiological (150 mM; *black bars*) or low (0 mM; *grey bars*) NaCl concentration for various times. Subsequently, virus suspension was resuspended at neutral pH and incubated with Proteinase K (see Materials and methods). Samples were run on polyacrylamide gels and the intensity of the HA1 band was quantified using AIDA image analysis software (see "Materials and methods"). Controls correspond to virus preincubated at neutral pH. The average ± standard error of estimate is given ($n = 3$)

The lag phase between acidification and the onset of membrane fusion was reduced at low ionic concentration. Markosyan et al. (2001) have concluded that the rate-limiting step in HA mediated fusion is the irreversible conformational change of HA. This suggests that the lag phase depends on the rate of the conformational change. Thus, a shorter delay time at low ionic concentration could be explained by facilitated mediated refolding of HA at low pH. The lag phase has been shown to depend also on the surface density of HA (Clague et al. 1991; Danieli et al. 1996; Blumenthal et al. 1996; Leikina et al. 2002). Analysis of fusion kinetics has led to the conclusion that the fusion pore is formed by several activated HA trimers. Depending on the approach and/or the underlying model of data analysis the number of HA trimers forming a fusion pore differs varying between 4 and 8 (Danieli et al. 1996; Blumenthal et al. 1996; Mittal and Bentz 2001). Based on that, it has been suggested that the lag phase reflects the formation of the fusion pore by several HA trimers and, thus, is dependent on the HA density and processes which determine the lateral rearrangement of HA trimers such as lateral diffusion. It is obvious that alterations of the HA density cannot account for our results. Furthermore, since HA trimers are densely packed in the viral envelope lateral diffusion and rearrangement of trimers and any effect of ionic

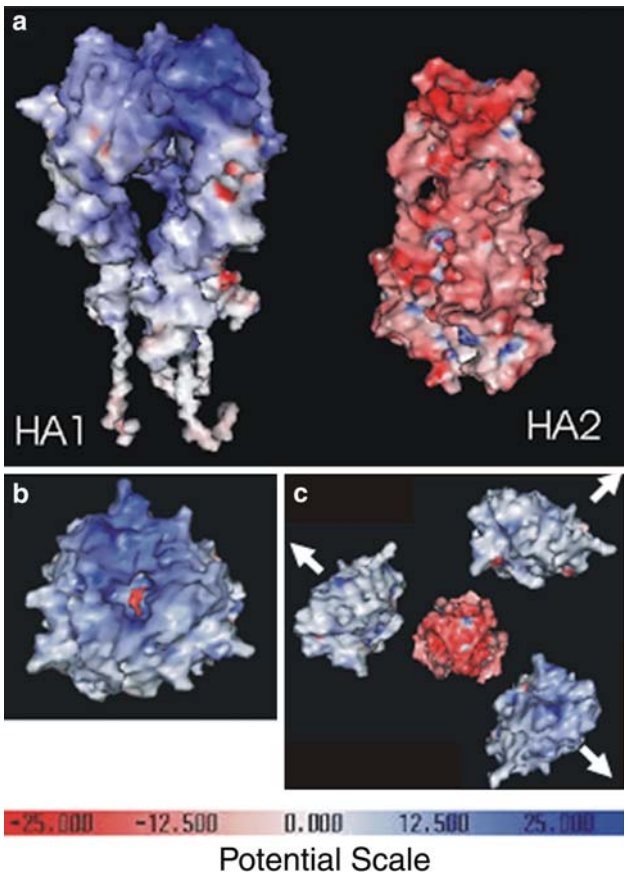

Fig. 6 Surface potential of the HA1 and the HA2 subunits. The surface potential of the HA 1 domain is positive (*blue*) while that of the HA2 domain negative (*red*) (**a**). Thus, the electrostatic force between three subunits either of HA1 or of HA2 is repulsive. However, the attractive electrostatic interaction between HA1 and HA2 domains accounts for the stability of the HA ectodomain (**b**, *top view* of the HA ectodomain). For further details, see text and Huang et al. (2002, 2003). Since the net charge in the HA1 domain is positive, increased protonation of this domain at low pH will lead to an even more positive surface potential 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**. Low ionic concentration may strengthen the repulsive force between the distal part of the HA1 subunits. Surface electrostatic potential of HA1 domain and HA2 domain was calculated with GRASP (Nicholls 1992). Electrostatic potential is colour-coded using a *sliding scale* indicated in the *lower part* of the figure (units in kBT/e). *Red* represents negative electrostatic potential, *blue* represents positive electrostatic potential and *white* is neutral

concentration on that should not be a determinant of fusion pore assembly.

