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Review

Architecture of the influenza hemagglutinin membrane fusion site

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Abstract

The mechanism of influenza hemagglutinin (HA) mediated membrane fusion has been intensively studied for over 20 years after the bromelain-released ectodomain of HA at neutral pH was first crystallized. Nearly 10 years ago, the low-pH-induced "spring coiled" conformational change of HA was predicted from peptide chemistry and confirmed by crystallography. Other work has yielded a wealth of knowledge on the observed changes in HA fusion/hemifusion phenotypes as a function of site-specific mutations of HA, or added amphipathic molecules or particular IgGs. It is becoming clear that the conformational changes predicted by the crystallography are necessary to cause fusion and that interfering with these changes can block fusion or reduce it to hemifusion. What is not known is how the conformational changes cause fusion. In particular, while it is generally agreed that fusion requires an aggregate of HAs, how the aggregate may act to transduce the energy of the HA conformational changes to creating the initial fusion defect is not known. We have used a comprehensive mass action kinetic model of HA-mediated fusion to carry out a "meta-analysis" of several key data sets, using HA-expressing cells and using virions. The consensus result of these detailed kinetic studies was that the fusion site of influenza hemagglutinin (HA) is an aggregate with at least eight HAs. The high-energy conformational change of only two of these HAs within the aggregate permits the formation of the first fusion pore. This "8 and 2" result was required to best fit all the data. We review these studies and how this kinetic result can guide and constrain HA fusion models. The kinetic analysis suggests that the sequence of fusion intermediates starts with protein control and ends with lipid control, which makes sense. While curvature intermediates, e.g. the lipid stalk, are almost certainly within the fusion sequence, the "8 and 2" result does not suggest that they are the first step after HA aggregation. The stabilized hydrophobic defect model we have proposed as a precursor to the lipid stalk can form and is consistent with the "8 and 2" result.

Keywords: Enveloped virus; Membrane protein aggregation; Hydrophobic

1. Introduction

It is generally believed that the energy released by the essential conformational changes of membrane fusion proteins are required to stabilize the defects that lead from two stable bilayers to the single bilayer of the fusion product [1,2]. While it is generally agreed that fusion proceeds from an HA aggregate [73,74,76,77], it is conjectured that lipid mixing is initiated either by a high curvature bending defect directly contacting the target bilayer [3–7] or a hydrophobic defect in the viral bilayer forming a target for lipid permeation from the target bilayer [8,9]. These two hypotheses agree on most points, including the necessity of lipidic stalks as one of the intermediates of fusion, whose formation appears to demark the transition from the protein-controlled steps to the lipid-controlled steps of fusion. The hypotheses differ on the direct target of the transduced energy of the protein's conformational changes, which is the site of evolutionary pressure on the conformational change in the first place. So, it is actually important to know the first step of destabilization. Unfortunately, despite the seeming difference in these two proposed mechanisms, no clear experiment has favored one over the other, largely because both posit at least two HA conformational changes. Furthermore, little is known about the energetics of a membrane fusion site, which is composed of a handful of HAs, some glycoprotein or ganglioside receptors, a hundred or so lipids and water. All the physical theories being used to model the site either neglect the proteins, due to computational constraints, or are being pushed beyond safe limits.

A fusion site is the first one of the HA aggregates to succeed in forming a fusion pore. These HA aggregates form rapidly subsequent to acidification [10]. Perhaps they are preformed on the virion due to close packing. HA

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aggregates have been visualized [11], but never correlated with fusion kinetics. However, the necessity of aggregation and estimates for the numbers of HAs in the aggregates has been inferred with increasing precision by kinetic studies [10,12–14,73]. In order to initiate first fusion pore formation, there must be at least eight HAs in the aggregate [10]. The next question is what makes the HAs aggregate and how does the aggregate form the first fusion pore?

There is a wealth of knowledge on the observed changes in fusion/hemifusion phenotypes as a function of site-specific mutations of HA, or added amphipathic molecules or particular IgGs [3,5,7,15–20,78,80]. The conclusions of these studies focus on the implied effect of the individual HA. The explicit or implicit assumption is that all HAs behave identically, i.e. all HAs within a particular fusion site have synchronized conformational changes. However, each HA can have one or more of at least four jobs with respect to the overall fusion process: binding to target membrane sialates, mediating self-aggregation, creating the initial bilayer defect, and allowing closer apposition of the bilayers so that bilayer merger can commence. The transmembrane domains appear to have a direct effect on this last job [5,8,18]. If each HA performs all jobs synchronously, within each HA aggregate, it would be difficult to determine how each job is affected by the mutation or additive? While cooperativity in the HA conformational changes appears to occur [21,38], the fusion kinetics are most simply modeled with a fusion site composed of HAs in different conformational states.

We approached this problem from the perspective of a protein folding landscape, wherein the HAs within the fusion site could be in different conformations and perhaps performing different jobs. Our kinetic model was constructed to have the data determine the answer. Furthermore, data was fitted exhaustively, so that all possible parameter sets that yielded best fits to the data were found. This meta-analysis of several key data sets on HA fusion, from different labs, showed that of the eight or more HAs comprising a fusion site, only two undergo the essential conformational changes needed for the first fusion pore to form [10,21,22]. Only two of the eight must do the "heavy lifting" to transduce their released energy to stabilizing the initial defect of fusion. The mechanism of initial lipid destabilization is unresolved, but the kinetic analysis has given the same "8 and 2" result for several different experimental systems, including intact virions. This result provides a new context within which to understand the effect of mutations on HA-mediated fusion.

