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Topical Review

The Energetics of Membrane Fusion from Binding, through Hemifusion, Pore Formation, and Pore Enlargement

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Abstract. The main steps of viral membrane fusion are local membrane approach, hemifusion, pore formation, and pore enlargement. Experiments and theoretical analyses have helped determine the relative energies required for each step. Key protein structures and conformational changes of the fusion process have been identified. The physical deformations of monolayer bending and lipid tilt have been applied to the steps of membrane fusion. Experiment and theory converge to strongly indicate that, contrary to former conceptions, the fusion process is progressively more energetically difficult: hemifusion has a relatively low energy barrier, pore formation is more energy-consuming, and pore enlargement is the most difficult to achieve.

Key words: Viral fusion proteins — Six-helix bundle — Trimeric hairpin — Lipid splay — Lipid tilt

Introduction

Fusion between membranes is a critical event in a multitude of cellular processes. It is central to exocytosis, intracellular trafficking, muscle development, healing of membrane wounds, fertilization of egg by sperm, and many other events. Fusion is also central to viral invasion of cells. Exhaustive studies have probably identified the minimal set of proteins required for fusion in exocytosis and intracellular trafficking [41, 43]. But it is only in the case of envelope viruses that all proteins required for fusion have been unambiguously identified [40]. The process of viral infection is initiated when a virion binds to a cell. Depending on the type of virus, the virion then either fuses directly to the plasma membrane (at neutral pH) or is taken up into an endosome where low pH induces fusion between the viral envelope and endosomal membrane. In both cases, fusion permits the virion to deposit its genome into cytosol. Within an infected cell, the viral genome and viral membrane proteins associate at localized positions along the plasma membrane. Budding of membrane at these sites releases new virus. The budded membrane becomes the new viral envelope. Since viruses have minimal genomes and do not encode for lipids, all lipids that form the bilayer membrane of the viral envelope are derived from the cell membrane of the infected cell.

The fusion of a viral envelope to a cell membrane can be monitored, but because the virus is small, techniques for study have been limited. By experimentally expressing the fusion proteins on the surfaces of cells, however, the cell membrane becomes, in effect, a giant viral envelope that can be fused to target cells that express the necessary receptors. Because both fusing objects are relatively large in this system, the fusion process can be readily recorded by a variety of techniques, including mixing of fluorescent dyes, transfer of reporter genes, and electrical capacitance measurements [24]. Because membrane fusion is characterized by two lipid bilayers merging into one, lipids must transiently rearrange at the sites of fusion. These rearrangements are controlled by fusion proteins. Experimental studies, combined with theory that describes lipid rearrangements, have led to our current understanding of the stages and dynamics of fusion. Recent developments have forced a reassessment of some prior inferences and led to some surprising conclusions that are contrary to former assumptions.

The Stages of Fusion

Membrane fusion proceeds through a series of intermediate stages. Studies strongly indicate that the main

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stages of viral fusion are: local membrane contact, creation of hemifused membranes, formation of a fusion pore, pore enlargement (Fig. 1). Of these stages, hemifusion remains the most enigmatic; it has been repeatedly observed to occur, and is largely accepted as a bona fide intermediate of fusion, but its relevance to the fusion process is still debated (*see* [17, 23, 61] for contrasting views). In hemifusion, the contacting lipid monolayers (e.g., the outer monolayers in fusion between cells) of the two membranes merge, while the noncontacting (the inner) monolayers remain intact. As a result of hemifusion, only a single bilayer membrane, rather than the original two, separates aqueous contents. This membrane, called the hemifusion diaphragm, is composed only of inner (rather than inner and outer) monolayer leaflets—one inner monolayer from each cell membrane. The hemifusion diaphragm is a pure lipid bilayer, devoid of integral membrane proteins; this must be the case because the extracellular portion of an integral membrane protein (the "ectodomain") cannot transfer into cytosol via a hemifusion mechanism. If, as is widely believed and posited here, fusion proceeds through hemifusion, then it is the disruption of the hemifusion diaphragm that causes formation of the fusion pore. It is not known whether a diaphragm remains molecularly small [31] before a pore forms, or becomes somewhat extended. Because proteins do not span the diaphragm, pore formation must occur either spontaneously in the diaphragm or by action of proteins that reside outside, but adjacent to, the diaphragm. If pore formation is spontaneous without protein involvement, either the energies necessary to create the pore are small or the alignment of the lipids within the diaphragm are distorted to the point that a large force is generated that promotes pore formation. If pore formation is induced by proteins, their location would suggest that they create the pore at the outer boundary of the diaphragm. In all cases, the membrane that lines the lumen of the initial pore would be bent out of its natural curvature, and pore expansion would relieve membrane strain.

It has often been assumed that hemifusion was the most difficult step to overcome energetically and that pore enlargement was the easiest. Why this might have been widely supposed is understandable. Careful biophysical measurements in the 1970s and 80s showed that repulsive forces oppose the approach of membranes at distances less than $\sim$ 3 nm [90]. Though there are several different physical phenomena that cause this repulsion [56], it is often referred to collectively as the "hydration force." Even after membranes are in local contact, the rearrangements for hemifusion to occur were thought to require considerable additional energy, since the acyl chains of lipids in the outer monolayers must somehow leave their comfortable hydrophobic environment and before they meet, negotiate whatever water separates the bound membranes. It was thought that once hemifusion was achieved, lipids could easily rearrange without exposing acyl chains to water, so that a fusion pore would readily form within the diaphragm. It was further expected that once a pore formed, fusion would be essentially completed and the pore would enlarge spontaneously—in other words, in this scenario all interesting aspects of the process would have been accomplished by the point of pore initiation.

While the stages of fusion appear to have been correctly identified, based on experimental studies and theoretical advances, notions about the relative energies required for each of the stages have had to be radically reassessed. The hydration force necessitates that considerable energy be expended to bring membranes into intimate contact over an extended area, but the energies needed to overcome the hydration force to bring membranes into contact locally are relatively modest [39, 57]. Also, experiments have shown that hemifusion is not difficult to achieve, and theory now demonstrates that through the deformations of lipid monolayer bending and lipid tilting, the initial connection of hemifusion—known as a "stalk"—requires modest energies. Further, experiments show that pore formation does not occur readily in either single bilayer membranes [19] or within hemifusion diaphragms [67], and theory is in accord with these findings [50, 51, 52, 65]. A fusion pore will even tend to close if further conformational changes of proteins are inhibited. Not only does pore enlargement require energy, but both experiment and theory now indicate that this final step in the process may be more energy consuming than the preceding ones.

