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Annual Review of Biophysics

Hemagglutinin-Mediated Membrane Fusion: A Biophysical Perspective

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hemagglutinin, membrane fusion, viral fusion, influenza cell entry, single-particle, modeling

Abstract

Influenza hemagglutinin (HA) is a viral membrane protein responsible for the initial steps of the entry of influenza virus into the host cell. It mediates binding of the virus particle to the host-cell membrane and catalyzes fusion of the viral membrane with that of the host. HA is therefore a major target in the development of antiviral strategies. The fusion of two membranes involves high activation barriers and proceeds through several intermediate states. Here, we provide a biophysical description of the membrane fusion process, relating its kinetic and thermodynamic properties to the large conformational changes taking place in HA and placing these in the context of multiple HA proteins working together to mediate fusion. Furthermore, we highlight the role of novel single-particle experiments and computational approaches in understanding the fusion process and their complementarity with other biophysical approaches.

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INTRODUCTION

Many biological processes rely on mixing the contents of two separate compartments. This mixing step requires fusion of the lipid membranes enveloping the compartments, a transition with an appreciable kinetic barrier. Fusion proteins act as catalysts to overcome this barrier so that fusion takes place within biological timescales (90). A classical example is the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. This group of proteins mediates fusion of vesicles in synaptic transmission between neurons (108); it is also involved in cargo transport between the Golgi apparatus and the endoplasmic reticulum and catalyzes fusion between the late endosome and the lysosome (58). Another example that has been the subject of intense study for decades and that has great significance to human health is the group of fusion proteins that mediate cell entry of membrane-enveloped viruses (48, 67). In this review, we focus on the influenza hemagglutinin (HA) fusion protein as a canonical example of a viral fusion protein and take a biophysical perspective in describing its mechanism of action.

The replication cycle of viruses relies on invading target cells and using them for the production of new virions. The viral genome, to be delivered to the nucleus of the target cell, is carried inside a protein capsid. Enveloped viruses are characterized by a lipid bilayer that envelops the protein capsid. Embedded in this membrane are the viral fusion proteins, which can be activated by binding to a specific receptor on the surface of the target cell or by a change in pH in the acidifying endosome (131). Upon activation, the fusion proteins establish a physical connection with the target cell by insertion of hydrophobic segments into the target membrane. Extensive refolding of the fusion protein brings the membranes in close proximity for fusion, resulting in the formation of a pore through which the viral genome is released into the cytosol of the target cell (21).

Three structural classes of viral fusion proteins have been identified (47). Class I fusion proteins (found in, for example, HIV-1 and influenza) consist of homotrimers that are primed by enzymatic cleavage, creating two distinct subunits. One is responsible for receptor binding and the other for fusion, with the fusion-active subunit containing primarily alpha-helical motifs. Class II proteins (in alpha- and flaviviruses and others) exist as hetero- or homodimers on the viral surface but

Influenza hemagglutinin (HA):
a class I homotrimeric glycoprotein of the influenza virus, responsible for target membrane binding and fusion

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trimerize upon activation. They are primed by cleavage of a partner protein and have a large amount of beta sheets in their structure. Class III (from, for example, rhabdo- and herpesvirus) represents a mixture of the other two (48). The focus of this review is on the class I fusion protein HA (118). Since the elucidation of its structure in 1981 (133), extensive research into the relation between its complex series of conformational changes and its ability to fuse two lipid bilayers has made HA the archetypal membrane fusion protein, serving as an example for the operating mechanisms of other fusion proteins (48, 117).

A vast amount of knowledge has already been acquired on HA, from structural information to kinetic data, and various experimental methods have been developed to reconstitute HA-mediated membrane fusion with careful control over binding and fusion. These studies and methods have made HA-catalyzed fusion into an ideal model system to understand the biophysical principles underlying protein-mediated membrane fusion. Additionally, HA is one of the primary targets for antiviral drugs against influenza (125). However, the ability of the virus to extensively mutate without losing function has thus far prevented the development of long-lasting vaccines. An improved insight into the fusion process as well as the intermediate protein and lipid conformations involved may help to identify conserved aspects of HA-mediated membrane fusion. Targeting conserved residues that are crucial for this mechanism provides a strategy for the development of a universal, rationally designed antiviral drug (127). Lastly, understanding the viral entry pathway can help in employing viral fusion mechanisms for more efficient delivery of targeted therapeutic agents. Such an approach is a potential route to better drug efficacy, since the escape of the agent from the endosome currently is a major hurdle for the delivery of such therapeutics (126).

This review combines recent structural and physical insights from experimental and computational studies to provide an up-to-date biophysical perspective on HA-mediated membrane fusion. We describe the pathways and energetics of the process, starting with the membrane rearrangements, followed by the conformational changes in HA. We then continue by discussing the role of multiple copies of HA in membrane fusion and conclude with suggestions for future research directions in this field.

