New and Notable

Filling Potholes on the Path to Fusion Pores

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Membrane fusion is at the heart of eukaryotic cell life. Carefully regulated membrane fusion is required for cell compartmentalization, for the import of large molecules into the cell, and for the export both of waste molecules and of signaling molecules that carry information to other cells in a community. The complex cellular machinery that carries out these functions is beginning to come into focus (Brunger, 2001), but a detailed molecular view of how these machines work on membrane lipid bilayers to accomplish a change in topology of cellular compartments is still lacking. The difficulty in dissecting this process lies not just in the complexity of the machines, but also in the task of defining experimentally a dynamic process operating in a semi-ordered system such as the lipid bilayer. Although experimental approaches are available, it has been popular for some time to treat this problem theoretically, in terms of simple models in which complex arrangements of lipid molecules are treated in terms of the material properties of arrays of lipid molecules as they occur in macroscopic semicrystalline lipid phases. Although this is clearly a vast simplification, it is probably, if properly parameterized, a useful zeroth order approach to the very difficult problem of estimating the free energies of presumed intermediates on the molecular path to fusion.

The widely accepted model for this process derives from the original proposal that two bilayers brought into close contact can merge their contacting (*cis*) monolayers in a toroidal "stalk" that joins half the lipid components of the two original bilayers (Fig. 2 *B* from Markin and Albanesi (2001)). The distal (*trans*) monolayers of this structure are not merged, which prevents continuity between the trapped aqueous compartments of the two fusing membranes. This has been termed the *stalk hypothesis* (Markin et al., 1984). Later, it was recognized that the stalk may exist in two different forms, an initial stalk and a transmembrane contact (Siegel, 1999). It was universally accepted that such structures must have a free energy substantially larger than that of the lipid bilayers from which it was proposed to arise. A systematic estimate of this energy was first made in terms of two major contributions: the energy associated with bending planar monolayers into toroids, and the interstice ("void") energy associated with the junctions where monolayers are peeled away from one another (Siegel, 1993). The latter was, in the initial level of approximation, estimated as proportional to the surface area of the interface between lamellar structures and a hypothetical void that represented the space unfilled by uniformly packed monolayers distorted to match the hypothesized nonlamellar stalk structure (Siegel, 1993). The bending energy was estimated in terms of the free energy required to distort a uniform lamellar monolayer from its spontaneous or "intrinsic" curvature to match the assumed stalk shape. The energy of the void was parameterized based on the properties of the phase transition from a lamellar to a nonlamellar phase (hexagonal phase) that was also viewed as composed of bent lamellar structures and voids. Based on this treatment, the free energy of the stalk was estimated to be so large (~200 kT) that there has been question as to whether such a structure could be part of the fusion process, although these calculations suggested that the stalk was the lowest-energy intermediate of several proposed fusion mechanisms (Siegel 1993). Thus, the stalk hypothesis was found to face an "energy crisis."

Two papers appear in this month's issue of the *Biophysical Journal* (Kozlovsky and Kozlov, 2001; Markin and Albanesi, 2001) that, along with another recent paper (Kuzmin et al., 2001), propose solutions to this energy crisis. Markin and Albanesi (2001) propose that the assumption of circular torroidal geometry for the stalk produced too large a bending energy. These authors show that it is possible to relax this assumption and define, in terms of two geometric parameters, a stress-free stalk that has the same average intrinsic curvature as the lipid mixture of which it is composed. Just as for the original stalk hypothesis (Markin et al., 1984; Siegel, 1993), one can question some of the geometric assumptions of this calculation. However, it is a reasonable result that the bending energy of a geometrically relaxed stalk can be reduced significantly from the original estimate that was based on a rigid (circular torroid) geometry. Even if the bending free energy of the stalk can be reduced to zero, the interstice or void free energy attributable to the intrinsic geometric mismatch between lamellar and nonlamellar lipid structures must persist. This is variously estimated as several tens of kT, still a formidable energy but much less imposing than the earlier estimates of the stalk energy.

The other two articles both ask whether it is appropriate to estimate the interstice energy in terms of the surface of the interface between a void and smoothly bent lamellar structures.

Submitted December 14, 2001, and accepted for publication December 14, 2001.