On the protein side, experiment has also overturned some previous assumptions. For many viral fusion proteins, folding into a "six-helix bundle" is a critical conformational change [103]. It had been assumed that the bundle would form early in the fusion process and in so doing, bring viral and target membranes close together; subsequent, more subtle, protein conformational changes or associations between proteins were thought to lead to pore formation. But experiment has shown that bundle formation occurs late in the fusion process [75, 97], and—unexpectedly—for some fusion proteins occurs after, rather than before, the pore has formed [68]. This means that conformational changes prior to the formation of the bundle must bring membranes together and cause them to merge, and that the considerable energy released by bundle formation is utilized for pore enlargement.

Structural Features of Fusion Proteins

Viral fusion proteins are always assembled from multiple monomers. Each monomer is synthesized as

a single polypeptide chain that is (for almost all viral types) post-translationally proteolytically cleaved, generally into two subunits. As a rule, one subunit, often referred to as the surface (SU) subunit, binds to receptors on target cells. The other subunit, the transmembrane (TM) subunit, is responsible for fusion. Each TM subunit is anchored to the viral envelope through a single membrane-spanning domain (MSD). All TM subunits also contain, within each of their monomers, a stretch of about 20 nonpolar amino acids, known as a "fusion peptide," that is essential for fusion. The fusion peptides have been shown to insert into target membranes [38, 87, 104], and in this way, the fusion proteins become anchored not only to their own membrane through MSDs but to the targets as well. Mutation experiments indicate that in addition to their membrane-anchoring function, fusion peptides also participate in inducing the lipid rearrangements of hemifusion and pore formation [26, 28, 92] and are involved in pore enlargement [99]. It is not yet known how fusion peptides induce these lipid rearrangements, even though for influenza virus hemagglutinin (HA) their structures have been determined within the protein and after insertion into membranes [37, 114]. Free energy is released upon insertion of fusion peptides into membranes [59], and this may contribute to the lipid rearrangements [28]. HA, which induces fusion at low pH, and HIV Env, which induces fusion directly to the plasma membrane at neutral pH, have been extensively studied and allow general principles of viral fusion to be specifically illustrated. For HA, the SU subunit is called HA1 and the TM subunit is HA2.

Influenza HA is the only fusion protein for which the uncleaved, cleaved (pre-fusion), and post-fusion structures are known. HA has an overall rod-shape, ~13.5 nm long. Cleavage of HA causes some conformational changes, though these changes are relatively minor [12]. But the subunits can now be induced to undergo further conformational changes that would not have been possible while they remained constrained as part of a single long chain. Experiments that monitor conformational changes indicate that prior to fusion, the HA2 subunits are clamped in place by the HA1 subunits. The TM and SU subunits of other fusion proteins also follow this pattern [3]. The conformations of the TM subunits are said to be "meta-stable" because at this point the triggers for fusion—low pH or binding of receptors to fusion proteins—lessen the associations between SU and TM subunits, allowing each TM subunit to be freed of its clamp, and to reconfigure [9]. For some viruses, portions of the SU subunit may transitorily interact with regions of the TM subunit, promoting further reconfiguration of the TM subunit (3). The conformational changes in HA2 induced by low pH are massive, leading to large-scale movements, as much as 10 nm, of some domains. These changes, including those of secondary structure, occur in several different regions of the subunit [7]. The transition from the cleaved, meta-stable state to the final conformation of HA occurs in discrete, sequential conformational steps [111]. The free energy released by each reconfiguration is thought to drive the lipid rearrangements necessary for each stage of fusion. While it is accepted that sequential conformational changes of fusion proteins allow free energy to be released in packets, rather than all at once, the concept of meta-stability has been challenged by the claim that for intact influenza virus, energy is not released when the fusion protein reconfigures [30]. For isolated ectodomains of HA, the energy released by the reconfigurations has also been measured, and it is large enough [96] to induce all the steps of fusion. This is consistent with the concept that meta-stability is a means by which proteins release the free energy utilized in fusion.

The conformational changes of TM subunits are regulated by interactions between proteins as well as those of subunits within the same protein. Several, perhaps many, proteins act cooperatively to generate the state of hemifusion and to create a fusion pore [4]. In the case of HA-mediated fusion, early low pH-induced conformational changes occur without interaction, while late conformational changes involve interactions between multiple copies of HA [70]. Recently, high-resolution electron crystallography has shown that for the fusion protein of Semliki Forest virus, six copies of the protein interact to form a ring [33]. In general, interacting proteins may create a functional network in which conformational changes release free energy in concert. Simultaneous conformational changes may allow energetically difficult lipid rearrangements to occur that would not be possible if proteins stochastically reconfigured independently of each other.

HA and HIV Env are members of a large class of viral fusion proteins, termed "class I," that form a structure known as a six-helix bundle; these proteins are assembled from three identical monomers in which the TM subunits entwine during the fusion process to form the bundle [25]. Forming the central core of the bundle are three N-terminal α-helices (one from each monomer), creating a triple-stranded "coiled coil." Each monomer bends into a "hairpin" so that the final structure of the fusion protein is a "trimeric hairpin." Three C-terminal segments pack into the three grooves of the coiled coil in an orientation anti-parallel to the N-terminal helices, completing the trimeric hairpin. All or part of the inserted C-terminal segments are folded into an α-helix. The α-helical folded portion of the trimeric hairpin is referred to as the six-helix bundle (6HB). Synthetic peptides that mimic the N-terminal (N-peptides) and C-terminal (C-peptides) helices spontaneously assemble among themselves into 6HBs [62]. It is notable that even though the 6HB can form spontaneously, this structure is not present in the

native, neutral pH, pre-fusion form of HA, but it is present in its low pH, post-fusion form [13, 114]. This leads to the concept that the meta-stable TM subunits fold into a 6HB and trimeric hairpin subsequent to the release of the clamp.

