Membrane fusion of Influenza and chikungunya viruses

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The importance of the stability of the influenza hemagglutinin globular bottom probed in single-particle membrane fusion assays

Abstract

The influenza hemagglutinin protein (HA) is involved in several key processes of cell entry by the influenza virus, including receptor binding and membrane fusion. To catalyze fusion, HA undergoes large conformational rearrangements to insert into the membrane of the target cell, to bring the cellular and viral membranes together, and to drive formation of a fusion pore so that the viral genome can enter the cell. During the past decades, the importance of the different domains of the HA protein to its fusogenic capability has been studied extensively. We describe here studies of a domain at the base of HA, the globular bottom, that is important in the refolding of HA after extension. The stability of the globular bottom has been suggested to influence the sequence of the conformational changes that are critical for fusion. We sought to determine the effect of destabilizing mutations in the globular bottom on the mechanism of fusion, using influenza A viruses with engineered mutations identified previously in all-atom molecular dynamics simulations. Using a fluorescence microscopy single-particle fusion assay, we find that the fraction of the population of viruses undergoing fusion correlates with globular bottom stability. Destabilized mutants were more susceptible to neutralization of fusion by HA-binding inhibitors. Also, we find that the globular bottom mutations affected HA incorporation during virus assembly, reducing the number of HA available per virion. Using a model in which influenza fusion relies on the stochastic occurrence of (near) simultaneous folding events, we postulate that this reduced HA incorporation explain the single-particle observations.

3.1 Introduction

3.1.1 Influenza membrane fusion

Influenza virus is the cause of yearly epidemics with the potential for the occurrence of a global pandemic. This yearly re-occurring threat is largely due to the ability of the virus to extensively mutate. The virus is enveloped by a lipid membrane, and fusion of this membrane with that of the target cell is a key step in its entry process. Understanding the fusion mechanism of influenza virus is paramount to the development of rationally designed antivirals that target this indispensable step in the viral replication cycle. High kinetic barriers prevent spontaneous membrane fusion to occur on a biological timescale.

The influenza hemagglutinin protein (HA) on the outside of the virus mediates influenza cell attachment and is the catalyst of fusion of the viral and host cell membranes, helping to overcome the membrane fusion barriers. HA is a homotrimeric protein that is part of a class of viral fusion proteins that also contains the HIV gp41 protein, a class defined by major alpha-helical structural motifs.

Upon attachment to the cell, the virus is taken up into an endosomal compartment. Maturation of the endosome causes the internal pH to reduce, which is the trigger for the HA to undergo large-scale conformational changes. The sequence of HA conformational changes is depicted in Figure 3.1. The acid-triggered HA opens up its top (Figure 3.1B, orange) and an amphiphilic fusion peptide (Figure 3.1, red) is projected towards the target membrane by formation of an alpha-helical coiled coil, leading to an extended intermediate bridging both membranes (Figure 3.1C). Subsequently, the base of the HA, termed the globular bottom (Figure 3.1, yellow), melts and zippers up along the coiled-coil core (Figure 3.1D), bringing the transmembrane domain and the fusion peptide together. The conformational changes mediate hemifusion of both membranes, where the proximal leaflets have merged (Figure 3.1D). The association of transmembrane domains and fusion peptides leads to the opening and expansion of a pore (Figure 3.1E) after which the genome can enter the cell and replication can commence.

Figure 3.1 The membrane fusion pathway mediated by influenza hemagglutinin. (A) The HA1 subunit (orange) binds sialic-acid moieties on target-cell receptors (dark brown). (B) After acidification, the HA1 subunits give way and the
The role of several domains in membrane fusion has been asserted through mutational studies. The globular head of HA, denoted HA1 (Figure 3.1A, orange), binds to cellular receptors and docks the virus. The HA1 domains need to be able to move away for fusion to take place, as was demonstrated by introducing cysteines that cross-linked the globular heads within an HA trimer.\textsuperscript{122,123} Release of the fusion peptide is rate limiting for extension, and forward and reverse mutations in the fusion peptide and its pocket have been found to affect the rate of release.\textsuperscript{32} Mutational studies of, and the use of peptides complementary to, the part of the globular bottom that zippers up along the HA trimer core have shown that the zippering is necessary for fusion to occur.\textsuperscript{134,135} Residues in the very end of the zipper are important for formation of an N-cap and are necessary for opening and enlargement of a pore.\textsuperscript{136} While the role of the zippering function of the globular bottom is now fairly well established, it is not clear how globular bottom stability affects the processes leading up to extension, for example potentially affecting the success rate of insertion.\textsuperscript{31,146}

3.1.2 Critical interactions in the globular bottom of influenza hemagglutinin

All-atom molecular dynamics (MD) simulations have identified key residues in the globular bottom that stabilize the domain vertically through an intra- and inter-trimer hydrogen-bonding network (Figure 3.2).\textsuperscript{219} Here follows a description of these simulations as they have been used as the starting point for the experimental work described in this Chapter. To reduce computing time, the molecular dynamics simulations used a truncated part of prefusion HA2, comprising residues 112 to 175. The stability of the globular bottom was probed by measuring the unfolding time under a constant force along the long axis of the protein, at the viral-membrane-proximal end of the domain while keeping the distal end fixed. Any vertical hydrogen bond that remained intact until macroscopic chain unfolding, and that strongly correlated with this unfolding event, was defined as being critical. Dissociation of a critical contact therefore was by definition necessary for macroscopic chain unfolding and represented the rate-limiting step in the unfolding process.