0006-3495/02/02/555/03 $2.00

Although it is unlikely that a true macroscopic void exists in intermediate structures leading to fusion, this construct was originally used as a self-consistent and convenient way to estimate the free energy associated with the inherent nonlamellar nature of the stalk (Siegel, 1993). The question is whether a better method exists for estimating this free energy. Kozlovsky and Kozlov (2001) and Kuzmin et al. (2001) both argue that this energy is better estimated in terms of the energy needed to bend lamellar structures so sharply that they accommodate the inherently nonlamellar nature of the proposed stalk structure. This requires *tilt* of individual lipid molecules and must occur at an energy price, as tilting lipid molecules relative to the bilayer normal expose their hydrophobic regions to water and elongate acyl chains. Similar to the void energy, the tilt deformation energy can be estimated from experimental data on phase transitions of lipid liquid crystals from lamellar-to-nonlamellar states.

Kozlovsky and Kozlov (2001) use the tilt approach to treat the deformation of monolayers in terms of a combination of splay (monolayer bending and/or tilt gradient) and uniform tilt of lipid molecules away from the local monolayer normal. The "tilt" formalism provides a means to account for the interstice energy without the assumption of a void. Figure 8 by Markin and Albanesi illustrates this approach relative to the traditional void approach. Kozlovsky and Kozlov describe the stalk in terms of two geometric parameters (width of the stalk base, interbilayer distance). This approach yields a minimum free energy for the stalk at an intrinsic curvature of $-0.1\ \mathrm{nm}^{-1}$ of 45–50 kT, which is approximately the same as one would estimate for the interstice energy based on the void model, as pointed out by Markin and Albanesi. Curiously, at intrinsic curvatures of $< -0.25\ \mathrm{nm}^{-1}$, the stalk of Kozlovsky and Kozlov becomes thermodynamically stable relative to planar bilayers.

In summary, one essential finding of both teams Markin/Albanesi and Kozlovsky/Kozlov is that the energy required to deform the monolayers into a stalk is substantially lower than calculated with the approach used previously. Basically, the source of the overestimate in previous models was the use of a simplistic geometric model. Both sets of authors show that a more realistically shaped stalk is likely to have considerably lower bending energy, which, relative to planar bilayers, can even become negative for bilayers of substantial negative intrinsic curvature. However, it seems that the interstice free energy still dominates the stalk free energy and we may still have to fill the potholes in the pathway to fusion pores.

There are tough problems that remain to model successfully the mechanism of fusion. In both these manuscripts, the authors calculate only the lower bound to the true activation energy for formation of fusion intermediates. Present models account only for the energy of intermediates viewed as static structures. Experimentally, the process of converting closely contacting membranes (initial static structure) to a fusion pore (final static structure) involves at least three kinetic steps and two intermediates (Lentz et al., 2000). The first and last of these steps involve changes in the topology of membranes and trapped compartments and thus demand dramatic rearrangements of lipid and water molecules. These are not likely to find description by the sort of continuum, macroscopic theories reviewed here, although some attempts have been made to do so (Kuzmin et al., 2001; Markin and Albanesi, 2001). The second step, interconversion of fusion intermediates, probably does not require changes in topology and may be amenable to such an approach. Nor have we really addressed the problem of how two membranes become distorted and so closely apposed that the initial conversion to a fusion intermediate can occur. Most importantly, experimental tests of these models need to be devised. Given the highly localized and dynamic nature of membrane fusion events, this will be very difficult. However, the same rules that govern the relative energies of different-geometry intermediates must also apply to the relative free energies of lipid molecules in lamellar and nonlamellar phases. Therefore, it is probably worth some effort to see which of the new proposals best describes the relative stability and formation kinetics of complex lipid assemblies such as inverted cubic phases. Clearly there is much lipid physical chemistry that remains to be done!

How do these new results help us understand the process of protein-induced membrane fusion? First, the resolution of the energy crisis increases our confidence that structures such as stalks do represent low-energy pathways to membrane fusion. Second, the dominant nature of the void or interstice energy suggests that we look to how parts of fusion proteins that reside in or interact with membrane bilayers might lower this free energy. This will require combinations of experimental and theoretical approaches. In doing so, it is appropriate to keep in mind that the stalk hypothesis is an hypothesis and that the actual mechanism of biomembrane fusion may be even more complex than suggested by this model.

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