MEMBRANE FUSION

Biological membranes consist of two amphipathic lipid monolayers that aggregate their lipid tails to form a hydrophobic layer. The delineating hydrophilic lipid head groups provide solvability to this planar aggregate. Fusing two separate membranes into one generally involves a hemifusion intermediate in which only the proximal monolayers have merged (17). Pore formation, through subsequent union of the distal monolayers, completes the fusion process. Zooming in on the process, several distinct intermediate states can be distinguished that have modest free-energy differences but that are separated by relatively high energy barriers. After introduction of the fusion pathway, we describe the methods for characterizing fusion intermediates and barriers with a focus on just the membranes, followed by a discussion of the physical origin behind these barriers and current barrier-height estimates.

Pathway

The canonical pathway of membrane fusion is illustrated in **Figure 1a**. Upon dehydration, in order to bring the two bilayers into close proximity, the nearest monolayers fuse to form a stalk. Radial expansion of the stalk creates a hemifusion diaphragm (HD) in which only the proximal leaflets have merged and the distal leaflets touch. Full fusion can proceed through pore formation within the hemifusion diaphragm or more directly from a minimally expanded stalk (18, 97). Alternative

Hemifusion diaphragm (HD):

a single bilayer formed by the distal monolayers after stalk expansion, when only the proximal monolayers have merged

Figure 1

(a) Schematic representation of intermediates in the canonical membrane fusion pathway and (b) the height of the energy barriers between them. The barriers for the single-step transitions directly to hemifusion and from there to pore formation are shown in blue. Several studies split the free-energy landscape into additional intermediate steps (indicated by the red, purple, green, and orange arrows in panel a) and associated barriers (indicated by correspondingly colored curves in panel b)—for example, stalk formation from an already dehydrated state (red), the formation of a hemifusion diaphragm from the stalk (purple), pore formation in the hemifusion diaphragm (green), and pore expansion (orange). Each of the barriers in panel b is drawn as a range between the maximum and minimum free-energy barriers reported in the literature, except for pore expansion, for which only qualitative data is available (1, 63, 64, 83, 93, 110, 120). The barrier estimates from these studies were selected on the basis of parameters most relevant to influenza fusion (see text). The barrier shape is schematic. Solid barrier lines are drawn only as guides to the eye, midway through each of the ranges of previously reported energies. To aid comparison of the barrier heights, the absolute free energies of all intermediate states are aligned at 0 kT. The arrows on the horizontal axis indicate contributions from protein-mediated events that can possibly lower the corresponding barrier (as discussed in the section titled Hemagglutinin Conformational Changes): zippering, fusion peptide (FP), and transmembrane domain (TMD). An overview of barrier data, including those displayed, can be found in **Supplemental Table 1**.

routes ensuing stalk formation, which involve lateral stalk expansion or a stalk–pore complex, are reviewed in References 36 and 88 and are treated only briefly here.

Methods

Cryo-electron tomography (cryo-ET):

three-dimensional imaging technique of biological samples under cryogenic conditions using transmission electron microscopy

Direct visualization of short-lived intermediates of membrane fusion at the relevant nanoscopic length scales demands experimental assays with very high temporal and spatial resolution. X-ray diffraction experiments have allowed for the visualization of stalk geometries and enabled the determination of the dehydration barrier through analysis of the interbilayer separation as a function of osmotic pressure (111). Hemifusion diaphragms have recently been observed using confocal microscopy on giant unilamellar vesicles (96) and in live cells (138). Hemifusion diaphragms (19) and extended areas of closely apposed membranes (44) have been imaged by cryo-electron tomography (cryo-ET). The kinetics of hemifusion and pore formation have been observed using optical tweezers (98) and fluorescence microscopy (83), methods that can be combined with single-particle tracking (100), as discussed later in this review.

These experimental assays are supplemented by modeling approaches to provide additional information on the molecular and energetic details of the fusion intermediates. Computational models can be divided into continuum elasticity theories (11) and particle-based numerical simulations (36). Starting from the Helfrich model of membrane bending (49), continuum elastic models have been formulated to incorporate lipid tilting (75), lipid splaying (73), membrane stretching (110), membrane dehydration, and saddle-splay deformation (72). In all these methods, energy minimization provides the optimal shape and free energy of fusion intermediates. Particle-based molecular dynamics (MD) simulations are based on the instantaneous interactions between individual atoms (31) or groups of atoms (55). An advantage of MD simulations is that the system can explore conformational space and reaction pathways in an unbiased and unguided manner, potentially resulting in alternative fusion pathways. A disadvantage is that many transition trajectories are needed to get an accurate estimate of the free energy (61). This is why, often, enhanced sampling methods have to be used (94, 119).

Barriers

Transitions between intermediate states of membrane fusion involve appreciable energetic barriers arising from unfavorable lipid interactions, such as dehydration of polar lipid head groups, generation of membrane curvature, and transient exposure of hydrophobic lipid tails to the aqueous environment. The height of these energy barriers depends on the membrane composition, tension, and initial curvature (18, 69, 70, 88), as summarized in **Figure 1b**. Because of the large number of variables involved, we consider only the canonical fusion pathway, using values reported for lipid compositions that are close to that of the influenza membrane envelope (41, 104) (approximately POPS:DOPE:cholesterol:sphingomyelin at molar ratios of approximately 1.5:1.5:5:2) and the epithelial cell membrane (112) (approximately POPC:POPE:cholesterol at approximately 2:1:1). A more comprehensive overview of barrier estimates can be found in **Supplemental Table 1**.