According to the MD simulations, the four most critical vertical hydrogen bonds were those between residues E128 (glutamic acid) and R170 (arginine), and E131 and R163 (Figure 3.2). The first of these pairs, E128 and E131, was located in the part of the globular bottom that stays around the HA base, whereas R163 and R170 end up in the leash that zippers up along the HA core. The residues were identified in the influenza A strain X31 (H3N2, A/Aichi/68).
Mutating these residues in the globular bottom significantly reduced the stability under pulling force, apparent as a change in unfolding time (Figure 3.3). The hydrogen bonding capabilities of E131 were disabled by introducing glutamine instead of glutamic acid, removing the contacts E131 had with R163 and E170. Introduction of alanine at R163 kept the helical propensity of that residue whilst also removing the capability for hydrogen bonding. Both mutations resulted in similar unfolding times, as expected for these binding partners. Furthermore, they significantly reduced the unfolding time compared to the wild-type structure. The double mutation E131Q-R163A did not result in a significant change with respect to the single mutations. Another residue making vertical contacts was E128, which bound with R163 and R170. The double mutation E128Q-E131Q did result in a significant speed-up of unfolding, as this removed all possibilities for R163 and R170 to make vertical contacts.

The reverse effect, globular bottom stabilization, was attempted in a different influenza strain. Residues E128, E131, R163 and R170 were found to be fully conserved across the X31 strain phylogenetic group, group 2. This group is defined by a conserved histidine at position 106. However, this conservation is not the case for group 1 viruses, such as PR8 (H1N1, A/Puerto Rico/8/34), where the residues identified in the simulations were present only sparsely and inconsistently. Therefore, a separate set of simulations characterized the unfolding time of the wild-type PR8 strain. It was found to be significantly faster than X31 wild-type (Figure 3.3). Introducing the networking residues from group 2 by mutations N128E, K131E and S163R resulted in a significant increase in the stability of PR8 HA, of magnitude comparable to X31 wild-type.
In summary, these in silico studies had previously identified several globular bottom destabilizing mutations for X31, and stabilizing mutations for PR8. A panel of these mutants was selected and grown by the lab of Dr. Tijana Ivanovic at Brandeis University for in vitro characterization in this study.

### 3.1.3 Single-particle fusion as a tool to probe the effect of the influenza hemagglutinin globular bottom stability on fusion

Taking the results from the MD simulations, we experimentally investigated the effect of the stability of the globular bottom on fusion of influenza virus. We used a controlled, in vitro assay based on fluorescence microscopy to observe the yield of hemifusion of single virus particles docked to a planar lipid bilayer. We probed differences in the fusogenicity of the virions. We did so by employing tagged HA inhibitors, counting their numbers bound to each virus particle, and correlating these inhibitor numbers with the observed fusion yield. Thereby, we obtained measures for the inhibitor-sensitivity of the different strains and the number of HA involved.

The combined analysis of biochemical and single-particle experiments showed that the major effect of the destabilized globular bottom was a reduction in HA density on the virus particles. We interpreted the single-particle results in the context of a numerical model of influenza viral fusion previously proposed by our group. This modeling showed that accounting for the reduction in the number of HA available per virus particle was sufficient to semi-quantitatively explain the data. Based on these results, we suggest studies to further test the molecular model of influenza fusion.
3.2 Results

We employed a previously established assay to visualize influenza viral fusion at the single-particle level, providing access to the population distribution of fusion events rather than an ensemble average.\textsuperscript{30,32,188,220} High-sensitivity fluorescence microscopy was used to observe single, fluorescently tagged virus particles fusing with a planar membrane.\textsuperscript{30}

![Figure 3.4 Single-particle assay.](image)

(a) Viruses were docked onto a planar lipid membrane supported by a coverslip. Virus membrane was labeled with a lipophilic dye (red) and antibodies were also tagged (green). A pH-sensitive dye provided a readout of the time of acidification. Laser-excited fluorescence was collected onto two different halves of a camera. (b) The left half (green, left) of the camera image shows the labeled antibody fluorescence, the right half (red, middle) shows the viral membrane label. Both sides co-localize since they result from the same virions. The fusion yield was determined by counting spots at the start (red, middle) and end of the experiment (red, rightmost). Arrows highlight six particles that have fused. Antibody intensity was extracted per spot directly after triggering of fusion by inflow of acidic buffer.

Briefly, a supported lipid bilayer was formed on the top surface of a microscope cover slip (Figure 3.4a), with sialic-acid containing receptors embedded in the membrane. A flow channel was used to flow reagents past the membrane. Virus particles were pre-docked to the membrane via the sialic-acid recognition of the viral HA. Rapid introduction of low-pH buffer provided the trigger for the viruses to fuse. Virus particles were labeled in the membrane with a
lipophilic dye, the escape of which into the target membrane (‘lipid mixing’) indicated that hemifusion of the viral and target membranes had been achieved. The yield of hemifusion was obtained by counting the number of viral-membrane-fluorescent spots at the start and end of the experiment (Figure 3.4b, right). Antigen-binding fragments crF8020 (Fabs), a fusion inhibitor binding to the X31 HA stem and preventing the conformational changes leading to fusion, were used with a fluorescent tag to enable quantification of the numbers bound to individual virions, and to enable correlation with the observed yield of fusion (Figure 3.4b, left).