The major portions of the extraviral or extracellular regions (ectodomains) of the TM subunits (without the subunits that provide the clamp) have been expressed in bacteria. These ectodomains are folded into their final, stable configuration, rather than their initial, meta-stable one [14], providing further support for the concept of meta-stability. The trimeric hairpins of these bacterially expressed ectodomains and the 6HB complexes created by N-peptides and C-peptides are highly thermostable [11, 62]. In cell-cell fusion, once the HIV Env bundle has formed, it does not dissociate [69, 98]. If trimeric hairpin formation is responsible for a major portion of the free energy released by fusion proteins, then the most energy-consuming steps of fusion would not be expected to occur subsequent to hairpin formation.

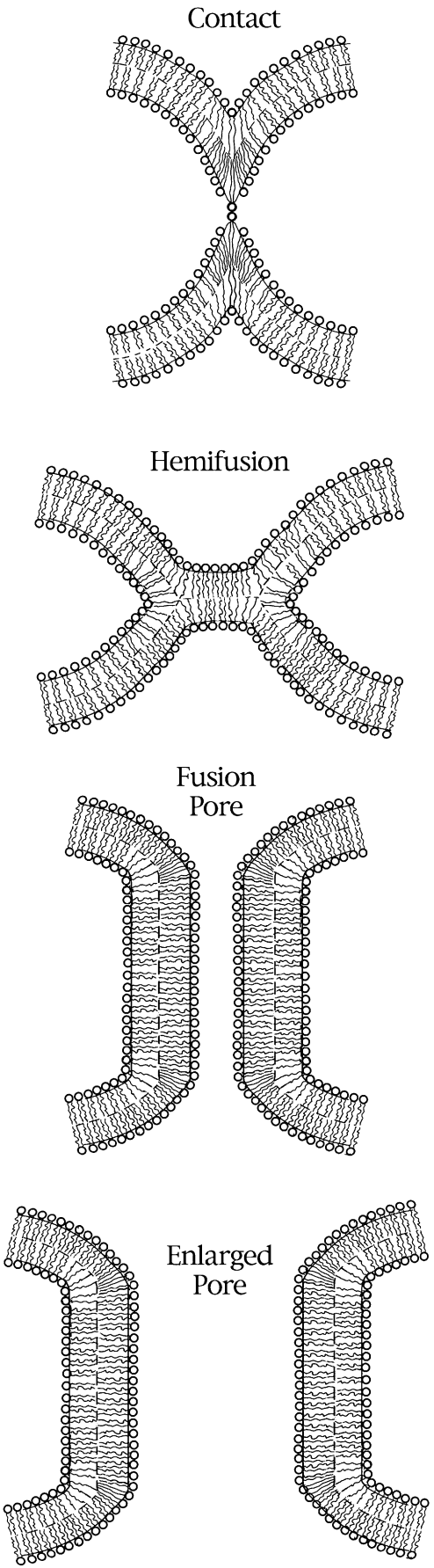
Because the trimeric hairpin is identified, by crystallography, as a common structure for class I viral fusion proteins, these proteins probably evolved from the same primordial precursor even though they do not have sequence homology. For a given virus, even one that rapidly mutates, the residues necessary for the hairpin and the bundle to form are largely conserved. Such observations immediately suggest that these structures are in some fundamental way central in the fusion process. In fact, a wide variety of experiments, including the use of synthetic C- and N-peptides to competitively inhibit fusion and mutation of critical bundle and hairpin residues, have convincingly verified this expectation [63, 81, 112]. It has been shown that inhibiting bundle formation inhibits viral infection and cell-cell fusion [8, 27, 64, 110].

In recent years, there has been considerable progress in the field of membrane fusion in identifying protein conformational changes. Important progress has also been made in understanding the manner in which lipid monolayers deform and the individual lipid may rearrange. One of the principal challenges facing our understanding in the field remains the task of discovering how these two elements work interactively to effect each of the steps of membrane fusion. Until this gap in our knowledge is overcome, we are faced with parallel streams of information, but no overarching connection between them. At this time we can only consider, for each successive step effusion, the roles of proteins and lipids separately.

Membrane Approach

For many proteins, the fusion peptides are near or immediately proximal to the N-terminal helices that form the central core of the 6HB, and the MSDs are similarly situated with respect to the C-terminal helices. For these proteins, which include HIV Env, the anti-parallel orientation of the N- and C-helices means that the three fusion peptides should be close to the three MSDs. (Because neither the fusion peptides nor MSDs are present in the crystal structure, their precise positioning with respect to each other is not yet known.) HA has a similar structural motif, except that much of its C-terminal region runs as an extended chain rather than as a helix. Crystallography shows that the central coiled coil is elongated by low pH and a very precise structure, known as an N-cap, is created that ties together the ends of the three α-helices of the coiled coil. The N-cap is near the fusion peptides, and C-terminal residues that interact with the N-cap are near the MSDs [13]. (Other fusion proteins, such as that of human T cell leukemina virus, also follow this pattern, [48].) Therefore, in the final trimeric hairpin structure of HA, fusion peptides and MSDs should be in proximity. For the classes of viral fusion proteins that do not form bundles, electron microscopy and crystallography indicate that the same motif of proximity holds [6, 33, 34, 79], strongly suggesting that this widespread if not ubiquitous motif serves critical functions. It is currently thought that one function is to locally bring the target membrane and viral envelope into contact, as the fusion peptides (inserted into the target membrane) and the MSDs (inserted in the viral envelope) approach each other. (It has also been proposed that the two membranes are brought together by tilting of ectodomains at a region adjacent to MSDs [105, 107].) Independent of how the protein reconfigures to establish local membrane contact, one (or both) of the membranes must project itself toward the other for contact to occur.

In order for a membrane to protrude locally, the individual monolayers must change their contours. Any contour changes of a monolayer are accomplished by a combination of just two independent deformations—bending and tilt. Bending can be understood by considering the different shapes—large, small, or relatively equal-sized areas of the headgroup as compared to acyl chains—of different lipid species (Fig. 2). Monolayers and membranes will spontaneously bend in one direction or another into a "spontaneous curvature" which occurs at its lowest energy. Work is required to bend a bilayer into a contour other than its spontaneous curvature. The energy required is analogous to that required to stretch or compress a spring. For a spring, this energy depends quadratically on the deviation of the stretched (or compressed) spring length from its spontaneous length, whereas for a bilayer it depends quadratically on the deviation of the mean of the two curvatures from the spontaneous curvature. (Any surface can be characterized by two radii of two



curvatures where curvature $\kappa = 1/\text{radius}$.) The proportionality constant, the bending modulus B, of a bilayer is $\sim 20\ k_B T$. For a monolayer, the same physics pertains, but the bending modulus is half that of a bilayer, $\sim 10\ k_B T$. The energy necessary for a membrane to bend into a protrusion can be readily calculated.