The first barrier in membrane fusion, the dehydration barrier, is formed by repulsive forces that have to be overcome to bring the bilayers into sufficiently close contact (<1 nm) (110). The formation of dimples on the membranes could lower this barrier by decreasing the area of close contact (75). As can be seen from **Figure 1b**, a dehydration barrier in the range of 30–90 kT has been estimated for influenza fusion (1, 64), depending on the specific geometry and lipid composition. This estimate includes the entire transition from unfused membranes to a stalk.

Once in a dehydrated state, stalk formation is initiated by the protrusion of a splayed lipid tail, establishing a lipid bridge with the opposing membrane (89, 120). Such protrusions are most favorable at an interbilayer distance of 0.9 nm and are more probable with increasing membrane curvature (123). Hence, the height of the barrier to stalk formation is dependent on the initial membrane separation and curvature, a fact that is often overlooked when citing quantities for this free-energy barrier (1). In dehydrated conditions, a remaining 15–30- kT barrier for stalk formation is estimated (**Figure 1b**) from MD simulations (63, 93, 120). This value corresponds well with estimates from experiments in the presence of high-molecular-weight polyethylene glycol or fusion proteins, such as SNARE (83, 98). Such protein mediation in membrane dehydration is discussed in more detail in the next section.

A stalk state can lead to a pore in a single step or through stalk expansion and subsequent formation and expansion of a hemifusion diaphragm. Estimates of the stalk-expansion barrier with membranes of physiologically relevant composition range from 14 to 33 kT (**Figure 1b**) (63, 83, 105, 110). This barrier arises from the opposing directions of intrinsic curvature between different lipids inside and outside the HD, specifically near the rim of the HD (110). During HD expansion, tension can build up along the rim until a pore forms (71). The energy required for

Continuum elasticity theories: continuum mechanics modeling of membrane deformations, generally based on the Helfrich model of membrane bending

Molecular dynamics (MD): a simulation technique to determine particle positions over time by integrating Newton's equations of motion using a predefined force field

Globular domain:

a compactly folded region of 47 residues per monomer at the viral membrane proximal part of the hemagglutinin stem

Fusion peptide (FP):

a 23-residue amphipathic peptide near the N-terminus of HA2 (the inner core of hemagglutinin) that can insert into the target membrane

Hydrogen-deuterium exchange with mass spectrometry (HDX-MS):

a technique to determine dynamic changes in peptide and protein conformations from the segment-specific amount of backbone amide hydrogens exchanged

expansion of such a rim-proximal pore increases with HD diameter (107), suggesting a limited window of opportunity for pore formation during HD expansion, as corroborated by observation of large, fusion-arrested HDs using cryo-ET (19). Starting from an HD with a diameter smaller than 10 nm, a pore formation barrier of 14–35 kT has been predicted (**Figure 1b**) (63, 110), which agrees well with estimates from experiments (83, 98).

The single-step formation of a pore from a minimally expanding stalk faces an estimated 90 to 120 kT (**Figure 1b**) (64, 110). The pathway through an expanding hemifusion diaphragm has lower barriers, but protein mediation and the specific conditions of membrane curvature and tension can favor the direct transition from a stalk to a pore (63, 98).

After its formation, the pore needs to expand for the virus to release its bulky contents into the host. Pore expansion has been reported to be energetically the most demanding step (17, 22), with membrane tension as the primary contributing factor (69), although some studies report no barrier for pore expansion (**Figure 1b**) (83, 110). Pore expansion in cell fusion was found to be highly dependent on the density of HA fusion proteins (HA density *arrow* in **Figure 1b**) (81) and similarly on SNARE density (135). Live-cell imaging has reported fusion-pore opening and closing (flickering) prior to full fusion, implicating the presence of cell-specific fission mechanisms that compete with fusion-pore opening (138). These observations emphasize the importance of the biological context involving membrane, protein, and environmental parameters. To distill the biophysical effects of each variable, dedicated experiments are crucial. Before we review an example of such an experiment, we first discuss the HA fusion protein in more detail.

HEMAGGLUTININ CONFORMATIONAL CHANGES

Influenza membrane fusion is mediated by the HA fusion protein. The prefusion (133) and postfusion structures of HA (**Figure 2, inset**) (9, 16) revealed that extensive conformational changes are involved in its fusogenic activity. Biochemical and computational studies have provided further information on the triggering mechanism and possible intermediate states, which has led to several hypothesized pathways of the conformational changes. As we discuss here, these structural states and transitions can be related to the intermediate states and energy barriers involved in membrane fusion.

Structure and Triggering

The HA glycoprotein is a 13.5-nm-long trimer that is synthesized as the inactive precursor HA0. It enters a metastable conformation after enzymatic cleavage, comprising two disulfide-bonded chains per subunit, HA1 and HA2 (**Figure 2, inset**) (133). HA1 (328 residues) forms the globular head of the protein and mediates attachment to sialic-acid receptors on the target cell by its receptor-binding domain. HA1 further plays a role in maintaining the protein in its metastable state at neutral pH by covering the fusion-active subunit, HA2 (221 residues). A triple-stranded alpha-helical coiled coil in HA2 forms the core of the protein, sitting on top of a small globular domain (**Figure 2, inset**) that contains the disulfide bond with HA1. The 23 residues near the N-terminus of HA2 make up an amphipathic fusion peptide that is tucked away in a hydrophobic pocket between the central alpha helices at neutral pH. The transmembrane domain at the C-terminus of HA2 anchors the protein in the viral membrane.