### 3.2.1 X31 hemagglutinin globular bottom mutants displayed decreased fusion yields

With the ability to visualize fusion at the single-particle level, we determined the probability to establish a hemifusion intermediate for the panel of globular bottom mutants described above. The virus particles were pre-docked to the planar lipid membrane and then rapidly acidified in the flow channel to pH 5.0.

![Figure 3.5 Yield of hemifusion of the panel of influenza A mutants of strains X31 and PR8. Virus particles were pre-docked to a planar lipid bilayer and acidified to pH 5.0. This induced the virions to fuse to the target, leading to dissipation of the membrane probe. The fraction of membrane-labeled spots at the start and end of the experiments was determined: the yield of hemifusion (see Figure 3.4). Triangles denote single trials. Bars show weighted mean±sem. P-values, relative to wild-type within strain, determined by Welch’s t-test, see Table A3.1; *: p<0.05, **: p<0.01, ****: p<0.0001, NS: not significant.](image-url)

For the X31 strain (Figure 3.5, left), the wild-type yield of hemifusion was determined to be 0.81±0.05 (mean±sem). All X31 mutants, with destabilized HAs, were observed to have significantly (see Figure 3.5 and Table A3.1) lower fusion yields than wild-type virus, with the single mutants having intermediate yields and the double mutants displaying low yields. The E131Q and R163A single mutants displayed similar, intermediate fusion yields of 0.59±0.04 and 0.61±0.08 respectively. Combining these two single mutations in the double mutant E131Q-R163A resulted in a low fusion yield of 0.28±0.02, and the double mutant E128Q-E131Q displayed a very low yield of 0.04±0.04. The wild-type PR8 strain (Figure 3.5, right) displayed a yield of 0.15±0.08, lower than that of wild-type X31. Introducing the residues identified in wild-
type X31 with the aim of stabilizing the PR8 strain HA globular bottom, i.e. mutant PR8 N128E-K131E-S163R, did not significantly change the fusion yield compared to PR8 wild-type (Figure 3.5 and Table A3.1). The reasons for this lack of improvement may be complex as the H1 HA amino-acid sequence is quite different from that of H3.

3.2.2 Globular bottom mutants were more susceptible to inhibitor-mediated neutralization of fusion

Previous reports combining numerical modeling and single-particle experiments have suggested that fusion is mediated by the assembly of a cluster of HAs that have inserted into the target membrane in a contact patch between the virus and target membrane.\textsuperscript{32,33} Furthermore, a large fraction of HAs has been determined to be non-participating in fusion.\textsuperscript{31} In the numerical model, the final yield of fusion is exclusively dependent on the number of HAs in the contact patch and the relative number of HAs that are able to participate, which taken together we denote as the effective number of HAs. Since in our experiments the fusion yield of the mutants was changed, we postulated that the effective number of HAs was different. By performing viral-fusion experiments on the different globular bottom variants while using varying concentrations of HA-binding inhibitors we controlled the number of participating HAs in a fusion reaction and studied how this number varied for different globular bottom variants.

Recent work demonstrated that binding of HA by the broadly neutralizing, stem-binding antibody CR8020 (group-2-specific, such as the X31 strain) and its Fab fragment crF8020 resulted in inhibition of HA-mediated viral membrane fusion.\textsuperscript{222} We used crF8020 to avoid the complications of bivalent binding of the antibody and used them labeled with a fluorescent dye to enable quantification of the number of inhibitors bound per virion. After incubation of the virus particles with these inhibitors and docking them onto the planar membrane in the flow channel, the fluorescence intensity per virion was measured. Using calibration experiments that were based on imaging individual labeled Fab fragments, the intensities were converted into numbers of inhibitors bound to the virus particle. By changing the concentration of incubation, different binding levels were achieved and we effectively titrated epitopes on the viral surface with inhibitors.

The batch of labeled inhibitors was identical to that used before (crF8020 labeled with AlexaFluor488 by Otterstrom et al.),\textsuperscript{30} and the assay was similar. The fraction of Fab without dyes, the dark fraction, had been measured before to be 0.90±0.01. The mean intensity of single crF8020-AF488, dependent on the specific optics used, was determined in order to calculate inhibitor numbers (see Figure A3.1). Keeping the final volume constant for all conditions, the different virus mutants were diluted to the same final concentration of viral protein (as determined with UV-VIS spectroscopy) and incubated for at least 45 min with the target concentration of inhibitor to allow binding saturation (as described before).\textsuperscript{30} Experiments without inhibitors followed the same protocol using buffer instead of inhibitor solution.
Figure 3.6 shows the observed yield of hemifusion versus the estimated median number of bound inhibitors. We tested the four variants of X31 virus that had fusion yield higher than 0.2. These displayed different profiles of inhibition. To facilitate comparison and for lack of a model at this point, we show linear regressions on the data. X31 wild-type was insensitive to inhibitor binding up to about 400 copies bound, with a yield of fusion comparable to conditions without inhibitor (Figure 3.6a). With higher inhibitor numbers, the yield of fusion was reduced, reaching near-complete inhibition of fusion in a range of around 500 to 1500 inhibitors bound.