To bend a spontaneously flat membrane into a portion of a sphere requires 2 ΩB of energy—independent of the radius of the sphere—where Ω is the solid angle of the bent portion. Therefore, an energy of 4 πB or $\sim 250\ k_B T$ would be necessary to bend one membrane into a hemisphere (solid angle 2 π), so that the resulting dome or "nipple" can locally contact the other membrane. The two curvatures of the base of the nipple have opposite signs, so the mean curvature is close to zero. Hence, the energy required to bend membrane into the shape of the base is small. But considerable bending energy is required to create the cap, for here both curvatures are positive. If the cap were subtended by a solid angle that was smaller than a hemisphere (i.e., if the cap was flatter), the bending energy would be correspondingly smaller. But if local contact with the apposing membrane is to be maintained, a flatter cap necessitates a larger area. A relatively flat cap would have more of its surface area near the apposing membrane and therefore would be more affected by local hydration forces. Thus, decreasing the bending energy to make local contact is at the expense of an increase in hydration energy. The solution to this energetic conundrum may be that local contact is established through lipid tilting in conjunction with membrane bending (Fig. 1, *contact*). With bending and lipid tilting, membranes can deform into pointlike, peaked protrusions at a low cost of energy [50]. If local membrane contact was achieved through contact of these protrusion points, the need to overcome hydration forces over an extended area would be obviated [17]. Whatever energy is used to create the protrusions would be elastically stored within the membranes and could, in principle, be tapped in subsequent steps of the fusion process.

Hemifusion

Even though viral protein-mediated hemifusion has been observed under many different conditions, it has

Fig. 1. The stages of fusion. Two membranes locally contact each other by the bending of their lipid bilayers and tilting of individual lipids. A single bilayer—the hemifusion diaphragm—separates aqueous compartments at the stage of hemifusion. Tilting of lipids fills in voids that would form at the rim of the hemifusion diaphragm if only monolayer bending occurred. Further lipid rearrangements create a fusion pore that connects aqueous compartments. Expansion of the pore completes the fusion process.

not been unequivocally demonstrated to be a bona fide intermediate of fusion. The major reason this issue has not been settled is that the lipid movement expected of hemifusion is not usually observed prior to aqueous continuity [92, 109, 118] (although the expected sequence has been observed in special cases [95]). Another reason for the controversy is that when lipid dye has been observed to move between membranes in the absence of pore formation, generally pore formation does not subsequently occur [16, 67, 78]. That is, hemifusion in which lipid movement is unrestricted is a dead-end state. The dead-end state is referred to as "unrestricted" hemifusion, while hemifusion that proceeds to fusion is termed "restricted" hemifusion. Restricted hemifusion is thought to occur only when a sufficient number of HAs have accumulated at a site; if they do not, the dead-end state would be the result [16, 23, 78]: in restricted hemifusion the MSDs of interacting proteins are thought to be too close together to allow for lipid to move between them, which would not be the case for dead-end unrestricted hemifusion. Since lipid movement cannot reveal a state of restricted hemifusion, other means are needed to determine if this state has formed.

Experimentally, one can indirectly detect the existence of a hemifusion diaphragm: Membrane-permeable weak bases accumulate in inner leaflets, as compared to outer leaflets, of cells [100], probably because their protonated cationic forms are electrostatically attracted to negatively charged inner leaflets. An amphipathic weak base that destabilizes a membrane will preferentially permeabilize the hemifusion diaphragm because the diaphragm is constructed of only inner leaflets; this high permeability is easily detected in cell-cell fusion as aqueous continuity between cytosolic compartments. Several weak bases are suitable [72]; chlorpromazine (CPZ) has become the standard. If the addition of CPZ to a fusion intermediate (for which lipid mixing does not occur) leads to aqueous continuity, that intermediate state is proposed to be restricted hemifusion [16, 76]. But because the nature of this state is not definitively known, it may be best to refer to such states as CPZ-sensitive.

Direct observation of the diaphragm has been attempted by electron microscopy in the case of influenza HA-mediated unrestricted hemifusion between cells [31]. Because unrestricted hemifusion permits lipid spread and its diaphragm is not restricted by a tight ring of protein, it would be likely that diaphragms of unrestricted hemifusion are as large or larger than those of restricted hemifusion. The thickness (70–100 nm) of the ultrathin tissue sections used in the electron microscopy study would have detected diaphragms greater than ~20 nm in diameter, but none were found [31]. Diaphragms of both types of hemifusion are probably much smaller than this. Stalks with angstrom-scale dimensions have been directly observed by x-ray diffraction in model lipid systems for several different lipid compositions [115, 116]. By inducing stacks of lipid membranes to transition from lamellar to non-lamellar phases, multiple periodic mergers occur, allowing the merged structures to be observed by x-ray diffraction. Although a lipid phase transition is not truly a membrane fusion event, such lipid rearrangements should be similar in many ways to the initial steps of fusion. The identified stalks (and potentially structures of other intermediate states) obtained on the scales of angstroms should help guide future experimental and theoretical studies.

Experiments in which the ectodomain of influenza HA was GPI-linked to the membrane were instrumental in leading to the current general acceptance of hemifusion as an intermediate of viral fusion [47]. Cells expressing GPI-HA were found to hemifuse—but not fuse—to both red blood cell and phospholipid bilayer membranes [46, 77]. Because the overwhelming majority of the amino acids of HA reside within the ectodomain and GPI-HA caused only hemifusion, it was thought that hemifusion was difficult to achieve because it required the massive and intricate structure of the ectodomain, and that pore formation and enlargement were easier to achieve because the inclusion of HA's small MSD was able to bring about these steps [2, 74].