2. The mass action kinetics of HA-mediated membrane fusion

Bentz [10] began the development of a comprehensive mass action model for HA-mediated fusion based on the fusion intermediates listed above and used it to analyze the electrophysiological data of Melikyan et al. [23] for first fusion pore formation between HA-expressing cells and ganglioside-containing planar bilayers. This data is the most rigorous for fusion and the basic "8 and 2" result was clear from its analysis. The model was extended in Mittal and Bentz [22] to extract consensus kinetic parameters for the data of Melikyan et al. [23], Danieli et al. [13] and Blumenthal et al. [14] for HA-expressing cells fusing with various target membranes. This required the explicit consideration of what jobs the HAs bound to sialates on glycophorin could accomplish. In Mittal et al. [21], protonation and inactivation of HAs was added to the model to analyze the data of influenza virions fusing with ganglioside-containing liposomes [24,25,72] and HA-expressing cells fusing with RBCs [19,26]. The key architectural results obtained first from HA-expressing cells, which have the problem of a distribution of HA cell surface densities, worked for the influenza virus fusing with target membranes, where the HA surface density is essentially uniform [27,28]. While rate constants varied, and the consensus-fitted parameters from these studies will be shown below, the "8 and 2" result holds for both virions and HA-expressing cells.

2.1. The division of labor for fusion: the minimal aggregate size and the minimal fusion unit

The mass action kinetic model includes only those steps that are generally accepted as essential, plus a rigorous distinction between the minimum number of HA trimers aggregated at the nascent fusion site (which is denoted ω) and how many of those trimers that must undergo a slow essential conformational change before the first conductivity can be measured across the fusing systems (which is called the minimal fusion unit and is denoted q). This "division of labor" was required to fit the kinetics of first fusion pore formation [10,23], which is the first measurable step of fusion and is therefore the most rigorous measurement.

This distinction allowed us to show that HAs bound to sialates on glycophorin could be members of the fusogenic aggregate, but not undergo the essential conformational change needed to form the first fusion pore [22]. This analysis resolved a long-standing question. Influenza HA contains both binding and fusion functions, and there was controversy about whether the same HA can execute both functions [12,19,29,30–32,79]. Ellens et al. [12] found that glycophorin-bearing liposomes bound equally well to both GP4f and HAb2 cells, implying the same number of HA-sialate contacts, but fused much more with the HAb2 cells. This proved that HAs need not be bound to sialate to induce fusion and suggested that a particular HA might not be able to perform both functions. Alford et al. [30] found that influenza virions fused more slowly as the ganglioside surface density in the target liposomes was increased above 10 mol%, suggesting that HA bound to ganglioside lost the

ability to sustain fusion. On the other hand, Millar et al. [32] found that detergent reconstituted virosomes could fuse with liposomes conjugated with Fab' fragments directed against HA1 at or near the site of sialate binding. Simply being bound to a large membrane-bound molecule did not stop HA from eventually mediating fusion.

However, it is not known how well these Fab' fragments mimic sialate binding. The IgG's used to generate the Fab' fragments used in Millar et al. [32] were screened to not inhibit the major conformational change of HA. Leikina et al. [19] found that soluble sialates slow the major conformational change of HA (X31 strain) and that RBC bound to HA(X31)-expressing cells fused faster following a neuraminidase treatment, i.e. with a reduction in HA-sialate contacts. The structural basis for sialate inhibition of the low-pH conformational change of HA is unknown at this time.

While the HAs bound to sialate on glycophorin may be part of the fusogenic aggregate (see below "The mass action model"), our analysis showed that two HAs within the fusogenic aggregate were unbound. It is not proven, but seems safe to assume, that this pair is the same as the two which undergo the essential conformational change needed to create the fusogenic defect in a timely fashion [22]. This suggested that the relatively weak HA-sialate binding constant could not evolve to a higher affinity, as that would inhibit HAs ability to mediate fusion. For fusion of the cells with ganglioside planar bilayers, calculations suggested that on average fewer than one of the HAs within the fusogenic aggregate are bound. This implied that HA binding to sialates is not necessary for fusion (see also Ref. [33]). It also implied that the binding constant of HA to ganglioside in a planar bilayer is at least 2–3 orders of magnitude smaller than that to glycophorin on RBC. This could be due to the difficulty for the HA1 binding site to reach sialates right next to the bilayer. This prediction can be tested. This is an example of the division of labor in fusion, i.e. there are two jobs for HAs within the fusion site and sialate bound HAs can fulfil only one of these jobs.

2.2. The mass action model

The current version of our mass action kinetic model of HA mediated fusion is shown in Fig. 1 [21]. It has been built through the post mortum analysis of data from several labs, which guarantees that the model is not too finicky and is focused on fitting only the most robust parameters. It is well known that PR8, X31 and (at high surface densities) Japan strains of the influenza virus hemagglutinin show inactivation of fusion capacity when the pH is low enough [21,34–38]. The mechanism of this inactivation is not known, but in order to obtain reliable rate constants for fusion for these HAs, we must take this inactivation into account. The data of Korte et al. [36,37] suggested that in addition to the protonation required for activation of HA, the protonation of a second site is required to allow HA inactivation of the PR8 and X31 strains. Obviously, there might be more than a single site on each HA monomer that must be protonated to initiate the conformational changes leading to fusion, just as more than one site might need to be protonated to initiate those conformational changes leading to HA inactivation. In step 1 of Fig. 1, we have considered only the sites with the smallest p*K*s, i.e. the last sites to be protonated as the pH is lowered. Since HA is a homotrimer, we do assume three identical and independent protonation sites per HA, regardless of function. These protonation reactions are assumed to occur instantaneously, relative to the protein conformational changes, and remain at equilibrium throughout the fusion process. Both of these assumptions are reasonable. The equations governing these equilibrium reactions are shown in Ref. [21].

We start with native HA at neutral pH (Fig. 1), denoted HA_{na}, which is first protonated to the fusion active form with the fusion peptide exposed, denoted HA_{fp}. If the pH is low enough, HA is protonated further to an inactivatible form, denoted HA_{fi}. Whether inactivatible HA does in fact inactivate will depend upon the pathway it follows, i.e. the relative rate constants. Both HA_{fp} and HA_{fi} can move to the next stage, wherein the fusion peptide is embedded into the proper membrane to initiate fusion, denoted HA_{em}. Whether that membrane is the viral membrane or the target membrane has been widely discussed, but is not germane to the kinetic model. This will be discussed further below.