Figure 3.6 Yield of fusion for different virus variants versus count of bound inhibitor. Virus was incubated with varying concentrations of inhibitor to reach a range of values of bound inhibitor (see Figure A3.3). Per trial (triangles), the median number of bound inhibitor over virions was determined as well as the yield of hemifusion (see Figure 3.4 and Methods). Virus variants: (a) X31 wild-type (b) X31 E131Q (c) X31 R163A (d) X31 E131Q-R163A. X-error bars show only errors from inhibitor intensity determination (Figure A3.1) and do not reflect population spread. Linear regressions (blue) are shown to aid comparison, and fitting was done without x-errors. 95% confidence intervals are indicated.

Introducing the single mutations E131Q or R163A not only reduced the yield without presence of inhibitors (as shown previously in Figure 3.5), but in addition, the point of half-maximal reduction decreased to about 250 inhibitors bound (Figure 3.6b and c). Concomitantly, complete inhibition occurred around 500 inhibitors bound. The double mutant E131Q-R163A was
observed to have a strongly reduced fusion yield under all conditions (Figure 3.6d), with an apparently slightly longer inhibition curve than the single mutants but a lower half-point than wild-type of around 400 inhibitors. Therefore, for a given number of inhibitors bound per virus particle the mutants showed lower yields than wild-type, and additionally were more susceptible to neutralization as implied by the shift in the half-maximum point.

3.2.3 The incorporation of mutated HA per virion was reduced and correlated with destabilization and fusion properties

The lower fusion yields without inhibitor and the increased susceptibility to neutralization suggested that the mutants had lower numbers of HAs on their surface available to engage in the fusion process. We therefore set out to determine the total number of HAs embedded on the virus particles.

HA incorporation was first determined by western blotting. Virus was loaded onto a gel and run denatured, and the quantitative fluorescence determined after fluorescent HA-antibody conjugation. To correct for loading differences, this value was normalized to the amount of M1 protein determined with an M1 antibody. The relative HA-to-M1 ratio, a proxy for the relative amount of HA per virion, is shown in Figure 3.7a (right y-axis) relative to the wild-type virus. Mutants were observed to have reduced HA incorporation, where E128Q-E131Q did not have detectable incorporation at all.

![Figure 3.7 HA incorporation of the virus mutants, and comparison of results across assays.](image-url)

(a) In order to determine the maximum number of Fabs that can bind to virions, these were incubated with a saturating 2 μM of Fab. Then, after
docking to a planar lipid bilayer in the single-particle assay, the Fab-fluorescence intensity per virion was determined and converted to Fab numbers (see text and Methods). The number of HA trimers per virion was then determined in Western blot and is shown normalized to the single-particle wild-type (red bars and right y-axis). Here, the fluorescent signal from antibody conjugation to HA was divided by the signal from an M1-antibody, to correct for differences in amount of virus loaded and to determine the virion relative HA-to-M1 ratio. All fluorescent Western blot X31 wild-type data are shown normalized to the single-particle wild-type and show mean±sem from 3 trials. (b) Correlation of HA incorporation (red) and fusion properties (green and cyan). The single-particle HA incorporation (red, same data as black bars in panel a), the single-particle yield of fusion without inhibitors (green, from Figure 3.5) and the EC50 (cyan, half-point of inhibition determined from Figure 3.6) are shown for the X31 mutants, normalized to wild-type. (c) Correlation plot of the single-particle number of HA per virion (same values as black bars in panel a) versus the unfolding times determined in silico (data taken from Boonstra,219 see Figure 3.3). Data from top to bottom: wild-type, E131Q-R163A, E131Q, R163A, E128Q-E131Q. Dashed line shows y = x. N/D: not detectable.

Because of its high sensitivity, we also studied HA incorporation in the single-particle assay using Fab binding as a proxy. The maximum number of Fabs per virion was determined by incubating the viruses with a saturating 2 μM of Fab in excess ratio and determining the number of Fabs per virion as described above. The estimate of the number of HA per virion (i.e. maximum number of Fabs divided by three, the number of epitopes per HA) is also shown in Figure 3.7a (left y-axis). Similar results were obtained as in the western blots. We highlight the E128Q-E131Q mutant that was determined to have about a ten-fold reduction in HA numbers: 24±11 HA proteins per virion, compared to 288±55 of the wild-type. Mutant virus particle sizes appeared similar in electron microscopy (Figure A3.2), indicating that the differences in HA incorporation measured in both assays above are due to different densities of HA in the membranes.

To compare the in silico prediction with the observations, Figure 3.7c shows the HA incorporation estimated in the single-particle assay correlated versus the unfolding time measured in the MD simulations. The level of destabilization of the globular bottom in silico and the resulting reduction in HA incorporation were strongly correlated, with a Pearson correlation coefficient of 0.93.

Then, to check if the HA incorporation could explain the observed properties of fusion, we show in Figure 3.7b the yield of fusion without Fab and the EC50 of inhibition (the half-points determined from Figure 3.6), normalized to the wild-type readouts. The HA incorporation and yield of fusion of the HA mutants were correlated. Moreover, HA incorporation appeared to be a good predictor of the EC50 of fusion inhibition. We therefore postulate a numerical model that may explain the fusion properties from the observed HA incorporations in the next section.