There were, however, indications that GPI-HA could induce continuity between aqueous compartments for either phospholipid bilayers [77] or RBCs [85] as target membranes. These observations were thought to be of little consequence because for bilayers as target, the aqueous continuity pathway did not have the electrical signature of a fusion pore, and passage of aqueous dye with RBCs as target was assumed to be caused by long-time instabilities of the unnatural hemifusion diaphragms. We now know that for more optimal conditions than had originally been used (i.e., lower pH, the higher temperature of 37°C), fusion pores form between GPI-HA-expressing cells and RBCs, but they do not enlarge [31, 67]. The occurrence of pore formation undermines the idea that creation of hemifusion consumes the bulk of free energy released by the complex conformational changes of the ectodomain. These experiments also demonstrate that pore growth is not spontaneous. Lastly, because pores do not enlarge in the absence of an MSD, we can conclude that the MSDs are involved in pore enlargement, possibly by coming into contact with fusion peptides.

The idea that pore formation and growth are energetically more difficult to achieve than hemifusion is supported by experiments within all the major model bilayer systems: Hemifusion can occur between hemispherical bilayer membranes [18, 83], liposomes and planar bilayers [10], liposomes [54, 88],

and bilayers adhered to substrates [39]. The hemifusion diaphragm connecting bilayers is generally stable, and appreciable external forces are required to destabilize it enough to form a pore that can enlarge [10, 72]. The addition of synthetic fusion peptides or a large trimeric fragment of the ectodomain of HA2 can also induce lipid mixing between liposomes or cells [29, 58, 91, 93].

The energy necessary to achieve hemifusion is sensitive to monolayer curvature. Adding lysophosphatidylcholine (LPC) to outer monolayers of membranes is the standard experimental means to inhibit hemifusion [15]. LPC has a large polar portion, in terms of cross-sectional area, relative to its single hydrophobic acyl chain. The incorporation of LPC into a flat monolayer will thus displace the headgroup region of that monolayer more than the acyl chain region, and will cause the monolayer to curve toward the hydrocarbon core, defined as positive curvature (Fig. 2). The incorporation into a monolayer of a lipid that has a small headgroup, such as oleic acid, will cause the monolayer to bend in the opposite direction, curving toward the aqueous phase, conferring a negative spontaneous curvature. Consider three lipids, LPC, DOPC, and DOPE (Fig. 2): For LPC, spontaneous curvature is quite positive ($\kappa_0 = 1/3.8\ \mathrm{nm}^{-1}$), for DOPC it is somewhat negative ($\kappa_0 = -1/8.7\ \mathrm{nm}^{-1}$), and spontaneous curvature of DOPE is much more negative than that of DOPC ($\kappa_0 = -1/2.8\ \mathrm{nm}^{-1}$) [32, 55, 106]. By calculation, to bend a monolayer containing 100 of each of these lipids into a flat sheet is $\sim 20\ k_B T$ for LPC, only $4\ k_B T$ for DOPC, and $\sim 40\ k_B T$ for DOPE. The standard explanation for the inhibition of hemifusion by addition of LPC is that LPC increases the energy required to bend two apposing monolayers into a stalk [15, 49, 66].

For a roughly hourglass-shaped stalk, one surface curvature is positive, the other negative. The bending energy would be at its minimum when the mean of the positive and negative curvatures equals the spontaneous curvature of the outer monolayers. The stalk should adjust its shape to minimize energy when the spontaneous curvature is altered by incorporation of LPC [65], but even with shape adjustment, explicit calculation shows that the positive curvature of LPC will make the stalk energy increase [50]. Because inner monolayers are essentially not deformed in the creation of the stalk, only the spontaneous curvature of the outer monolayer is energetically significant in stalk formation.

An activation barrier must be surmounted in order to create a stalk because the usual lamellar arrangement of lipids of contacting monolayers must be transiently disrupted. The height of the activation barrier should, a priori, be the chief determinant of the ability of the stalk to form. Any disruptions within contacting monolayers should expose hydrophobic portions of the lipids to water [57]. Hydrophobic surfaces separated by water attract each other [42, 53]. The work required to bend the monolayers into the activated state will also contribute to the height of the barrier. The activation barrier that must be surmounted to achieve a stalk has been determined from a balance of hydrophobic and hydration energies (ignoring the elastic energy of deformation). For zero spontaneous curvature of outer monolayers, the activation barrier is $\sim 40\ k_B T$ (52). By assuming reasonable values for the parameters that determine the pre-exponential factor for surmounting a barrier, a 40 $k_B T$ barrier can be surmounted spontaneously in a few seconds [52]. It is unlikely that barriers larger than 40 $k_B T$ can be spontaneously overcome.

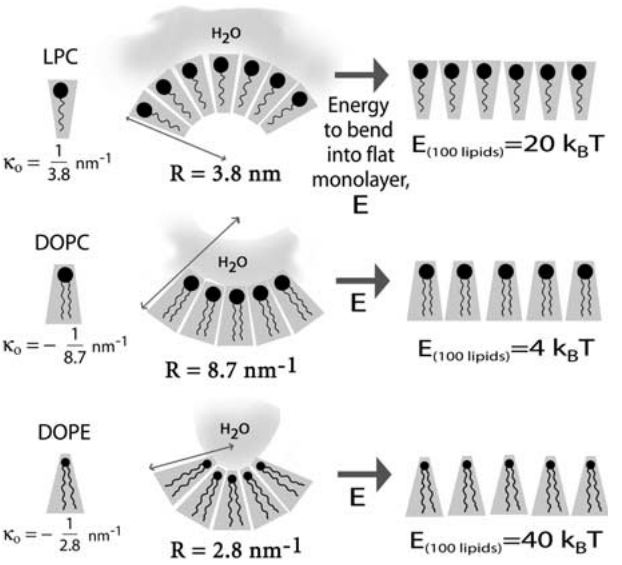


Fig. 2. Energy is required to bend lipid monolayers. The energy required to bend a lipid monolayer from its spontaneous curvature to a flat sheet is illustrated for three lipids: palmitoyl lysophosphatidylcholine (*LPC*), which has positive spontaneous curvature; dioleoylPC (*DOPC*), which has a somewhat negative spontaneous curvature; and *DOPE*, which has a more severe negative spontaneous curvature.