We surmise that low ionic concentration affects the electrostatic interaction between the HA subunits in the trimer of influenza virus X31 and, thereby, the conformational change. The relevance of electrostatic interactions for the stability of the native nonfusogenic state of HA protein has been shown by a molecular modelling approach which was supported by experimental work (Huang et al. 2002, 2003). Based on the crystal structure of HA (X31 strain; subtype H3) at neutral pH (PDB: 1HGF) the approach unravelled that the surface potentials of HA1 and HA2 are essential for the stability of HA. The HA1 surface is positively charged, while the HA2 has a negatively charged surface (Fig. 6a, b). Thus, at neutral pH, the electrostatic interaction between trimeric subunits of HA1 or of HA2 is repulsive. For example, HA1 subunits alone would not be able to form a stable trimer. However, the interaction between HA1 and HA2 subunits is attractive enabling the formation of a stable trimer (Fig. 6a, b). At low pH an enhanced protonation of amino acid residues, such as the negatively charged Glu or Asp residues, reduces electrostatic attraction and thereby weakens the tight association of the globular HA1 head domains which relocate sideways (Fig. 6c; Huang et al. 2002, 2003). The relocation of the HA1 subunits enables subsequent steps of conformational changes towards a fusogenic state. Indeed, the tight association of the HA1 distal domains in the neutral pH form is considered to function as a 'clamp' for HA2 subunits (Carr et al. 1997), which prevents the formation of the extended trimeric coiled-coil structure. Previous studies have provided strong evidence that the dissociation of the HA1 distal domains is an important step of the conformational change of HA (Godley et al. 1992; Kemble et al. 1992). In those studies it was shown that covalent linkage of the HA1 distal domains by disulfide bonds prevented the conformational change and the associated exposure of the fusion sequence. Only after breakage of these bonds, the fusion activity was restored.

We suggest that the reduced shielding of the positive surface charge at low ionic concentration enhances repulsion between the distal globular domains of HA and, thus, reduces the stability of the trimeric HA ectodomain. As a consequence the conformational change at acidic pH would be facilitated in a media of low ionic concentration. Furthermore, due to the densely packed trimers in the viral envelope, interaction of activated trimers could support the refolding of adjacent trimers and thereby the formation of a fusion pore. Markovic et al. (2001) have concluded that the degree of HA activation rises with increasing surface density and, thus, HA activation exhibits positive cooperativity. This provides a way by which synchronized release of conformational energy of adjacent HA molecules is achieved circumventing that the first activated trimer at the fusion site would be already

discharged by the time the next trimer undergoes a conformational change. Thus, at low ionic concentration the shorter lag phase may not only result from a facilitated conformational change of HA. A more synchronized activation of HA trimers may contribute as well which may also explain an enhanced fusion extent. On the other hand, such a cooperative interaction would also enhance inactivation of HA in the absence of the target membrane. Leikina et al (2002) observed that irreversible refolding of HA is accelerated for higher HA densities. They suggested that the irreversible conformational change and inactivation of HA are associated with interactions of activated HA trimers.

Enhancement of fusion at low ionic concentration may also be a consequence of altered surface potential of the membrane surface and the HA ectodomain. The membrane surface potential of red blood cell membranes and liposomes is negatively charged. For red blood cell membranes the surface potential is essentially determined by sialic acid residues of the glycocalyx. In case of liposomes the negatively charged head group of PS and the sialic acid of GD_{1a} contribute to the surface potential. While shifting the pH in the range between 7.4 and 5.0 has only a minor influence, the surface charge of PS containing liposomes (Papahadjopoulos 1968) and protonation of sialic acid residues of GD1a (Rodrigues et al., 1996) decreasing the ionic strength leads to a more negative surface potential of red blood cells (Herrmann et al. 1986) and liposomes with negative charged lipids (Eisenberg et al. 1979). Therefore, lowering the ionic concentration may enhance attractive interaction between membrane surfaces and the ectodomain which has a positively charged surface potential at neutral pH (see Fig. 6), which in turn could modulate the fusion behaviour. However, the inactivation behaviour and the proteinase K assay clearly show that the ionic concentration directly affects the conformational change.

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