3.2.4 Reduction in HA incorporation explains trends observed yields and inhibitor sensitivities

We observed a strong correlation between destabilization of the globular bottom in X31 virus and decreased HA incorporation. HA incorporation then correlated with the observed fusion yield, and the sensitivity to Fab neutralization. As the particle sizes had not changed, the availability of HA in the contact patch in contact with the target membrane should be quite different
due to the differences in HA density. We therefore considered the number of Fabs bound to the virions relative to their HA numbers, and determined the effect of these Fabs in the case of a reduced contact patch in our current model of influenza fusion. First, the median number of inhibitors bound to the observed particles was determined for single trials as a function of the concentration (Figure A3.3). The maximum number of inhibitors that can bind was determined by fitting a hyperbolic function (also see Table A3.2). The observed yields were then plotted versus the bound number of inhibitors, normalized to the maximum binding determined from the fit (Figure A3.4). To facilitate comparison with the model described below, logistic curves were fitted to the data (Figure A3.4) and are shown in Figure 3.8e.

![Figure 3.8 Simulation of the effect of varying HA incorporation on fusion yield.](image)

We then ran numerical simulations of the current numerical model of HA-mediated membrane fusion as briefly described above. The region in contact with the target membrane was considered: a contact patch of $M$ HA proteins (Figure 3.8a). A fraction of 65% of HAs was...
randomly assigned to be non-participating in fusion (as modeled before for H3 strain)\textsuperscript{31} and varying numbers of inhibitors were randomly bound over the available epitopes. HAs could mediate fusion if not inhibitor-bound (i.e. all three epitopes were unbound), and if not non-participating. Fusion was modeled only to ensue if a cluster of fusogenic HAs of size 3 or larger was present anywhere in the contact patch (Figure 3.8b), otherwise the virion was non-fusing (Figure 3.8c). The fusion yield was determined as the fraction of virions that was able to fuse. The only parameter we then varied with respect to Ivanovic et al.\textsuperscript{31} was the contact patch size $M$.

The simulations showed that a decreased contact patch led to a reduction in fusion yield without inhibitors (Figure 3.8d, y-axis intercepts). Also, the smaller contact patch led to an increased sensitivity to the inhibitors, where the fusion yield curves shifted downward and leftward. These phenomena arise from the decreased chance to have a cluster of participating HAs available as the contact patch becomes smaller and the bound fraction of epitopes higher. Comparing the data (Figure 3.8e) to the numerical simulation (Figure 3.8d), we see that the initial inhibition trends might be explained by a reduction in contact patch size, that is, a reduced number of HAs available per same-sized virion. The tails of the experimental distributions, however, do not match with the model. In conclusion, the decreased baseline yield and increased sensitivity to inhibitor binding of the HA globular bottom mutants appear to be at least partly explained from the decreased incorporation of mutant HA on the virions.

3.3 Discussion

We investigated influenza mutants that displayed destabilization of the HA globular bottom in silico and found that these had reduced fusogenicity in vitro. Boonstra et al.\textsuperscript{219} previously identified the residues critical for the stability of the globular bottom. We used viruses expressing the point mutations identified in that study. In a single-particle assay, we found that the destabilized mutants had reduced yields of fusion. Furthermore, by counting the number of inhibitors over single virions, these mutants were determined to be more susceptible to neutralization by inhibitors. We characterized the relative number of HA over the virions and found that the mutations lead to decreased HA incorporation. In conclusion, the decreased baseline yield and increased sensitivity to inhibitor binding of the HA globular bottom mutants appear to be at least partly explained from the decreased incorporation of mutant HA on the virions.

The in silico unfolding time, a measure for stability, and the HA incorporation as determined in vitro strongly correlated (Figure 3.7c). This unfolding time was reduced when residues were mutated to prevent them from forming a hydrogen-bonding network. The mutated HA unfolded 2.3-fold to 6-fold faster compared to wild-type protein (Figure 3.3). We similarly measured that the virus mutants had reduced numbers of HA incorporated into the virions (Figure 3.7a). Comparing these two measurements (Figure 3.7c) shows that the two quantities are strongly correlated: HA with destabilized globular bottom incorporate less well into virions. Notably, E128Q-E131Q, for which insufficient material was available to determine the fusion
properties, was the most destabilized and had the least HA incorporated. Identifying the exact cause of the HA incorporation reduction is beyond this work, but we speculate that HA trimerization may be affected. As Figure 3.2 shows, most of the hydrogen bonds from residues E128, E131 and R163 are inter-chain, so between HA monomers. Therefore, removing any of these hydrogen bonds may partially disrupt trimerization, in the end leading to less HA expressed on the virions. Alternatively, interactions between HA trimers, or HA-NA interactions might be reduced, affecting HA density in viral budding.

Several fusion properties correlated with HA destabilization (Figure 3.7b). Comparing the yields of fusion, the single-point mutants X31 E131Q and R163A displayed decreased yields, reflecting that these residues are partners in the same hydrogen bond. However, the double mutation E131Q-R163A further reduced the fusion yield, in line with the in silico findings that E131 also interacts with R170, and R163 with E128. A negligible fusion yield was observed for E128Q-E131Q, corresponding to removal of all possible vertical hydrogen bonding. Comparing with HA incorporation (Figure 3.7b), especially the EC50 of Fab inhibition closely matched the trends, indicating that it is the number of inhibitors bound relative to the number of HA available that determines the stoichiometry of fusion inhibition.