Inactivation is any conformational modification of HA after protonation that renders it nonfusogenic, denoted by HA_{in}. Therefore, in step 1 of Fig. 1, we assume that both species HA_{fp} and HA_{fi} can inactivate at different rates. While not necessary for our kinetic analysis, it seems simplest to consider inactivation to be the fate of an HA that undergoes the essential conformational change, either in the absence of target membrane or in presence of a target membrane when that HA is not a member of a fusogenic aggregate [8,9].

In step 2 of Fig. 1, aggregation is assumed to occur rapidly compared with subsequent bilayer destabilization, supported by the analysis in Ref. [10], and remains at equilibrium. The nucleation mechanism was assumed solely because it would predict the minimum number of HAs needed to form a fusion site, not because it is particularly realistic. This minimal aggregate size is called a fusogenic aggregate, which has been fitted as $\omega \geq 8$ [10]. Other, more realistic, distributions would yield larger numbers for the minimal aggregate size [10,39].

In step 3 of Fig. 1, the HAs within the fusogenic aggregate independently and identically undergo the essential conformational change. Once q of them have done so, then the fusogenic aggregate can form the first fusion pore (FP), as shown in step 4. Note that while $q \leq \omega$, it is otherwise independent of ω [10]. The first fusion pore is measured by conductivity [23] or transmembrane electrostatic potential changes [14]. This transforms to a lipid channel (LC), monitored by the spread of fluorescent lipids

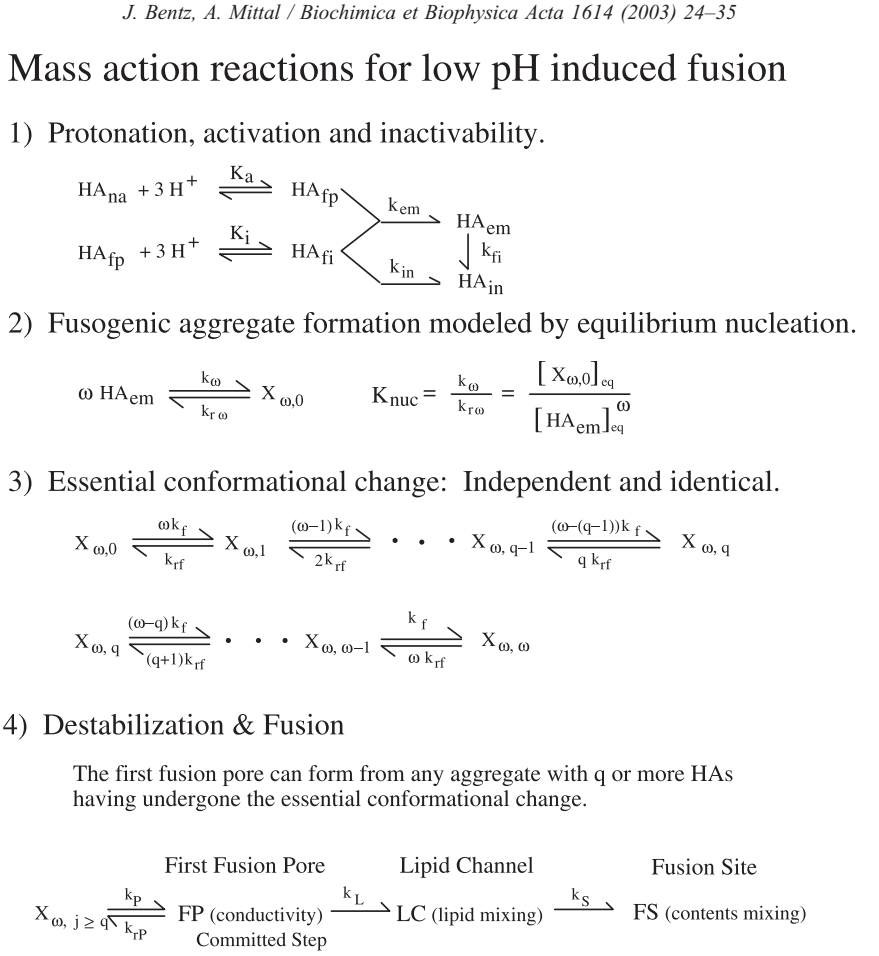

Fig. 1. The comprehensive kinetic model for influenza hemagglutinin mediated membrane fusion. The protonation reactions shown in step 1 are assumed to occur instantaneously, relative to the protein conformational changes, and remain at equilibrium throughout the fusion process. Stoichiometrically, we are concerned only about the last protonation site for each of the monomers in the HA trimer. HA trimer in native form (HA_{na}) is protonated to expose the fusion peptide (HA_{fp}). According to the model proposed in Ref. [8] and extended in Ref. [9], the exposed fusion peptide embeds into the viral membrane (HA_{em}) or is further protonated to become an inactivable species (HA_{fi}). HA_{fi} can either inactivate directly resulting in HA_{in} (HA incapable of being a part of the fusion mechanism) or can proceed as a part of HA_{em} species. In principle, all HA_{in} derived from the HA_{em} species should come from HA_{fi} species. Step 2 represents nucleation aggregation that is at equilibrium, assumed to guarantee the smallest estimate for the number of HAs in a fusogenic aggregate. Following protonation, the HA_{em} aggregate of size ω forms, rapidly denoted as $X_{\omega,0}$. ω is the minimal size for a fusogenic aggregate and a lower bound of eight was found by kinetic analysis in Ref. [10], i.e. $\omega \geq 8$. At step 3, ω denotes the number of the HAs within the fusogenic aggregate which can undergo the essential conformational change, independently and identically with a rate constant of k_f. Thus, the overall rate constant for the first reaction would be ωk_f. These conformational changes continue for each HA until q of them have occurred, $X_{\omega,q}$. q is called the minimal fusion unit, as it equals the minimum number of HAs that have undergone the essential conformational change needed to stabilize the first high energy intermediate for fusion. At step 4, the fusogenic aggregate can transform to the first fusion pore, which is observed as the first conductivity across the apposed membranes. The first fusion pore, FP, evolves to the lipid channel, LC, demarked by mixing of lipids, which evolves to the fusion site, FS, demarked by aqueous contents mixing.