The drop in pH to a value between 5 and 6 in the maturing-to-late endosome (76) activates a series of conformational rearrangements in the protein. Computer simulations have indicated that protonation of residues around the fusion peptide, in particular residue Asp112 (86), is the major trigger to release the fusion peptide from its pocket (**Figure 2, ii**) (27, 139). Hydrogen-deuterium exchange with mass spectrometry (HDX-MS) experiments have further shown that

Figure 2

Schematic representation of the conformational changes in influenza hemagglutinin (HA) and the corresponding membrane rearrangements. Only two subunits of the trimer are shown, and HA1 is omitted in columns *iii–vi* for clarity. The protein structures in the inset show the transition from the prefusion [114; Protein Data Bank (PDB) code 1HGF] to the postfusion state (16; PDB code 1QU1), with one monomer highlighted in each of the states. In all pathways, HA binds to cell receptors (*i*, brown) and is activated by low pH, inducing release of the fusion peptide (FP) (*ii*, red) and dissociation of HA1 (*ii*, orange). Next, the relative rates of extension ($k_{\text{extension}}$) and foldback (k_{foldback}) determine the nature of the hypothesized fusion pathway. In the canonical productive pathway, for $k_{\text{extension}} > k_{\text{foldback}}$ (1), coiled-coil formation in the B loop (blue) enables HA extension and insertion of the fusion peptide into the cell membrane (*iii*, 1), followed by foldback of the hinge region (purple) and the zippering mechanism upon unfolding of the globular domain (black) to overcome the dehydration barrier (*iv*, 1) prior to stalk formation (*v*, 1). The FP and transmembrane domain interact to facilitate pore formation (*vi*, 1). Two alternative pathways have been proposed. For $k_{\text{extension}} < k_{\text{foldback}}$ (2), foldback before extension enables insertion of the fusion peptides in both the virus and cell membranes (*iii*, 2), before simultaneous coiled-coil formation and zippering brings the membrane into close contact (*iv*, 2), again followed by stalk (*v*, 2) and pore (*vi*, 2) formation. Nonproductive refolding occurs when extension happens simultaneously with foldback ($k_{\text{extension}} \approx k_{\text{foldback}}$, 3), giving the fusion peptides no opportunity to insert into the target membrane (*iii*, 3). Instead, they are directed toward the viral membrane (*iv*, 3), into which they insert, thereby inactivating HA (*v*, 3). Parts of figure reprinted from Reference 5, copyright 2016, with permission from Elsevier.

reversible release of the fusion peptide (35) precedes the dissociation of the interface between neighboring HA1 subunits within the trimer (39), the latter being a necessary step for function (43, 65). Protonation of residues at this HA1–HA1 interface (27) and increased electrostatic repulsion between HA1 subunits drive their dissociation (28, 52, 139), while they remain bound to the receptors (Figure 2, *ii*) (113).

Heptad repeat:

repeating pattern of hydrophobic and charged amino acid types in the protein sequence typical for alpha-helical coiled coils

Pathways of the Conformational Change

After fusion-peptide release and HA1 dissociation, HA2 undergoes extensive conformational changes before entering the postfusion state (9, 16). Depending on the rates of the conformational changes of individual segments, two pathways have been proposed that successfully bring the two membranes together for fusion.

In the first productive pathway (**Figure 2, ①**) (18), the unstructured B loop folds into a coiled coil [with rate $k_{\text{extension}}$, proposed to be independent of pH (51)]. This coiled-coil structure extends the existing coiled coil, bringing along the fusion peptide for insertion into the target membrane (**Figure 2, iii, ①**) (122). This conformational change forms the elusive extended intermediate, a state that thus far has escaped structural characterization. Only recently, direct indications of the existence of the extended structure have been observed in a cryo-ET study (10). Intriguingly, the strong coiled-coil propensity of the B loop region is suppressed during the folding of HA in the endoplasmic reticulum, and extension becomes possible only after priming by enzymatic cleavage (15). This highlights the metastability of the prefusion structure and suggests a so-called spring-loaded mechanism (12).

The second structural change involves partial unfolding of the central helix from the point where the fusion peptides initially were tucked away. Here, the hinge region at the bottom of the central helix folds back toward the remaining coiled coil, at a rate that is lower than the initial HA extension ($k_{\text{foldback}} < k_{\text{extension}}$) (**Figure 2, iv, ①**). The tendency toward this foldback transition is another example of a built-in structural metastability in the prefusion structure, owing to a shift in the coiled-coil heptad repeat (115). From the extended intermediate, with both membranes connected through the protein structure (**Figure 2, iii, ①**), the foldback seems possible only once the globular domain has sufficiently unfolded. The unfolded globular domain subsequently packs as a so-called leash into the grooves of the coiled coil, zippering up along a ladder of distinct hydrophobic patches (**Figure 2, v, ①**). The refolding then culminates in stabilizing N-cap interactions (16, 102) and fusion peptide and transmembrane domain association for pore formation (**Figure 2, vi, ①**) (13, 77). Indirect evidence for this pathway comes mainly from the inhibition of fusion by peptides that bind to the extended intermediate of the HIV fusion protein (132), an approach that also works with peptides targeting HA, albeit at much higher peptide concentrations (80).