If a stalk does indeed form by connecting transient hydrophobic patches between bound membranes, the energy to create these patches should contribute strongly to the activation barrier. The easier it is for lipid headgroups to cover the acyl chains from water, the more difficult it would be for a hydrophobic patch to form within a monolayer. The large headgroup of LPC should aid in this cover and thereby raise the activation barrier between the bound state and the stalk. Thus, LPC may cause the inhibition of hemifusion by hindering formation of hydrophobic patches in addition to or instead of raising the energy of the stalk. Brownian dynamics and Monte Carlo calculations are beginning to be applied, approaches that could definitively identify the most energy-consuming processes of fusion [80, 84].

Pore Formation

Intermediates of fusion can be captured by creating conditions that are suboptimal, so that fusion cannot proceed to completion. Ideally, a single condition, such as temperature, is made suboptimal. To check that a functional, rather than a dead-end, state has been isolated, the suboptimal condition is returned to optimal, and full fusion—defined as formation of a fusion pore that fully enlarges—must occur. Functional CPZ-sensitive states have been captured and characterized [16, 76, 117]. If the fusion process is readily arrested at restricted hemifusion and more optimal conditions are required in order for the process to advance, the subsequent step of pore formation must be more demanding than restricted hemifusion.

When bending contacting monolayers into a stalk, a packing problem presents itself. With bending alone, interstices, or voids, must occur within the enclosure formed by the monolayers, and the large energies associated with these voids would prohibit contact between distal monolayers, necessary for formation of the hemifusion diaphragm, [101, 102]. In the field, this has been referred to as the "energy crisis." There had been considerable debate about how interstices are avoided when membranes actually fuse. Most investigators now think that it is accomplished not only by bending monolayers, but also by tilting of lipid [50, 51,52, 65], although other explanations have been advanced [71]. Through lipid tilt, monolayer and membrane shapes that are not energetically feasible through bending alone - such as sharp protrusions - can be created at modest energies [50].

When bending, the direction of the long axes of the lipids (the "director") remains perpendicular to the water-lipid interface; in tilting, the director inclines with respect to this interface. Bending and tilt are separate, independent modes of deformation. In the strict definition [35, 36], lipid tilt is a displacement by an angle θ from the monolayer normal, with acyl chains stretched out so as to not alter the monolayer thickness (Fig. 3, *middle panel*). Because monolayers are almost volumetrically incompressible, the cross-sectional area occupied by a lipid is constant under tilt and thus tilt is an independent deformation. But the theory is formal, founded on tensor calculus, and requires detailed reasoning to obtain a tilting modulus. An intuitive understanding of tilt and the energy required to stretch acyl chains can be achieved, however, through a heuristic approach, which leads to exactly the same final equation for energy to the order that calculations are performed (the harmonic oscillator approximation) and to the same tilt modulus.

In the heuristic approach, a lipid molecule is treated as if it were a rigid object rotated by an angle

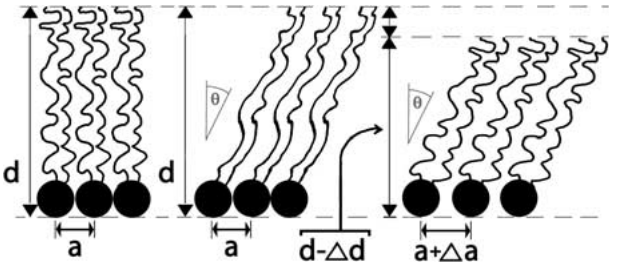


Fig. 3. The deformation of lipid tilt. Lipid tilt is the inclination of the acyl chains from the perpendicular (*left* to *center panel*) so as to maintain the thickness of the monolayer. Constant thickness necessitates that the acyl chains elongate without altering the area occupied by each lipid (i.e., the volume per lipid does not vary for an incompressible monolayer). This deformation is energetically equivalent to lipid inclination without altering the length of acyl chains (*right panel*). For an incompressible monolayer, the consequent decrease in monolayer thickness necessitates an increase in the area per lipid. The exposure of a portion of the hydrophobic acyl chains to water requires an expenditure of energy $E = \sigma\theta^2/2$ where $\sigma \sim 40$ erg/cm^2 and θ is the angle of tilt. The acyl chains of the tilted lipids have been somewhat shortened in the right panel in order to visually emphasize the decrease in monolayer thickness. Lipid tilt allows membranes to readily assume contours that would be energetically prohibitive if accomplished by monolayer bending alone. Lipid tilt is probably essential for membrane contact and for avoiding interstices within the hemifusion stalk (Fig. 1).

θ [52]. The lengths of the acyl chains are not altered, but the monolayer thickness decreases. Lipid volume must be conserved through an increase in the area per lipid. This increase in area means that when lipids tilt, portions of their hydrophobic acyl chains become exposed to water (Fig 3, *right panel*). The surface tension of the interface that separates the acyl chains and water (roughly at the plane of the phospholipid glycerol backbone) is $\sigma \approx 40$ erg/cm^2 for most lipids [94]. Therefore, the energy of tilting can be described by the surface tension (an energy per unit area) multiplied by the areas of the acyl chains that are exposed to water, yielding $E = \sigma(1 - \cos\theta)$. For a small angle of tilt, $E = \sigma\theta^2/2$. $\sigma = 40$ erg/cm^2 is thus the tilt modulus and only $\sim 20\ k_B T$ is required for 100 lipids to tilt 15°.

The energy needed to expose hydrophobic acyl chains to water is thus equivalent to the energy necessary to stretch acyl chains in the tilt deformation. Stretching allows acyl chains to fill in spaces that cannot be filled in by smooth bending, and by tilting, large voids would not form within the stalk. The originally proposed model of the stalk has therefore been somewhat modified; the hemifused membranes are connected by tilting as well as bending and the existence of voids is obviated.

When a monolayer bends, the acyl chains incline with respect to each other. In contrast, if lipids tilt at a constant angle, the acyl chains do not incline relative to each other. But if lipids tilt to different extents along a monolayer surface, the acyl chains do incline

with respect to each other. The inclination of acyl chains relative to each other is known as "splay." Splay is the sum of bending and a non-uniform lipid tilt. In the most general theories, splay replaces the simpler, more limited concept of bending [50]. The generalization is conceptually straightforward, with spontaneous curvature replaced by spontaneous splay. By splay of lipids, energetically unfavorable interstices do not form either within a stalk or at the rim of a hemifusion diaphragm (Fig. 1. *Hemifusion*).

