Non-Equilibrium Large-Scale Membrane Transformations Driven by MinDE Biochemical Reaction Cycles

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Abstract: The MinDE proteins from E. coli have received great attention as a paradigmatic biological pattern-forming system. Recently, it has surfaced that these proteins do not only generate oscillating concentration gradients driven by ATP hydrolysis, but that they can reversibly deform giant vesicles. In order to explore the potential of Min proteins to actually perform mechanical work, we introduce a new model membrane system, flat vesicle stacks on top of a supported lipid bilayer. MinDE oscillations can repeatedly deform these flat vesicles into tubules and promote progressive membrane spreading through membrane adhesion. Dependent on membrane and buffer compositions, Min oscillations further induce robust bud formation. Altogether, we demonstrate that under specific conditions, MinDE self-organization can result in work performed on biomimetic systems and achieve a straightforward mechanochemical coupling between the MinDE biochemical reaction cycle and membrane transformation.

Introduction

Living systems are based on self-organization, by which the interactions of a system’s components generate emergent structures and functions. Self-organization supports morphological changes through energy dissipation, enabling evolutionary adaption to varied environmental conditions and exercise complex tasks characteristic of life. One paradigmatic feature of self-organization is pattern formation that can be found throughout all kingdoms of life. Because of its compositional simplicity, the Escherichia coli (E. coli) Min system has been extensively studied in vivo, in vitro, and in silico, and has become a model system for protein pattern formation. The Min proteins oscillate from pole-to-pole within the rod-shaped E. coli cell, regulating the position of the FtsZ ring that orchestrates cell division. MinD, MinE and ATP are necessary and sufficient to form Min patterns on lipid membranes. MinD is an ATPase that dimerizes in its ATP-bound state. Upon ATP binding and dimerization, MinD gains sufficiently high affinity to bind the membrane via the simultaneous presence of two individually weak membrane targeting sequences (MTS). MinE exhibits a latent cytosolic conformation and an active conformation in the presence of membrane-bound MinD, which induces MinE recruitment to the membrane. ATP hydrolysis in MinD, and subsequent dissociation of MinD and MinE from the membrane. Importantly, MinD and full-length MinE need to interact with phospholipid membranes for pattern formation. During membrane binding, the diffusion constants of the proteins are locally decreased, inducing dynamic instability as needed to form the self-organized patterns. The membrane can thus be considered as a catalyst for Min pattern formation. First Min protein reconstitution systems used supported lipid bilayers (SLBs) as the model membrane, where MinD and MinE self-organization yielded protein waves with MinE accumulating at the rear of propagating MinD zones. However, due to the tight adhesion of the membrane to its support, it was not structurally affected by the protein waves. Even in the case of free-standing giant unilamellar vesicle (GUV) membranes deposited on top of SLBs, there was no indication of any mechanical effect by the Min proteins propagating across them. In contrast, when encapsulating MinD and MinE proteins into osmotically deflated GUVs, dramatic reversible membrane deformations could be achieved, pointing to the possibility of mechanical forces generated by Min proteins. However, similar transformations have also been observed in systems approaching equilibrium by simple membrane binding of proteins without any energy dissipation when the surface tension is sufficiently low. Although Min-induced shape changes in vesicles have in fact also been observed in (near-)isotonic conditions, close to spherical GUVs with relative high tensions are no ideal model systems for investigating membrane transformations in more detail. Thus, in order to explore the potential of Min proteins to actually perform mechanical work, we sought to establish a new model membrane system supporting large-scale changes in membrane morphology. In previous studies, we fabricated dipeptide (Diphenylalanine, FF) crystal-supported phospholipid membranes, in which the disassembly of the dipeptide crystals deformed the supported lipid membranes into reservoirs of various shapes. The most prominent of these were long membrane tubes and layers of membrane...
sheets, reminiscent of flattened vesicles, on top of a SLB.[9a,b]
When reconstituting the dynamic Min proteins on these
membrane structures, we observed reversible growth and
shrinkage of the structures in concert with the Min oscilla-
tions. Structures directly at the SLB surface experienced
adhesion, frustrating relaxation into their original shapes and
thus supporting continuous growth. Suspended structures well
above the SLB support, where adhesion is absent, periodically
relaxed back to their original sizes, indicating the presence of
restoring forces, against the Min oscillations perform
mechanical work. Furthermore, at reduced concentrations of
negatively charged phospholipids in the membrane or Mg2+ in
buffer, we also observe robust bud formation on flat
membrane sheets, reminiscent of the complex action of
membrane sculpting proteins in cells.