Two other pathways are possible from the moment of activation, depending on the relative rates $k_{\text{extension}}$ and k_{foldback} . The second productive pathway was predicted by MD simulations of HA2 using a structure-based bias (85), later supplemented by unbiased all-atom MD (**Figure 2, ②**) (86). For values of $k_{\text{extension}}$ that are sufficiently smaller than k_{foldback} , rapid foldback before complete unfolding of the globular domain leads to a symmetry-broken intermediate (**Figure 2, iii, ②**). Diffusion-limited insertion of fusion peptides in both the target and viral membrane would allow for the bundling of energy from both coiled-coil formation and zippering (**Figure 2, iv-vi, ②**). No experimental evidence has confirmed the existence of this pathway yet.

In the nonproductive pathway (**Figure 2, ③**), foldback happens almost simultaneously with extension ($k_{\text{extension}} \approx k_{\text{foldback}}$), directing the fusion peptides away from the target membrane before they can insert (**Figure 2, iii, ③**). Irreversible insertion of the fusion peptides into the viral membrane, as demonstrated by unbound virions after acidification (128, 130), causes inactivation of HA (**Figure 2, v, ③**). Such nonproductive refolding would occur stochastically after HA activation, so a single HA protein cannot a priori be called (non)productive. As appears from fusion-kinetics experiments combined with stochastic modeling, the majority of HAs may refold nonproductively (57), suggesting that $k_{\text{extension}}$ is indeed close to k_{foldback} or that other factors hinder HA activation or fusion-peptide insertion. Additionally, a difference in binding affinity of the fusion peptide with

either the target or the viral membrane could influence the fraction of nonproductively refolding HAs, and changing this affinity by using different lipid compositions might provide a way to test the nonproductive pathway hypothesis.

There are several arguments to assume that $k_{\text{extension}} > k_{\text{foldback}}$, thus favoring the first pathway for productive refolding. The folding rate of a cross-linked coiled-coil dimer is about $3 \cdot 10^4 \text{ s}^{-1}$ (30). Although the folding rate for the extension of the larger trimeric coiled coil, $k_{\text{extension}}$, would probably be somewhat lower than this, it would still be orders of magnitude higher than the rate constant for complete HA rearrangement. In the absence of a target membrane, the latter rate is about 5.8 s^{-1} at pH 4.9 (74), although this value may be different in the context of a native virion and target membrane. Furthermore, it has been suggested that B loop extension is guided by receptor-bound HA1 (53, 57), thus increasing $k_{\text{extension}}$ with respect to unconfined folding. Similarly, the presence of HA1, not modeled by Lin et al. (85, 86), could hamper symmetry breaking and thereby decrease k_{foldback} (53). Finally, HDX-MS studies have shown that, during activation, fusion peptide and B loop dynamics already increased before HA1 dissociation, essentially giving coiled-coil extension a head start (39).

Surmounting Membrane Fusion Barriers

The connection between membrane fusion intermediates and specific conformational states of HA is not fully clear. It has been shown that the zipper mechanism of HA and formation of the N-cap at the end of the coiled coil deliver a significant amount of energy for dehydration of the fusion site and stalk formation (indicated by the zipper *arrow* in **Figure 1b**) (7, 16, 102). The amount of energy available from HA refolding has recently been computed to be about 34 kT per HA (6). Estimates of the energy supplied by other individual fusion proteins range from 47 to 71 kT for HIV (59, 91) and 35 or 65 kT from partial or complete SNARE complex formation, respectively (38, 84). Not all this energy will be used efficiently, so it is plausible that multiple fusion proteins will be required to surmount all the membrane fusion barriers shown in **Figure 1b**.

Interactions of the fusion peptide with the membrane are essential for fusion, as mutations in the fusion peptide can completely inhibit fusion or halt the process at hemifusion (2). The fusion peptide can lower the barrier to stalk formation (FP *arrow* in **Figure 1b**) by increasing the probability for lipid protrusions (62, 79) and by promoting the strong negative curvature in the stalk by its inverted wedge shape (42, 87, 121). Computational studies indicate that fusion peptides form transmembrane bundles (106) and induce positive curvature, thus stabilizing pores instead of stalks (37). However, the latter studies used structures derived from a shorter 20-amino-acid sequence that displays a more elongated boomerang shape (46), which could cause the difference in observations. In addition to the fusion peptide, the viral-membrane proximal region of HA (the region between the ectodomain and transmembrane domain) might be involved in stalk formation but its exact role and structure are still undetermined.