Several models have been advanced to describe the mechanism of pore formation from the state of a modified stalk. Some assume that a pore forms directly from a modified stalk without expansion of the diaphragm [52], whereas others assume expansion [50, 65]. Because inner monolayers must be deformed to create the pore, here the spontaneous curvature of the inner (as well as outer) monolayers will significantly affect pore formation. All of the models indicate that for zero spontaneous curvatures of monolayers, the energy of the pore is about 40 k_BT higher than that of the modified stalk (*see* [52] for an explicit calculation). The activation barrier that separates modified stalks and pores has not yet been considered, but the very existence of an activation barrier would mean that pore formation requires more energy than stalk creation.

Pore Enlargement

HA does not require cytoplasmic tails for pore formation and their absence does not greatly affect pore enlargement [45, 73]. As we have seen, GPI-HA can induce pores that do not enlarge, showing that MSDs are necessary for pore enlargement. For many fusion proteins, including HA [74], HIV Env [113], and others [86, 108], MSDs from proteins unrelated to fusion can substitute for those of the viral proteins, and fusion proceeds as well as normal. Also, truncating the MSDs of HA from either end or from the middle is without consequence, as long as the remaining segments still span the membrane [2]. For Env of Simian Immunodeficiency Virus, truncation of the MSD does not hinder pore formation, but does prevent pore growth [60]. In general, a wide latitude of amino-acid sequences of MSDs supports fusion, but point mutations within the MSD can be deleterious to pore formation or to enlargement [22, 76, 108]. For example, a point mutation within the MSD of HA has been found that allows pores to form, but not to enlarge, exhibiting the same behavior as pores formed from GPI-HA. Because many amino-acid sequences serve well, whatever aspect of the MSD that controls pore enlargement must be rather broad. This motif may not be structural per se, but rather, may relate to the ability of the MSD to mingle well with fusion peptides in the trimeric hairpin structure.

Measurement of temperature dependencies and the use of synthetic C- and N-peptides that compete against bundle formation demonstrate that even after a fusion pore has formed, HIV Env has not completed its refolding into a 6HB, and significant energy barriers must still be surmounted for pore enlargement to occur [68]. A recombinant protein consisting of the HIV Env N-segments and C-segments provides a model for the bundle of native Env. Differential scanning calorimetry and circular dichroism experiments indicate that the free energy of association into a 6HB is $\sim$30 k_BT/bundle for the recombinant protein [44]. Folding of fusion proteins into bundles should thus release considerable free energy. Most importantly, we now know that for HIV Env the original assumption that the 6HB forms early in the fusion process to bring membranes together is incorrect. In fact, it forms late—after creation of the pore. Initial fusion pores are not stable and can close. The 6HB stabilizes the pore against closure and may be required for pore enlargement. The full membrane continuity conferred by a fusion pore may be necessary for MSDs and fusion peptides to come together [75, 97]. These notions of pore enlargement and bundle formation would be strengthened if it were found that preventing the proximity of MSDs and fusion peptides prevented HIV Env from folding into a trimeric hairpin and/or inhibited pore enlargement. Mutations have been made in the region between the MSDs and C-segments of the 6HB of HIV Env, and some of these mutants hinder pore enlargement but not pore formation [82]. Perhaps pore growth was prevented by limited approach of MSDs toward fusion peptides.

The relation between pore formation and proximity of MSDs and fusion peptides has been investigated for influenza HA. Interaction of a small structure, the N-cap, with only a few C-terminal residues, should be key for bringing the MSDs and fusion peptides of HA into close proximity. It has been found that mutation anywhere within the N-cap can cause fusion to become arrested at a CPZ-sensitive stage [5]. But mutation has been without effect at all the interacting C-terminal positions except one, an isoleucine residue. Mutation here, to more polar residues, resulted in either fusion arrested at a CPZ-sensitive state or, less frequently, at a small pore that did not enlarge and tended to close [5]. When isoleucine was mutated to a nonpolar residue, alanine, a nearby residue had to also be mutated for fusion to be inhibited [89]. Structurally, the hydrophobic side chain of this critical isoleucine fits into a deep hydrophobic cavity within the coiled coil, a cavity walled at its end by a hydrophobic surface of the N-cap. Insertion of this isoleucine residue into the cavity appears to be essential for completing trimeric hairpin formation [5]. It therefore appears that MSDs and fusion peptides do not have to approach each