Results and Discussion

Min Oscillations Induce Reversible Length Change of Free-
Standing Membrane Tubes

In all our assays for creating FF crystal-templated
membrane structures, the coverslip was decorated with an
SLB in advance and the FF crystal-templated membrane
structures and the SLBs shared the same lipid composition.
For its composition, we used DOPC50%/DOPE20%/ DOPG30%, the negative net charge of which is necessary
for the formation of Min patterns.[10] The FF-crystal supported
membranes were produced and the crystals dissolved as
described in SL.[9a,b] The types of structures produced could
be regulated by the exact time points of protein addition. When
initiating Min protein oscillations before dissolving the
crystals, the supported membranes on the crystals deformed
upon crystal dissolution into membrane tubule networks
suspended between local protein-lipid aggregates (Scheme 1,
Figure 1 a, Figure S1, Movie S1). These local aggregates could
be used as fiducial markers to quantify changes in tube length.

During MinDE reaction cycles involving reversible
MinD-ATP membrane binding, dramatic local stretching of
the tubes up to 20% of their initial length could be observed,
resulting in large-scale deformation of the network (Fig-
ure 1 b,c, Movie S2). The length increase followed the rise in
local concentration of the membrane-bound MinD. MinD-
ATP binds to the membrane with its C-terminal MTS, which
forms an amphipathic helix, and is assumed to align parallel to
the membrane surface.[11] The hydrophobic residues of the
helix penetrate into the hydrophobic interior of the bilayer,[13]
MinD-ATP is further known to form higher-order structures
on membranes.[12] Therefore, MinD-ATP binding increases the
length of the membrane tubule by directly increasing
membrane surface area. This area increase is likely accom-
modated by inducing membrane elastic stress (thereby
shrinking the tube diameter), although we cannot rule out the
possibility of lipid extraction from the adjacent
membrane-protein aggregates, which would in this case act as
tropic springs (Figure S2).

Importantly, however, these length changes induced by
MinD-ATP binding were fully reversible, and the tubes
spontaneously retracted back to their original length after
MinD dissociation (Figure 1 c), due to either membrane
elasticity or lipid retraction into the spring-like aggregates.
This indicates that MinD-ATP binding indeed exerts a me-
chanical force to repeatedly increase the length of the tube
(Figure S3). To estimate this force, we assume for simplicity
that the elastic response is determined by the membrane
itself. If we thus simplify the tube extension and define
a point force $F$ to be applied to the tube, $F$ can be calculated
as $F = 2\pi \gamma R$[12] where $\gamma$ is the membrane bending rigidity and
$R$ is the radius of a membrane tube. Adopting a typical value of
$\gamma = 40$ pN nm$^{-1}$[13] and a tube radius of 300 nm as shown in
Figure 1 c, we obtained a force of $F = 0.84$ pN. For tubule
extension of 1 μm, the work $0.84 \times 10^{-10}$ N m was done, that is
204 $k_B T$. It is worth mentioning that MinD-ATP binding can
change the membrane spontaneous curvature, which will
reduce the force required to extend the tube.[12a]
Formation of Flat Vesicles by Dissolving FF Crystals

When FF crystals were dissolved in the absence of proteins, SLB-supported flat vesicles were obtained (Figure 2a; Movie S3). They featured bulging rims, and stacks of double bilayers in the middle (Figure 2b), often attached to membrane aggregates as membrane reservoirs. Fluorescence recovery after photobleaching (FRAP) measurements revealed that the apparent lipid diffusion in the flat vesicle stacks was higher than in SLBs, indicating increased membrane fluidity (Figure S4). The diffusion ratio between flat vesicles (1.1 μm²/s⁻¹) and the SLB (0.3 μm²/s⁻¹) was around 3.6.

Formation of flat vesicles has been documented as a result of vesicle spreading in a tank-thread motion on moderately adhesive substrates, such as MgF₂, avidin or Al₂O₃ surfaces. Hydrating a lump of phospholipid with high salt concentration has been shown to generate flat vesicles on top of a single bilayer. High ionic strength shields the repulsive electrostatic interactions between negatively charged membranes, and KCl has been shown to promote adhesion between lipid membranes. Moreover, divalent cations can bind two negatively charged lipid membranes by charge bridging. Therefore, the high cation concentration in the buffer (25 mM Tris, pH 7.5, 150 mM KCl and 5 mM MgCl₂) promotes membrane adhesion and facilitates membrane spreading.

To systemically elucidate the flat vesicle formation conditions in our system, we varied coverslip treatments, as well as lipid and buffer conditions. After passivating the coverslip with PLL-PEG (Poly(L-lysine)-graft-poly(ethylene glycol)) resulting in no SLB to support the membrane spreading, the FF crystal-supported membranes deformed into membrane aggregates and tubules (Figure S5d). Flat vesicles formed at 10 mol%, 30 mol% and 50 mol% DOPG (Figure S5a–c) and their spreading speed appear to be tightly controlled by cation concentrations (Figure S5f–h, k). We further explored how FF crystal dissolution contributed to the flat vesicle formation. It has been shown that FF induced membrane permeation and depolarization at a sub-critical concentration (non-assembled FF), which can facilitate its diffusion across the membrane. In fact, we detected FF monomers in the solution by UV measurement (around 1 mM, see detail in SI). Diffusion of FF across the membrane will generate an osmotic shock, and in response, water influx into the flat vesicle. Because the high cation concentration promotes membrane adhesion, we propose that the inflowing water during FF crystal dissolution aids flat vesicle spreading.