The mechanisms that drive stalk expansion and pore formation remain unclear (*question marks* in **Figure 1b**). Point-like forces, such as those between the transmembrane domains of SNAREs (105), might exist between transmembrane fusion-peptide bundles and transmembrane domains of HA (93, 106). These forces could cause a thinning and widening of the stalk (26). Hemifusion diaphragm expansion could also be driven by increasing membrane perturbations when fusion peptides associate with the transmembrane domains (TMD + FP *arrow* in **Figure 1b**) (13, 77) as well as increased membrane tension from HAs pulling the membrane around the fusion site (82). Finally, it has been shown that part of the transmembrane domain is necessary for pore formation and enlargement (3, 66, 92).

Although it is clear that the large conformational changes in HA serve to bring the two membranes into close contact and that the fusion peptides and transmembrane domain play important roles in further local membrane remodeling, the molecular details and individual energetic contributions remain elusive. We proceed by summarizing what has been learned about these aspects from recent experimental studies.

SINGLE-PARTICLE CHARACTERIZATION OF INFLUENZA FUSION

Over the last four decades, assays ranging from cell–cell and liposome–virus fusion to biochemical and structural studies have greatly improved our understanding of HA-mediated fusion (reviewed in 5, 45). More recently, novel methods that focus on the observation of fusion at the level of individual particles have resulted in significant new insight into the mechanisms of HA activity and the manner in which multiple HAs work together. For example, single-particle tracking in cells has allowed the visualization of the route of influenza entry into cells (76, 78; reviewed in 8) and reconstitution of fusion of fluorescently labeled viral particles with artificial target membranes has enabled the study of fusion kinetics (23, 34, 56, 129). Such in vitro single-particle approaches, with their ability to control reaction conditions and their high kinetic resolution, provide important insight into the biophysically relevant aspects of fusion and are the focus of this section.

Single-Particle Kinetic Assays

Single-particle assays allow the observation of multiple steps in the fusion pathway within a single experiment for many individual virus particles simultaneously. Therefore, rather than observing an ensemble average, the full population distribution is obtained. Further, such single-particle trajectories provide access to short-lived intermediate states, information that would be lost in the bulk experiments owing to the asynchronicity and dephasing of events. The analysis of single-particle data using stochastic modeling has brought new insights of influenza fusion, which we describe below, and has successfully been applied to other viral fusion systems (14, 68).

Bottom-up and controllable design is a key aspect of in vitro single-particle assays, relying on the use of purified components and model membranes. In the most commonly used design (**Figure 3a**), a planar, supported lipid bilayer serves as the fusion target (24, 34). Assembling such a supported bilayer in a flow channel allows for a synchronous reduction of the pH to trigger the viruses to fuse (23, 34, 129). The use of fluorescent tags enables tracking of multiple observables simultaneously, and the low background needed for single-particle sensitivity is achieved by employing total internal reflection fluorescence microscopy (4). The synchronous triggering of the fusion reactions is achieved by acidification of the immediate environment and monitored by the use of a pH-sensitive probe (**Figure 3b**). With a lipophilic dye incorporated in the virus membrane, the association of viral particles with the target membrane can be directly visualized. Such experiments have demonstrated the rolling of influenza particles along the membrane under the force of the flow and the subsequent cessation of this movement (**Figure 3c**). These two events have been interpreted as the weak association of HA1 with sialic-acid membrane receptors and the insertion of the fusion peptide into the membrane, respectively (56). Escape of the dye into the target membrane indicates lipid mixing and reports on the formation of a hemifusion state (**Figure 3d**). By encapsulating an aqueous dye inside the virus, pore opening can be detected when the content label dissipates into the space underneath the supported lipid bilayer (**Figure 3e**). Other possible readouts are the stoichiometry of the fusion proteins or their inhibitors (**Figure 3f**) and the ordering phase of the target membrane (136). With respect to inhibitors, the advantage of single-particle observation is that the influences of affinity and concentration are

Figure 3

Single-particle assay and observables. (a) The ~ 100 -nm-thin layer of laser excitation resulting from total internal reflection is used to excite fluorescent labels while minimizing background fluorescence from solution. (b) Multiple probes can be tracked concurrently for the same virus particle. (c) The binned distribution of times from pH drop to arrest of single, rolling particles (associated with fusion-peptide insertion). (d) Time distribution from pH drop to hemifusion, as detected by the membrane label escaping into the target membrane. (e) Times from hemifusion to opening of a pore, as reported by content-label escape. (f) By using tagged inhibitors, the fusion yield (fraction of the population achieving hemifusion) was correlated with the observed number of inhibitors bound to an individual virion. The line represents a general logistic model with 95% confidence bands (99). Examples of observables in panels c–f adapted from References 34, 56, and 99. Parts of figure reprinted from Reference 5, copyright 2016, with permission from Elsevier.

bypassed by directly counting the number of inhibitors bound. Future extensions may be able to clarify the full sequence of events from docking to genome release, by tagging the viral capsid or genome. Multicolor alternating laser excitation (60) could enable the simultaneous readout of more observables.