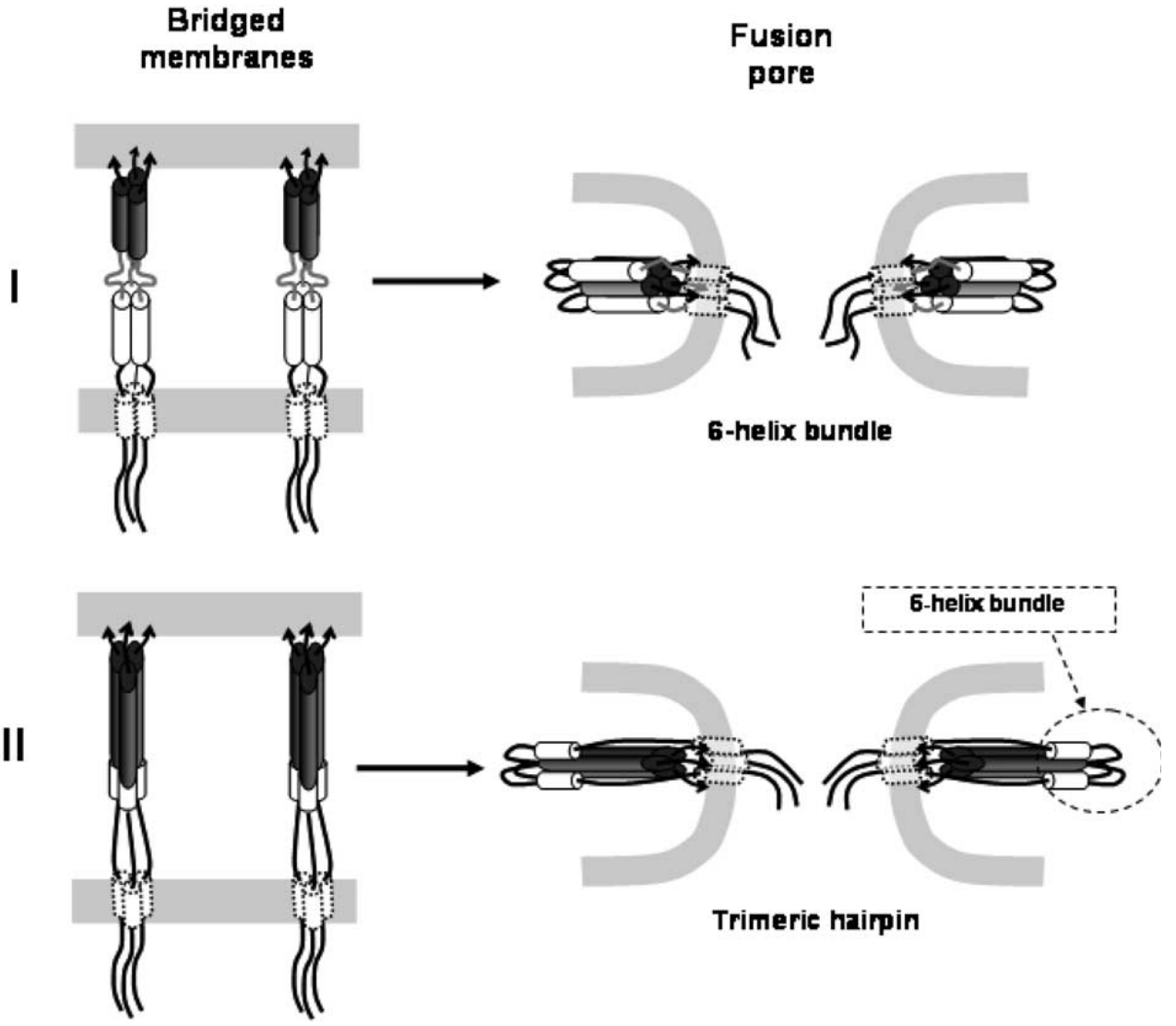


Fig. 4. The trimeric hairpin forms late in the fusion process. The grooves of the coiled coils (*left panels*, *shaded cylinders*) of fusion proteins become exposed prior to hemifusion. Insertion of fusion peptides (*arrows*) into the target membrane creates a bridge between the target membrane and viral envelope. For some proteins, such as HIV Env, the trimeric hairpin is largely comprised of the 6HB (*I*, *right panel*); bundle formation brings the fusion peptides and MSDs (*dotted cylinders*) into proximity. For other proteins, such as influenza HA, the 6HB (*open cylinders* packed into shaded coiled coil) comprises only a portion of the trimeric hairpin (*II*, *right panel*); trimeric hairpin formation is necessary for fusion peptide and MSD proximity.

other to their maximum extent for HA to create restricted hemifusion or pores, but they must do so to induce pore enlargement. In summary, completing the folding of HA into its final trimeric hairpin structure is necessary for the late stages of fusion [5] (Fig. 4).

The wall of a small fusion pore should have a higher concentration of fusion proteins than an enlarged pore; as the pore enlarges, growth should be increasingly controlled by membrane elasticity. If the geometry of the pore wall were not constrained by protein, it would assume a shape in which its mean curvature did not deviate from the spontaneous one—a catenoid shape if spontaneous membrane curvature were zero—and the pore would spontaneously enlarge. If proteins restrain pore geometry, membrane mechanics can give rise to energy barriers. These barriers have been calculated for a toroidal pore wall (a hemisphere of revolution) [20, 21]. In the absence of membrane tension, the pore will spontaneously enlarge only if the spontaneous curvature κ_0 of the membrane that comprises the wall is quite negative. (Membrane tension always helps promote pore growth [21].) Because CPZ has positive curvature and accumulates in inner leaflets of cell membranes, adding it to solution makes membrane curvature more negative. Adding CPZ to cells in a state of hemifusion should thus not only promote

pore formation, but enlargement as well, as is experimentally observed [1, 16, 72, 76]. If $\kappa_0 = 0$ (as would be the case if the two monolayers of the wall were identical), an appreciable energy barrier, >50 k_BT, must be surmounted for the pore to grow. If κ_0 is even slightly positive, the energy preventing pore growth increases indefinitely with pore radius and the pore will never fully enlarge. Thus, theory is in accord with observation: the energy barriers against pore growth can be quite large, larger than the barriers against stalk formation and pore formation.

How Might Sequential Conformational Changes of the Fusion Protein Account for Progressively more Energetically Difficult Steps of Fusion?

To create a trimeric hairpin, the three MSDs within a trimer must separate from each other and move into contact with the fusion peptides at the end of the coiled coil. In principle, the three MSDs could move either simultaneously or sequentially. If simultaneous, the protein must be able to assume a conformation that would allow all three movements at once. But if sequential, the protein has more freedom, allowing more conformational possibilities. If each MSD moves sequentially, it is likely that the protein becomes more constrained after each movement. This would naturally account for larger energy barriers with each sequential step of fusion. To appreciate how the steps of fusion could proceed, consider a simple model: Before any of the MSDs move into proximity with the fusion peptides, the coiled coil projects the fusion peptides into the target membrane. The viral envelope and target membrane are brought into contact by the movement of the first MSD toward the fusion peptides. This movement requires that one C-terminal segment pack against the central coiled coil. The inserted fusion peptides and other portions of the fusion protein disrupt the lipid arrangement of contacting monolayers, and thus hemifusion is a low-energy-requiring process. By the time of hemifusion, the fusion proteins are no longer independent of each other, but rather are already configured in a ring around the fusion site. A fusion pore is created when the second MSD intermingles with the fusion peptides. This step is less favorable energetically because the proteins are more constrained. When the second C-terminal segments pack against the central coiled-coil, the interacting proteins move outward from the fusion site because the pincer-like motion forces a lengthening of the proteins along the axis of the hemifusion diaphragm. Outward protein movement increases membrane tension. In addition, the intermingling of fusion peptides and MSDs destabilize the hemifusion diaphragm. It is one of these forces, or some combination of both, that causes the pore to form. Movement of the third MSD (of each protein of the ring) to complete refolding into a trimeric hairpin is the most energetically difficult conformational change. The pincer movement of the third C-terminal segment further pulls the proteins outward, ensuring that the pore remains open and enlarges.

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