[3,13–15,24–26]. Finally, the fusion site (FS) can be formed, as monitored by aqueous contents mixing of fluorescent molecules [14], provided there is not too much leakage of contents [24,40,75].

3. Consensus kinetic parameter estimates

Table 1 shows the collected parameters fitted thus far [10,21,22,26]. For each data set, we generated all best fits. The consensus is defined as the subset of values that can fit all data sets. While it was significant that the three independent data sets of HA expressing cells fusing with target membranes could be explained, i.e. have similar fitted parameters, with a single kinetic model, there remained two important questions. First, in terms of these key fusion site architecture parameters, are the results of HA-expressing cells applicable to the virion fusing with target membranes? Second, while the kinetic analysis assumed a single homogeneous average surface density for each cell line, because of computational time constraints, the HA-expressing cells probably have an inhomogeneous distribution of HA surface densities. The question, then, was whether the key fusion site architecture parameters would remain largely unchanged once the distributions were incorporated into the analysis?

Our results showed, for the first time, consensus quantitative agreement on the fusion site architecture for the PR8 influenza virus and Japan-influenza HA expressing cell

Table 1
Summary of fitted rate constants for HA fusion

q	Fusion intermediate	Protein, k_f (s^{-1})	Fusion pore, k_p (s^{-1})	Lipid channel, k_l (s^{-1})
2	FP [23]	$(0.3-2)\times10^{-4}$	$(0.3-7)\times10^{-4}$	n.d.
	LC [13]	$(4-4.8)\times10^{-3}$	$(4.5-8)\times10^{-4}$	$(3-5)\times10^{-2}$
	LC [14]	$(4-4.5)\times10^{-2}$	$(0.03-1)\times10^{-4}$	$(2.5-2.6)\times10^{-2}$
	LC [72]	3	5.4×10^{-3}	1.5×10^{-1}

Parameters from exhaustive fitting of different kinetic data on HA mediated fusion using steps 3 and 4 of the model in Fig. 1. For a minimal aggregate size of eight HAs ($\omega=8$ [10]), a minimal fusion unit of two HAs ($q=2$) fit the data of HA-expressing cells (with Japan strain) fusing with (1) ganglioside containing planar bilayers at 37 °C [23]; (2) erythrocytes at 28–29 °C; and (3) erythrocyte ghosts at 37 °C. The same minimal fusion unit fit the data of PR/8 strain virions fusing with ganglioside containing liposomes [72]. See text for details.

lines. Evidently, since the minimal aggregate size $\omega=8$ and the minimal fusion unit $q=2$ are obtained from ratios of fitted parameters, the effects of the expected distributions of HA surface densities on the HA-expressing cells are not very significant. We will discuss this finding in "Proposed mechanism of HA-mediated membrane fusion".

We found that fitting all the PR/8 viral fusion data simultaneously and selecting the best-fit parameter sets yielded only two convergent solutions for parameters [21], as opposed to ranges for parameters [10,22] because more curves were being fitted simultaneously. By providing more data, with less experimental noise, steps 3 and 4 of the kinetic model in Fig. 1 are able to extract very robust estimates for the kinetic parameters.

We find that as the experimental systems provide higher surface density of HAs in the area of contact, the value of the average rate of the essential conformational change of HA, k_f, increases. As can be seen from Table 1, k_f for the virus is $\sim 3\ s^{-1}$, which is 1–2 orders of magnitude faster than HA-expressing cells fusing with RBCs, where surface density of HA in the area of contact is increased due to accumulation resulting from HA-glycophorin binding [22]. Blumenthal et al. [14] used 37 °C, as compared to 28–29 °C used by Danieli et al. [13], which explains much of the difference in the fitted values for k_f. The value of k_f for the virus is 4 orders of magnitude faster than HA-expressing cells fusing with ganglioside containing planar bilayers [10,22], where very little HA binding and accumulation occurs.

The increase of k_f with HA surface density suggests cooperativity, which is not yet incorporated into the kinetic model, since its mechanism is not yet known. An increased HA surface density should yield more and larger fusogenic aggregates [10,39]. Based upon our current knowledge, while more fusogenic aggregates would not promote any cooperativity, larger aggregates might. Markovic et al. [38] found that the overall refolding rate of Japan, X-31 and Udorn HA increases with increasing surface density, as assayed by subsequent DTT dissociation of the HA. They suggested cooperativity as the mechanism. The avenue of this cooperativity could well through the fusion peptides embedded in the viral or HA-expressing cell bilayers [8].

Both k_p and k_l are an order of magnitude faster for the virions fusing with target membranes than what we previously found for HA-expressing cells fusing with target membranes. The differences might simply be HA-strain dependent. Since the liposomes used by Shangguan et al. [24,25] were similar in composition to the planar bilayer used by Melikyan et al. [23], the differences are not likely to be due to target membrane properties. Also, the similarity in rate constants for lipid mixing and contents mixing found here for HA mediated fusion and by Lee and Lentz [41] for PEG-induced fusion of phosphatidylcholine liposomes supports the idea that subsequent to stable fusion pore formation, the evolution of fusion intermediates is determined more by the lipids than by the proteins. This is consistent with a lipidic stalk being common to bilayer fusion mechanisms, see Kozlovsky et al. (2002) [81] for a discussion.

Mittal et al. [21] found a pK_a of 5.6–5.7 for activation both PR8 and Japan strains of HA and a pK_i of 4.8–4.9 for inactivation of HA for the PR/8 strain of HA. This provides an incentive to investigate key histidine, aspartate or glutamate residues common to all strains. While the pK_a of the histidine side chain is closest to the value we find, glutamate and aspartate pKs could be increased by hydrophobic or negatively charged neighbors.