Influenza Fusion Mediated by a Cluster of Stochastically Inserted Hemagglutinins

Measurements of the time between acidification and hemifusion for single influenza particles showed a rise-and-decay distribution (**Figure 3d**), with the mean fusion time shortening with decreasing pH (23, 34, 54). Also, the arrest times for the particles to stop rolling exhibit a rise-and-decay distribution (**Figure 3c**) (56). This non-single-exponential behavior indicates that attaining arrest and hemifusion is not a single-rate process but rather requires multiple steps of comparable rates to complete. The observations that fusion is mediated by HA, that HA activation is pH dependent (29, 39), and that HA can be driven into the postfusion state by high temperature (109) have led to the development of a model explaining the single-particle observables (arrest and hemifusion) as resulting from stochastically inserting HAs without the necessity of inter-HA interactions. The key steps in this model are summarized in **Figure 4a**.

Ivanovic et al. (56) found that the rate-limiting step in the conformational change of HA was fusion-peptide release. Hence, the change of HA from the prefusion to the extended state was modeled as a transition into a deep potential well with a single energy barrier. Reduction of

a Key states of influenza fusion **d** Nonproductive HAs **Figure 4**

Influenza fusion modeled by influenza hemagglutinin (HA) cluster formation after stochastic insertion and sensitivity to fusion inhibitors. (a) The key states of fusion: a virion is docked to receptors and rolls along the surface while HA insertions take place stochastically in the contact patch (schematically shown as a simplified grid of $M = 19$ trimers; realistic estimates are $M = 50\text{--}150$, where M denotes the number of HAs potentially interacting with the target membrane). Individual HAs insert independently with rate k_{insert} , a function of pH. A certain number of insertions N_{arrest} (example of 3 shown) arrests the particle. Insertions continue until a sufficiently large local cluster N_{cluster} (example of 3 shown) is formed. Hemifusion proceeds rapidly after cluster formation (i.e., k_{hemi} is large compared to previous steps). Finally, a pore opens with rate k_{pore} as directly observed (see **Figure 3e**). (b) Using $N_{\text{arrest}} = 3$ as an example, the distribution of arrest times arises as the convolution of the three single-exponentially distributed insertions, resulting in a rise-and-decay distribution. (c) The requirement to have N_{cluster} -inserted HA neighbors convolves over the number of insertions with their time distributions (arising in the same way as in panel b) to form the hemifusion time distribution. (d) The graph shows the fusion yield (the fraction of the virus population undergoing fusion) as a function of the number of fusion inhibitors bound to individual virus particles, as modeled in Reference 57. Data for two different strains are shown, differing markedly in their sensitivity to these inhibitors. The half-maximum points are indicated (dagger and asterisk). The small number of inhibitors necessary to effectively inhibit fusion is explained by the presence of a large fraction (2/3 to 3/4) of nonparticipating HAs (gray in pie charts), thought to arise from nonproductive HA pathways (see **Figure 2**). Strain 1 requires more inhibitors (dotted in pie charts) to reach half-maximum fusion yield than strain 2, because it has a larger fraction of productive HAs (blue in pie charts). The difference in yield without inhibitors is also explained by the change in the nonproductive fraction of HAs. Parts of figure reprinted from Reference 5, copyright 2016, with permission from Elsevier.

this barrier by protonation enables the metastable HA to extend, driven by thermal fluctuations. This model describes HA insertion by a single pH-dependent rate $k_{\text{insert}}(\text{pH})$ and was able to quantitatively explain available data (137).

The observations from the single-particle assays can be summarized as follows. Under low-pH conditions and hydrodynamic flow associated with the acidic buffer exchange, the virus particle rolls along the surface, forming and breaking weak receptor bonds, followed by HAs extending and inserting into the target membrane. The contact patch M , the number of HA proteins able to interact with the target membrane, is estimated to contain $M = 50\text{--}150$ HAs, depending on particle geometry (56). A certain number of inserted HAs within this patch, N_{arrest} , arrests the particle by providing sufficient anchoring. The arrest distribution then arises as the convolution of the single-exponentially distributed, independent insertions (**Figure 4b**), approximating a gamma distribution for $M \gg N_{\text{arrest}}$ with rate parameter $M \times k_{\text{insert}}$ (56, 137).

Insertions continue stochastically and hemifusion ensues when a first, local cluster of N_{cluster} -inserted HAs has formed. The cluster contains a sufficient number of HAs that together are able to overcome the membrane fusion barrier. The formation of this first cluster is regarded to immediately result in hemifusion; in other words, k_{hemi} (see **Figure 4a**) is large compared to the rate of previous steps—there is no data available separating these states (57, 137). The probability distribution of the number of insertions that have happened prior to the formation of a first, critical cluster is Gaussian for a sufficiently large contact patch M (137). The observed hemifusion distribution then results from the combination of the geometric requirement to have formed a cluster and the time distributions of the insertions, generally resulting in a slightly right-skewed Gaussian distribution (**Figure 4c**). After hemifusion, a pore opens with a rate k_{pore} (34), as discussed in more detail in the previous sections.

Recent work indicated the presence of a large fraction of HAs that is not involved in fusion (see **Figure 4d**) (57), which is thought to arise from nonproductive HA refolding pathways (as described in the section titled Pathways of the Conformational Change). Single-particle experiments with tagged HA-binding inhibitors (antibody fab fragments) (99) showed that the number of bound inhibitors required to reach half-maximum fusion yield is a small fraction of the total number of HAs on a virus particle (**Figure 4d, right pie charts**). Furthermore, the two influenza strains investigated differed markedly in their response to their respective strain-specific inhibitors and had different yields without inhibitors (**Figure 4d, graph**). These observations are explained by assuming a large fraction of nonproductive HAs in the native virus, where, for some strains, fewer inhibitors are necessary to effectively inhibit fusion because of an even larger nonproductive fraction. Different strains also appear to require different cluster sizes to overcome the membrane hemifusion barrier (25, 57), additionally influencing the sensitivity to fusion inhibitors.