Using the current numerical model of HA-mediated fusion, we therefore proposed that the observed curves of fusion inhibition can in part be explained through a reduced contact patch size. As our viruses had both HA mutations and HA density differences, we cannot draw a narrower conclusion from this work. The relative Fab occupancy (Figure 3.8e) trends show similar half-maximum points, as a result of the correlation of HA incorporation and EC50 of inhibition. In the numerical model (Figure 3.8d), reduction of the contact patch lead to increased inhibitor sensitivity and reduced baseline yield. Comparing model and data, the differences in baseline yield might be due to factors not considered in the model: the larger spacing of HA may make clustering more difficult, or the fraction of HA that successfully refolds to drive fusion may be affected. Then, the experimentally determined Fab occupancy may be an overestimate by a factor of 2, as estimated in previous work, explaining the differences in scale of the model and experiment axes. Furthermore, the saturation of binding of Fabs due to their mutual steric hindrance may depend on the density of epitopes on the surface, which implies that we overestimate the length of the mutant curve tails compared to wild-type.

In conclusion, we investigated the effect of mutations determined in all-atom molecular dynamics at the level of whole, single-virus particles and showed that these mutations likely have a direct effect on the incorporation of HA into the viral membrane during virus assembly and thus adversely affect the efficiency of fusion. Future work should address two main uncertainties identified here in the numerical model of influenza fusion. First, obtaining absolute numbers of HA available per virus particle will allow better estimates on the parameters of inhibitor occupancy and contact patch size. The combination of single-particle, cryo-EM and atomic force microscopy assays could address this issue. Second, influenza viruses with varying
densities of wild-type HA on their surface will allow to refine the estimate on the fraction of HA not involved in fusion, and if HA diffusion or spacing play a role in the fusion process.

3.4 Acknowledgements

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3.5 Methods

The single-particle experiments were performed to match the work by Otterstrom et al.\textsuperscript{30} as closely as possible. While a more detailed explanation of the methods can be found there, a brief overview follows here. Materials were purchased from Sigma-Aldrich unless noted otherwise.

**Virus and R18 labeling.** Influenza A strains A/Aichi/2/1968 (X31, subtype H3N2) and A/Puerto Rico/8/1934 (PR8, subtype H1N1) and the specific HA mutants were produced in and received from the Ivanovic lab at Brandeis University, USA. The viruses were UV-irradiated in order to render them non-infectious: 254 nm UV light from a Herolab NU-3 type lamp was irradiated for 28 min at 635 µW/cm² into open 0.5-mL Eppendorf tubes containing 4 µL of sample. For labeling of the viral membrane, an appropriate amount of virus stock was diluted to 0.5 mg/mL in PBS-EDTA (PBS with 0.2 mM NaEDTA, henceforth referred to as PBS only) in a volume of 20 uL. 0.1 µL of 0.2 mM R18 (Rhodamine C18 sodium salt, ThermoFisher) in DMSO was added, and all components were carefully mixed by pipetting up and down. After standing at room temperature for 1 h in darkness, the virus-R18 suspension was added to a PBS-pre-equilibrated G-25 column (ThermoFisher) and allowed to enter the bed, 480 µL of PBS was added and the eluate discarded. Then, virus fractions per two drops were collected by eluting with 1mL of PBS, and the fractions of most concentrated virus were combined to about 350 µL total volume. The most concentrated fractions were determined by imaging 3 µL droplets on a bare coverslip, and comprised fractions 2-6 or 3-7.

**Inhibiting Fab characterization.** crF8020 Fab fragments, and the AlexaFluor488-labeled version crF8020-AF488, were the same stocks as used in Otterstrom et al.\textsuperscript{30} stored at -80 °C. Detailed methods and characterization are described in that reference. The labeled, and therefore visualized, fraction was determined there to be 0.90±0.01 and we corrected for this.

**Coverslip and flow cell.** Experiments were performed in a 5-channel PDMS-chip self-adhering to a glass coverslip like before. Briefly, polydimethylsiloxane (PDMS, Sylgard 184; Dow Corning) was mixed with the curing agent in a 10:1 ratio, poured on a mold and allowed to harden for two days at room temperature. PDMS chips were cleaned before use by sonication with 0.5% v/v Triton-X and 70% ethanol for 10 min each. Cleaning involved an additional step of sonication with 1M NaHCO₃ for re-use. Glass coverslips (thickness #1.5; Marienfeld) were cleaned by sonication in detergent, acetone, ethanol (each 30 min) and 1M KOH solution (10 min) successively, and subsequently rinsed with large amounts of water. Polyethylene tubing (Bioseb) provided the connection between buffer reservoirs, the flow cell and the syringe pump (NE-1000; New Era Pump Systems Inc.). Flow channel dimensions in the imaging region were 0.2 mm height x 0.5 mm width.

**Proteoliposome preparation.** Liposomes were formed by extrusion through 200 nm-pore membranes (Avanti Polar Lipids), and composed of a 0.75 : 0.25 : 2.5x10⁻⁵ ratio of DOPC : cholesterol : biotin-DOPPE (Avanti Polar Lipids). Liposomes were solubilized with Triton-X and the receptor protein GYPA (Glycophorin predominantly glycophorin A) was added in an approximate ratio of 1:27000 GYPA:lipid, correcting for GYPA content per the manufacturer’s specification sheet.
**Microscope specifications.** All experiments were performed on an inverted microscope, under near-total-internal-reflection conditions so that an even irradiation of the sample was assured. 488-nm and 561-nm lasers (Sapphire model, Coherent) excited the AlexaFluor488 dyes and R18 molecules, respectively. Fluorescence was split and collected onto two halves of an EM-CCD camera (C9100-13, Hamamatsu) to enable co-localization of Fab and viral-membrane signals (also see Figure 3.4).