Fitting inactivation data of Leikina et al. [19], Japan HA-expressing cells and RBC, and Shangguan et al. [25], PR8 virions and 10 mol% ganglioside PC liposomes, both gave an estimate for the rate constant for inactivation as $k_{fi}\sim 2\times10^{-4}\ s^{-1}$ [21]. This is about the same as $k_f\sim 1\times10^{-4} s^{-1}$ measured by Bentz [10] for the data of Melikyan et al. [23], wherein Japan HA expressing cells fused with ganglioside containing bilayers. The simplest interpretation of k_f and k_{fi} is that they measure the same event, e.g. formation of the extended coiled coil, in the presence and absence of a target membrane, except that k_f also refers to a "successful" conformational change, which helps the first fusion pore to form. We would expect k_{fi} to measure the basal rate for an unbound and unaggregated HA.

4. HA conformations

During infection, virus bound to the cell surface is endocytosed and exposed to low pH, which produces at least three new conformations in HA, as depicted in Fig. 2. The native structure of HA (Fig. 2, conformation 1) is based upon the crystal structure of the bromelain-released hemagglutinin ectodomain, BHA [42]. Upon acidification, exposure of the amino terminus of HA2, known as the fusion peptide, occurs (Fig. 2, conformation 2). This change is rapid compared to fusion and is required to promote fusion between the viral envelope and the target membrane [43–47,82]. The second conformational change leads to the

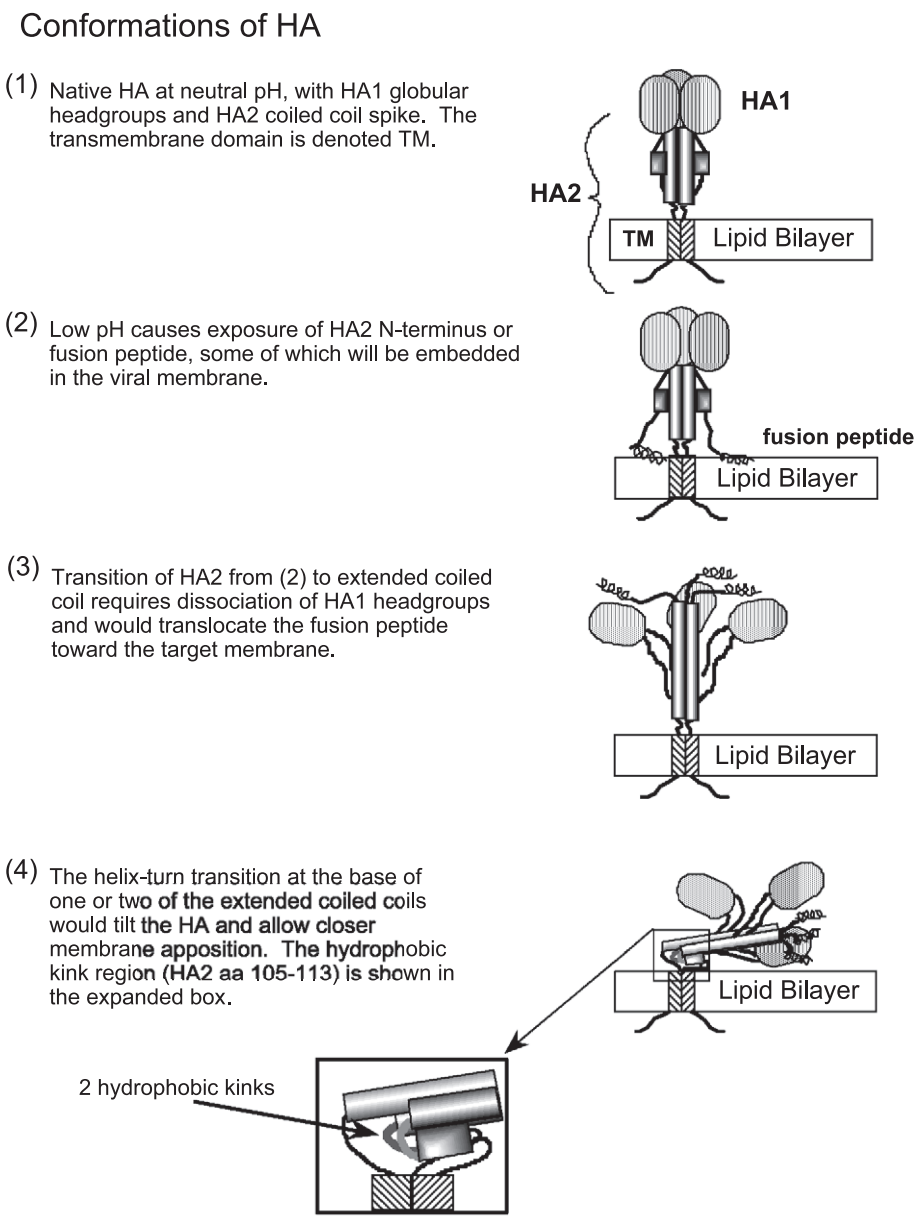

Fig. 2. The conformations of HA. (1) This is the native conformation of BHA, adapted from Ref. [42]. The globular HA1 headgroups sit on top of the spike-like HA2. Regions of α-helix and coiled coil are shown as cylinders. The bottom aggregate of α-helices is the transmembrane domain, striped and denoted TM. (2) Low pH releases the HA2 N-terminus, also known as the fusion peptide, which is shown here as embedded in the viral envelope, although other states are also possible. (3) The transition to the extended coiled coil by HA2, causes dissociation of the HA1 headgroups. (4) The helix-turn transition at the base of the original coiled coil of HA2 found in the crystal structure of TBHA2 [85] is shown. This transition has not yet been proven for membrane-bound HA.

formation of the extended coiled coil of HA2 (Fig. 2, conformation 3), which was predicted by Carr and Kim [48], proven for the crystallographic structure of a fragment of BHA (TBHA2, residues 38–175 of HA2 and 1–27 of HA1 held together by the disulfide bond) by Bullough et al. [49] and morphologically observed on the intact virus by Shangguan et al. [25], as discussed in Bentz [10]. In addition, Qiao et al. [16] showed that site-directed point mutations predicted to inhibit the formation of the extended coiled coil did inhibit the fusion of erythrocytes to HA expressing cells. This conformational change would extract the fusion peptide from the viral bilayer and relocates it more than 10 nm towards the target membrane. New stretches of coiled coil structure are formed.