The details have not yet been resolved, but the cluster size, fraction of nonproductive HAs, and HA activation rate seem to be system parameters that influenza may vary in response to evolutionary pressures in order to achieve efficient cell entry, while at the same time avoiding immunogenic detection and maintaining stability outside of the cell.

FUTURE DIRECTIONS

In improving our understanding of HA-mediated membrane fusion, the combination of single-particle experiments and stochastic modeling has enabled the identification of several important parameters, such as the independence of HA triggering by low pH and the action of multiple HAs in a cluster to catalyze hemifusion. Nonproductive pathways of HA refolding appear to play an important role, as probed with neutralizing antibodies. These parameters of influenza fusion can be described in a unified way, and this approach allows us to appreciate the strategy of viral

Contact patch: the region of the virus particle interacting with the target membrane

fusion or even fusion catalysis in general: The large membrane fusion barrier is conquered by first overcoming small kinetic barriers of the fusion proteins to insert into the membrane, after which their catalyzing capability and energy of refolding are utilized to drive fusion. Even though details vary, this mechanism appears universal across all classes of enveloped viruses (14, 57, 68, 137) and may very well be extended to other fusion systems.

For efficient entry of viruses into cells, just like for the functioning of these cells themselves, the timescale at which membrane fusion occurs needs to be synchronized with other biological processes. As described in this review, the influence of the high kinetic barriers of dehydration and pore formation in determining this timescale became evident from the determination of the kinetics and thermodynamics of membrane fusion. To elucidate the relative importance of the factors that determine these barriers, the field will benefit from further integration of experiment and computation. However, owing to a huge variety in system parameters (as evident from the diverging data in **Figure 1b** and **Supplemental Table 1**), there is a need for more structured, collaborative studies that coordinate to closely mimic the parameters involved in influenza membrane fusion. By combining insights from *in vitro* and *in silico* assays, such collaborative studies will aid direct comparison of membrane fusion barrier heights between different approaches and can further determine the relative importance of lipid composition, initial curvature, membrane tension, and, in particular, fusion-protein mediation between fusion intermediates.

To accomplish such future studies, high-resolution experimental assays have become available that have proven to be powerful tools for access to intermediate states in the pathway to fusion, especially when augmented by modeling approaches. Emerging experimental tools are cryo-EM/ET (20), HDX-MS (33, 40), fluorescence microscopy (95, 100), and single-molecule force spectroscopy (50, 59, 134) as well as combinations thereof (116). Meanwhile, computational approaches become more powerful in time and length scale (31), while novel computational (101, 103, 124) and analytical (32) methods can probe free-energy landscapes governing protein conformational changes. With these tools, the synergy between experimental and theoretical approaches at the molecular level has come within reach. Increasing the complexity of *in vitro* and *in silico* assays toward *in vivo* conditions, one step at a time, will lead to a better understanding of the factors governing influenza fusion and ultimately of all membrane fusion in living cells.

SUMMARY POINTS

1. A number of experimental assays, supplemented by membrane-structure or free-energy calculations from computational approaches, allow for the determination of the membrane fusion pathway and the estimation of the barriers between fusion intermediates.
2. Influenza hemagglutinin (HA) refolding proceeds either through two hypothesized productive pathways that involve fusion-peptide insertion into the target membrane or through nonproductive refolding with the fusion peptide failing to insert.
3. Specific HA conformational changes and membrane interactions lower the barriers between membrane fusion intermediates.
4. The combination of single-particle assays and stochastic modeling has identified major parameters in the fusion of influenza virus: the HA activation rate, the HA cluster size to overcome the membrane fusion barrier, and the fraction of nonparticipating HAs.
5. Influenza fusion kinetics are determined by the action of multiple hemagglutinin proteins with a large role for nonproductive pathways.

FUTURE ISSUES

1. A structured, collaborative approach, involving experimental and computational assays that use the same system parameters, is needed to enable a more direct, quantitative comparison of membrane fusion barrier heights.
2. There is a need for an experimental assay to determine the pathway of membrane fusion after stalk formation, either proceeding through radial expansion or lateral elongation of the stalk.
3. There is insufficient information on the amount of free energy that is available from specific conformational changes or membrane interactions of influenza hemagglutinin (HA) and the membrane intermediates that they relate to.
4. The relation between the amino acid sequence of HA and the parameters of fusion, such as HA extension rate, productivity, and cluster size, is still poorly understood, as is how these different parameters are balanced against each other under evolutionary pressure.
5. Although pore expansion is reported to be the energetically most demanding step in the fusion pathway, the parameters defining the kinetics of the processes from pore formation until genome release are still largely unknown.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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137. The paper consolidates current biophysical understanding by providing an analytical model of influenza fusion.

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