**Fusion experiment.** After rinsing with PBS, proteoliposomes were drawn into the channel and left for 45 min for the liposomes to spontaneously form a bilayer. The channel was then washed with PBS. Bilayer integrity was confirmed with fluorescence recovery after photobleaching (FRAP). The virus was incubated at room temperature with or without fabs for at least 45 min to assure binding saturation. 30 The following steps were then performed directly after eachother and with about the same timings: all of the virus mixture was drawn in; fluorescein-streptavidin (Thermo Fisher) at 0.27 µg/mL was drawn in to bind to the sparse biotin-decorated lipids in the bilayer and provide a readout of the local pH conditions; rinsing with PBS; lastly, pH 5.0 buffer (10 mM citric acid, 0.2 mM NaEDTA) was drawn in quickly to acidify the channel and trigger the viruses to fuse. Acidification typically happened at 7 to 9 min after introducing the virus into the channel and was complete within seconds. Fusion movies were taken with 200 ms frame exposure time for 4 min, and the first and last five seconds were imaged with the 488 nm laser off to allow rapid virus particle localization and fusion yield determination.

**Data extraction and analysis.** Virus particles were detected in the R18 channel with the 488 laser off by using a discoidal averaging filter, and coordinates translated to the Fab channel. The time of acidification ("pH drop") was determined by fitting an error function to the fluorescein background signal in the 488 channel. The illumination profile was corrected for by filtering out peaks and fitting a gaussian to the whole field of view. A 7x7 pixel region of interest was used to extract R18 and Fab signal intensities per virus particle, and background was globally corrected. Hemifusion was detected from a dequenching or dissipating virus spot signal. Fusion yield, the fraction of the virus population undergoing hemifusion, was determined by counting the number of virus particles at the beginning and end of the movie without 488 illumination on, by using a threshold combined with manual inspection. Single Fab intensity was determined (Figure A3.1) by flowing a low concentration (roughly a pM) of fabs diluted into the same pH 5.0 buffer into a clean flow channel, rinsing, and imaging the single spots under the same conditions as in a fusion experiment. This intensity was then used to calculate the number of fabs bound per virus particle averaged over the first 20 frames after the pH drop.

**HA incorporation by fluorescent western blot.** 40, 80 and 120 ng total viral protein was loaded to each lane, for wild-type and mutant viruses (E131Q, R163A, E131Q-R163A). Viruses were loaded after treatment with PNGaseF (NEB) according to manufacturer’s guidance, to remove the potentially different glycosylations from the variants. 10% polyacrylamide gels were used. After protein transfer to the 0.2 µm PVDF membrane, the membrane was cut between the HA1 and M1 bands and the two gel parts were probed separately until the secondary antibody addition when they were combined in the same dish. The following primary antibodies were used. Antibody A2, mouse monoclonal antibody against HA1, was produced and purified from hybridomas (hybridomas were a generous gift by Judith White). The M1 antibody is from Abcam, clone 23936. Both primary antibodies were used at 1:10 000 dilution. The secondary antibody used was Goat anti-mouse IgG Dylight680 (Invitrogen) used at 1:20 000 dilution. Then, fluorescence was detected using a Li-Cor Odissey fluorescent imaging system. Fluorescence signal with background correction was quantified using ImageJ. The linear dependence of the detected fluorescent signal on the input protein amount was confirmed. For each loaded protein amount, the HA1/M1 ratio was determined. This ratio for the WT virus was set at 100%, and the rest were expressed relative to WT. Error bars are standard deviation of this measurement for the three different protein amounts loaded.

**Electron microscopy of virion sizes.** Electron microscopy was performed on a FEI Morgagni TEM, using 80 kV, tungsten-filament, and a 1k CCD. The grids were from SPI supplies, formvar/carbon copper grids.
Prior to sample application, grids were glow discharged at 20 mA for 30 s. Main virus stocks were diluted between 1:5 and 1:90, depending on the starting virus concentration, and 3 ul was applied to the grid for 30 s. Staining was done with 2% phosphotungstic acid (PTA) at pH 7.5. Grids were washed once quickly with stain, stained for 30 s, then dried by vacuum suction and imaged.

**Simulations.** The model proposed in Ivanovic et al.31 was used. The region in contact with the target membrane was considered, the contact patch, of $M$ HA proteins. A fraction of 65% of HAs was randomly assigned to be non-participating in fusion (as modeled before for H3 strain31) and varying numbers of inhibitors were randomly bound over the available epitopes. HAs could mediate fusion if not inhibitor-bound (i.e. all three epitopes were unbound), and if not non-participating. Fusion was modeled only to ensue if a cluster of fusogenic HAs of size 3 or larger was present anywhere in the contact patch, otherwise the a virion was nonfusing. The fusion yield was determined as the fraction of a virions that was able to fuse. The parameter we then varied with respect to Ivanovic et al.31 was the contact patch size $M$. The patch size $M$ for wild-type was set to 97 HAs; the other patch sizes, modeling the mutants, were 55 (E131Q), 43 (R163A) and 31 (E131Q-R163A). These numbers were based on the western blot HA density and assuming that patch size scales with HA incorporation. The wild-type number of HAs per virion was taken to be 375 and number of Fab epitopes to be three times that value. Number of Fab epitopes for the variants were thus 1125 (wild-type), 652 (E131Q), 512 (R163A), and 388 (E131Q-R163A).