The crystal structure of TBHA2 [49] also shows that the C-terminal end of HA2, where the native coiled coil flares out to accommodate the fusion peptide in the native state, flips up in helix turn between residues 106–112 of HA2 and forms an antiparallel α-helical annulus from residues 113–129 of HA2, i.e. at the base of the extended coiled coil (see Fig. 2, conformation 4). This helix-turn transition has been proposed as the essential conformational change to

cause the initial destabilization of the apposed bilayers [1,49–55].

Kim et al. [56] have argued that the helix-turn region, which they term the kink region of HA2 (aa 105–113), is important for fusion, while Epand et al. [57] and Leikina et al. [19] have found that the FHA2 fragment (the equilibrium structure of aa 1–127 of HA2, which runs from the fusion peptide to the end of the annular α-helix, with the extended coiled coil in place and the kink exposed) mediates membrane destabilization and lipid mixing in a pH-dependent fashion. FHA2 reversibly aggregates at low pH via the kink region [19,56,58], almost certainly due to hydrophobic amino acids.

5. Proposed mechanism of HA-mediated membrane fusion

Based upon the fitted kinetic parameters of HA fusion from a variety of experimental systems, we can construct a mechanism which is consistent with all of the data examined. HA contains a sialate binding site within the HA1 subunit that can bind to glycosylated proteins and gangliosides [59]. This provides a wide range of target receptors. Once bound to the target cell, the influenza virion is endocytosed. Acidification of the endosome protonates HA, which is the signal for fusion. This initiates the cascade of conformational changes leading to merger of the viral and endosomal membranes. After the signal for fusion, the fusion peptide is exposed on the N-terminus of these proteins [52,60–62].

While there has been a long literature proposing that the exposed N-terminal of HA next inserts into the target membrane to start fusion, it is more accurate to say that HAs can have their fusion peptides either suspended between the membranes or embedded in the target bilayer or in their own bilayer [4,25,63,64]. The fraction in each state probably depends upon time and the real question is: Which state or which sequence of states is on the ‘‘fusion pathway’’?

The common speculation follows from Skehel and Wiley [1], which had the extended coiled coil bring the fusion peptide to the target membrane bilayer, to hold the membranes in apposition, presumably about 15 nm apart. The helix turn then brought the bilayers together [49], stabilized by the N-cap formation at the C-terminus of the extended coiled coil [7,51]. This mechanism has been proposed for HIV fusion also (see [71]). While the N-cap structure is quite interesting, for it to form in vivo would require the HA transmembrane domain to dissociate and reform over the N-terminus of the extended coiled coil. While this is the most stable form of the EBHA2 fragment in solution, it is unclear how HA2, with its transmembrane domain embedded in the viral bilayer, would accommodate, or even get to, this structure. This model has not yet been used to construct a pathway by which the lipids of the target bilayer reach the viral bilayer.

Alternatively, Kozlov and Chernomordik [4] and Bentz [8] have proposed that those HAs whose fusion peptides embed initially into the viral/HA-expressing cell envelope are on the fusion pathway. Having the fusion peptide reach the target membrane represents a later step in the process. Thus, in Fig. 2 (conformation 2), the fusion peptide is shown to be embedded in the viral envelope and this is the population we follow.

The next step is aggregation of the fusion proteins. As discussed previously, we have shown that rapid aggregation of eight or more HAs followed by a slow ‘‘essential’’ conformational change of two of the HAs within the aggregate fitted key kinetic data from different laboratories. The cause of this rapid HA aggregation is not known. It could be adhesion of the fusion peptides within the aqueous space [65], although this would seemingly render the fusion peptides useless for bilayer destabilization.

Kozlov and Chernomordik [4] have proposed a novel mechanism for HA aggregation based on membrane curvature minimization. It begins with the fusion peptides embedded in their own (viral or HA-expressing cell) membrane and under tension created by the formation of the extended coiled coil. How does a protein create the tension needed to pull at a membrane, causing it to distort, and thereby induce HA aggregation, as proposed by Kozlov and Chernomordik [4]? The formation of the extended coiled coil is simply a sequence of heptad repeat binding steps, each of which extends the coiled coil another 10 Å, or so, and shortens the line to the fusion peptide by as much as 14 Å [9]. The formation of the next heptad repeat of the extended coiled coil should proceed by a somewhat random conformational search, which gets stuck by the formation of heptad repeat. These proteins proceed down a free energy pathway by falling through a sequence of quasi-irreversible steps, like a ratchet. Each step is made essentially irreversible by a large positive activation energy for return.

According to Kozlov and Chernomordik [4], the heptad repeat of a single HA has insufficient binding energy to hold up the bilayer against the curvature energy. However, when several HAs are close, the net curvature to the bilayer needed for all of them to sustain one (or more) heptad repeat binding reactions is sufficiently small to permit the action. Once a ring of HAs have formed their first heptad repeats of the extended coiled coil, this holds the bilayer curvature in place and the aggregate is held in place. Other HAs can diffuse into the aggregate and form their next heptad repeat, thereby increasing the tension and the bilayer curvature. Their model then proposes that the bilayer dome formed inside the circular HA aggregate is high enough to contact the target bilayer, where fusion would relieve the high curvature at the top of the dome.

As discussed in Bentz [8], this model has no role for the kinetic finding that two of the HAs in the aggregate do heavy lifting. The dome is formed by the tension formed by all of the HAs in the aggregate. We have coupled our kinetic results with the curvature model of Kozlov and Cherno-

mordik [4] and found that self-consistency is reached when the HA aggregates have 9–12 HAs [39]. Furthermore, the dome height is at least 4–5 nm from the target bilayer, and even further is the receptor for HA is a glycoprotein, like glycophorin. Fig. 3A shows the maximum curvature that can be induced in the viral membrane as a result of a collar of nine HA trimers from this calculation, with other HAs in the aggregate bound to glycophorin receptor. Even if the target membrane in the area of contact is curved maximally to approach the curvature of a 30-nm SUV, then also, the viral membrane curvature is not big enough to introduce outer monolayer contact. Thus, its seems unlikely that fusion follows simply by an HA-aggregate-induced bending of membranes. This is a wide gap for the lipids to cross. It suggests that there might be more to the mechanism of how the bilayers start mixing their lipids. This mechanism should include a role for the two HAs that undergo the slow, essential conformational change.

Beginning with the model of Kozlov and Chernomordik [4], there is an appealing segue with the model proposed in Bentz [8]. With the fusion peptides inserted into the viral membrane, the HAs will aggregate and begin to form the dome toward the target membrane. The collar of HAs will tighten until the site becomes lipid flow restricted, a step preceding lipid mixing [3,66,67]. Then the dome can grow no further.

5.1. High-energy conformational change

The question is whether the fusion peptide can remain embedded until the dome reaches the target membrane. Kozlov and Chernomordik [4] estimated the energy it takes to remove an alpha helical hydrophobic peptide from a bilayer was too great, while recognizing that this is a very crude calculation, and hypothesize that this membrane curvature increases until a dome from the viral bilayer reaches the target membrane. However, as argued in Bentz [8], there seems to be enough energy in the formation of the extended coiled coil to extract the HA fusion peptide from the viral envelope when the molecularity of the site is considered. For example, within the HA aggregate, the surface density of fusion peptides is very large and aggregates of fusion peptides could form [83], substantially reducing the energy needed to extract one of them from the site. The release of the fusion peptide from the viral bilayer would likely precipitate the rapid completion of the extended coiled coil for that HA and the translocation of that trio of fusion peptides to the target membrane. The HA transmembrane domains are required to be part of the

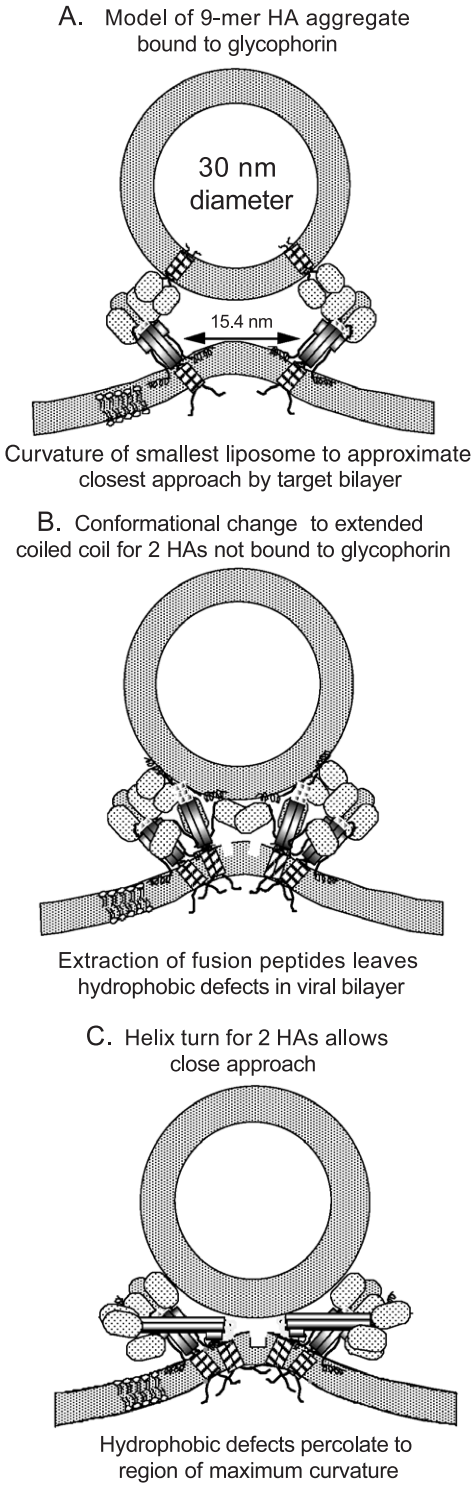

Fig. 3. Domes, hydrophobic defects and membrane fusion. (A) A typical dome created in the viral bilayer by an aggregate of nine HAs was calculated in Ref. [39], based on the formulations in Ref. [4]. Even for the maximum achievable curvature in the target membrane, shown by a 30-nm SUV, the outer monolayers are still substantially separated. Thus, membrane fusion seems unlikely to be initiated simply by bilayer–bilayer contact. (B) Extraction of fusion peptides from the viral bilayer creates hydrophobic defects. (C) The hydrophobic defects percolate to the top of the dome, thereby reducing the elastic curvature energy, creating a hydrophobic cavity. The structures in (B) and (C) are stabilized by transmembrane domains of HA and subsequently, by the low-energy conformational change exposing the hydrophobic kink region to the defect. When the hydrophobic defect at the top of the dome is big enough, viral inner monolayer lipids are engaged [68]. Lipids from the outer monolayer of the target membrane heal the defect initiating membrane fusion.

hydrophobic defect, because the HA aggregate must be closely packed enough to restrict lipid flow (see Fig. 3 and Ref. [8]). Having the transmembrane domains as part of the surface of the hydrophobic defect lowers the free energy required for its creation.