### 3.6 Appendix

![Figure A3.1 Intensities of single, tagged crF8020 molecules.](image)

**Figure A3.1 Intensities of single, tagged crF8020 molecules.** crF8020 molecules were used labeled with AlexaFluor488 dyes, the identical batch as used in earlier work.30 The inhibitors were introduced into a flow channel and absorbed nonspecifically to the glass surface. Using conditions identical to that used in the fusion experiments, the intensity per inhibitor spot was extracted from a 7x7 pixel region. These were corrected for background and inhomogeneous illumination. A binned histogram of these single intensities is shown, together with the population mean (dashed line).
Figure A3.2 Scanning electron microscopy images of X31 variants by phosphotungstic acid stain. See Methods for procedure. Scale bars, 100 nm.
Figure A3.3 Median bound number of crF8020 versus concentration of crF8020 incubation. Virions were incubated with the indicated concentration of Fab and docked to the planar bilayer for imaging. The extracted number of Fabs bound is shown as the medians of the virion population per trial (triangles). Hyperbolic fit shown with 95% confidence interval (see Table A3.2 for fit parameters).
Figure A3.4 Yield of fusion versus normalized binding of inhibitors. This figure shows the data from Figure 3.6 scaled to the mean maximum inhibitor numbers that could bind as determined in Figure A3.3. Due to the uncertainty in determining the maximum inhibitor numbers, values large than 1 resulted for some conditions. X-errors were calculated from the error of inhibitor intensity determination and from the fit of the maximum binding level. Logistic curve fits with 95% confidence intervals are shown for (a) X31 wild-type, (b) X31 E131Q, (c) R163A, and (d) E131Q-R163A.
Observable: Fusion yield
Compared conditions: Strain wild-type vs. strain mutants
Null hypothesis: Equal means
Hypothesis test: Two-sided weighted Welch’s t-test (t-test for unequal variances)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Variant</th>
<th>Weights (number of particles)</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>X31</td>
<td>Wild-type</td>
<td>406, 101, 199, 793, 464</td>
<td>(reference)</td>
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<tr>
<td></td>
<td>E131Q</td>
<td>64, 43, 85</td>
<td>0.0035</td>
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<tr>
<td></td>
<td>R163A</td>
<td>171, 125, 83, 97</td>
<td>0.036</td>
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<tr>
<td></td>
<td>E131Q-R163A</td>
<td>58, 33, 50</td>
<td>5x10^-5</td>
</tr>
<tr>
<td></td>
<td>E128Q-E131Q</td>
<td>126, 68, 49</td>
<td>3x10^-6</td>
</tr>
<tr>
<td>PR8</td>
<td>Wild-type</td>
<td>64, 45, 12</td>
<td>(reference)</td>
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<tr>
<td></td>
<td>N128E-K131E-S163R</td>
<td>53, 94, 140</td>
<td>0.19</td>
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</tbody>
</table>

Table A3.1 Significance testing results of Figure 3.5.

A. Yield of fusion versus Number of Fab bound (Figure 3.6)

Fit function: \( y = A_1 + A_2 \times x \)

<table>
<thead>
<tr>
<th>Virus variant:</th>
<th>Parameter:</th>
<th>Wild-type</th>
<th>E131Q</th>
<th>R163A</th>
<th>E131Q-R163A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-intercept A1</td>
<td>0.71±0.06</td>
<td>0.62±0.05</td>
<td>0.58±0.04</td>
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<tr>
<td>Slope A2 (10^-5)</td>
<td>-4.6±0.9</td>
<td>-9±2</td>
<td>-12±2</td>
<td>-3.2±0.9</td>
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</table>

B. Yield of fusion versus Normalized inhibitor binding (Figure A3.4)

Fit function: \( y = A_1/(1 + (x/x_0)^p) \)

<table>
<thead>
<tr>
<th>Virus variant:</th>
<th>Parameter:</th>
<th>Wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-intercept A1</td>
<td>0.79±0.06</td>
<td></td>
</tr>
<tr>
<td>Center/EC50 x0</td>
<td>0.49±0.06</td>
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<tr>
<td>Power p</td>
<td>3±1</td>
<td></td>
</tr>
</tbody>
</table>

C. Bound number of inhibitors versus Inhibitor concentration (Figure A3.3)

Fit function: \( y = P_1 \times x/(P_2 + x) \)

<table>
<thead>
<tr>
<th>Virus variant:</th>
<th>Parameter:</th>
<th>Wild-type</th>
<th>E131Q</th>
<th>R163A</th>
<th>E131Q-R163A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horizontal as-</td>
<td>1074±75</td>
<td>550±41</td>
<td>271±37</td>
<td>531±64</td>
<td></td>
</tr>
<tr>
<td>ymptote P1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vertical asymp-</td>
<td>67±20</td>
<td>37±11</td>
<td>23±13</td>
<td>18±9</td>
<td></td>
</tr>
<tr>
<td>tote P2</td>
<td></td>
<td></td>
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Table A3.2 Fitting functions used and resulting fit parameters for Figure 3.6, Figure A3.4 and Figure A3.3.