While the formation of the extended coiled coil of HA2 would extract the fusion peptides from the outer monolayer of the viral envelope, for those HA in isolation or in aggregates not in the state of restricted lipid flow, the evacuated space in the viral outer monolayer will be quickly filled by lipid diffusion. As a result, little or no significant change will happen on the viral envelope. Later on, for isolated virions, significant blebbing of the envelopes occurs [25]. Presumably, the fusion peptides of all the HAs undergoing the formation of the extended coiled coil would embed in the target membrane, which would explain the hydrophobic binding of virions to target membranes, e.g. liposomes, which occurs before fusion [3,8,10,45,68]. However, for those fusion peptides embedded initially within the site of restricted lipid flow (caused by fusogenic aggregate), the evacuated space cannot be refilled instantly, since the aggregated HA transmembrane domains and the remaining embedded fusion peptides would block the flow. Thus, a hydrophobic defect would be created at the site of extraction of this fusion peptide (see Fig. 3B), stabilized by the large aggregate of HAs, like a dam [8].

To probe the meaning of a persistent hydrophobic defect in a bilayer, Tieleman and Bentz [69] performed molecular dynamic simulations of up to 10 ns of a planar PC bilayer from which lipids have been deleted randomly from one monolayer. In one set of simulations, about half the lipids in the defect monolayer were restrained to form a mechanical barrier. In the second set, lipids were free to diffuse. The question was simply whether the defects in the monolayer caused by removing lipids (the ones shown in Fig. 3B left by removing the embedded fusion peptide) would aggregate together, forming a large hydrophobic cavity, or whether the membrane would adjust in another way.

It was found that in the absence of mechanical barriers, the lipids in the defect monolayer simply spread out and thinned, with little effect on the other ‘‘intact’’ monolayer. In the presence of a mechanical barrier, the behavior of the lipids depends on the size of the defect. When 3 lipids out of 64 were removed, the remaining lipids adjust the lower half of their chains but the headgroup structure changed little and the intact monolayer is unaffected. When 6–12 lipids were removed, the defect monolayer thins, lipid disorder increases, and lipids from the intact monolayer move towards the defect monolayer.

Our simulation showed a coupling of the intact monolayer with the defect when the defect perimeter lipids were fixed and six or more lipids were removed. This break point is interesting since two bilayer-embedded HA fusion peptides would occupy about the same surface area as 6 or so lipids, so that their removal would create about the same-sized hydrophobic defect. So, in the proposed fusogenic HA aggregate [8,9], the removal of two embedded fusion peptides from the center of the fusogenic aggregate would create a large enough hydrophobic defect to cause the intact monolayer acyl chains to enter into the defect area of the defect monolayer because of the hydrophobic transmembrane domains.

Provided that the ‘‘dam’’ of HAs remains intact, this scenario is reasonable and provides a first guess as to the molecular definition of ‘‘engaging the inner monolayer’’, which can distinguish between the defects which lead to fusion from those which lead only to hemifusion. That is, amphipathic peptides [9,19], low surface densities of HA [15] and some mutant HAs [5,15–18] only disturb the outer monolayers, leading to hemifusion. Here we have an example of how a hydrophobic defect in the lipid defect monolayer, if surrounded by a hydrophobic mechanical barrier, will engage the inner/intact monolayer.

Clearly, we are a long way from fusion and from evidence for the formation of a hydrophobic cavity. Obvious in Fig. 3B is the large distance between the bilayers. Likewise, these simulations run 10 ns, which leaves a long time for other events to occur. But it is a beginning. These simulations assumed a planar bilayer, since the extension to curved membranes is not yet theoretically feasible. If the beginning point for the fusogenic aggregate is a lipid dome tightly ringed by the aggregated HAs, then there is a positive elastic energy at the peak of the dome, due to its curvature [4]. One way to minimize the elastic energy of the curved dome would be to percolate the hydrophobic defects to its peak, thereby creating a single cavity, which would ameliorate the curvature free energy with little effect on the hydrophobic free energy, as shown in Fig. 3C.

5.2. Low-energy conformational change and the hydrophobic kink

It has been recently shown that the kink formed by the helix turn of HA mediates the low pH-dependent aggregation of the HA fragment known as FHA2, which comprises amino acids 1–127, i.e. the fusion peptide through the C-terminus of the six helix bundle at equilibrium [56,58]. This aggregation appears to be hydrophobically driven, perhaps following the protonation of aspartates within the kink region. This same fragment induces hemifusion between liposomes [57] and between cells [19]. Mutants in the kink region or lacking the fusion peptide do not induce lipid mixing.

A useful function of such a hydrophobic kink in fusion is readily apparent in the model of fusion shown in Fig. 3C. The formation of one or two helix turns would bend HA, allowing the close approach of the membranes. Just as importantly, the hydrophobic kink would now form a hydrophobic collar just above the hydrophobic defect. Bilayer lipids making an excursion from the target membrane would be stabilized by this hydrophobic collar and

thereby have a substantially greater chance of reaching the hydrophobic defect, i.e. begin lipid mixing. Like an enzyme, the hydrophobic collar would stabilize the transition state of fusion.

For the viral fusion proteins using coiled coils and folding something like HA [1,84], this hydrophobic kink should be near the C-terminus of the N-heptad repeat region or near the N-terminus of the C-heptad repeat region. Interestingly, Peisajovich et al. [70] have claimed to find a "second fusion peptide" in the Sendai virus F1 protein near the C-terminus of the N-heptad repeat domain.

It has been shown that the helix turn of HA [7] and its presumed equivalent in HIV [2,71] are required for fusion. All of the models that propose that the formation of the extended coiled coil simply tethers the membranes together predict that the helix turn is also required for fusion [1,2]. In fact, this is what the model of Fig. 3 would predict for HA, as explained in detail in Ref. [8]. Our hypothesis is also explicitly two-step, wherein a high-energy conformational change creates the defect and a low-energy conformational change is required to communicate that defect to the target membrane. If either step is blocked, then fusion is blocked. If the target bilayer is too far from the hydrophobic defect, then eventually the aggregate will disperse as the remaining HAs undergo the essential conformational change and lipid lateral diffusion fills the defect region [8]. As stated at the beginning, the question of whether bilayer fusion initiates with the contact of the dome with the target bilayer or with a HA aggregate stabilized hydrophobic defect providing a sink for lipids permeating from the target bilayer has not yet been answered. We believe that the consequences of both models must be compared with the detailed kinetics of fusion before clarifying experiments will emerge.

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