Min Oscillations Promote Flat Vesicle Spreading and Tubule Deformation

After flat vesicle self-spreadning upon FF crystal dissolution ceased, initiating Min oscillations initiated further continuous expansion of flat vesicles on SLBs (Figure 2c–e, Movie S4), the morphology of which however differed from the osmotically driven self-spreadning. MinD-ATP binding first deformed the rim of the flat vesicles into tubule-like structures, thereby temporarily reducing the covered area. When the tubules subsequently retracted back into the flat vesicle, however, the membrane area was significantly increased as compared to before (Figure 2f). It is well known that peripheral binding of proteins or polymers to membranes can induce dramatic membrane deformations. However, these transformations are usually thermodynamically stable once binding reactions reach a steady state. Here, energy dissipating Min oscillations induced reversible membrane tubule deformation and, in combination with additional surface forces to prevent elastic retraction, promoted a progressive membrane spreading phenomenon that is not achievable under equilibrium conditions.

Further spreading of the membrane reservoir generated two-layer flat vesicle stacks, and different layers can be identified through their distinctive boundaries (Figure S6). In contrast to the limited tubulation of the bottom layer flat vesicle (Figure 2d), the second layer showed even more robust tubule deformation (Figure S6; Movie S5). The reduced tubulation of the bottom layer flat vesicle likely results from their closer interactions with the substrate.

Min Oscillations Drive Membrane Extension from Membrane–Protein Aggregates on SLBs

Moreover, we were able to observe the “de novo” membrane extension into flat vesicles from membrane-protein aggregates upon Min oscillations. We quantified it...
defining the aggregates as starting points (zero points) of membrane spreading. The membrane-protein aggregates were obtained by dissolving FF crystals with protein binding to the FF crystal-supported membranes as described above (Figure 1a). Besides acting as pillars for free-standing membrane tubules, some isolated membrane-protein aggregates were located on SLBs (Figure 3a). Min oscillations were able to extract membrane from these aggregates with increasing area (Figure 3b).

We quantified the membrane spreading dynamics by analyzing the membrane area changes over time (Figure 3c). Consistent with the previous results,[14] the spreading speed can be considered constant at early times. Linear fitting yielded the spreading speed driven by Min oscillations with a mean value of 0.24 μm² s⁻¹ (30 mol% DOPG; n = 5; 2 independent experiments). The speed did not change markedly at 50 mol% DOPG (0.22 μm² s⁻¹; n = 4; 3 independent experiments), but Min oscillations were not able to extract membrane at 10 mol% DOPG (3 independent experiments; Figure 3d), which may be due to reduced membrane adhesion and protein binding to the membrane.[10b] The speed was reduced after deleting MgCl₂ from the buffer (0.13 μm² s⁻¹) and further decreasing KCl concentration to 100 mM (without Mg²⁺ in the buffer; 0.08 μm² s⁻¹; Figure S7). It should however be noticed that 2.5 mM MgCl₂ was always supplied from ATP solution, as described previously for the generation of Min oscillations.[20] The spreading speed was also reduced at higher Mg²⁺ concentration in the buffer (15 mM; 0.09 μm² s⁻¹; Figure S7). It has been shown that high concentrations of divalent ions cause flat vesicle shrinkage, due to long-range repulsive double-layer forces[15] and even rupture flat vesicles due to high adhesion energy,[14] which could contribute to the decreased speed at high Mg²⁺ concentrations. Thus, membrane spreading driven by Min oscillations is controlled by both, lipid composition and cation concentrations.

Vesicle expansion on SLBs, driven by free energy released by ATP hydrolysis during Min oscillations and aided by membrane adhesion, has to overcome several forces. These are the elastic force of the flat membrane, the entropic force induced by the aggregate as mentioned above, as well as several friction forces, including the sliding between two monolayers, the friction between the two bilayers, and dissipation occurring by the forced penetration of water.[14b] Among them, the best studied is probably the sliding between two monolayers.[14b] When membrane moves with a constant velocity v, the friction F = πbhν, where b is the interfacial drag coefficient and h is the thickness of the monolayer.[14a] Assuming b = 5 × 10⁶ Ns m⁻¹,[19] h = 2 × 10⁻⁹ m[14a] and v = 0.24 μm² s⁻¹, we estimated F = 0.75 pN (see details in S1). Considering the other potential opposing forces, this value would be the lower limit of what needs to be overcome by the Min proteins, being in the same range as what has been estimated above (0.84 pN).

**Min Oscillations Promote Budding of Flat Vesicles at Reduced Concentration of DOPG or Mg²⁺**

In order to deform the bottom layer of flat vesicles, adhesion forces between the flat vesicle lower surfaces and SLBs need to be overcome. To induce a transformation of this layer, we reduced the concentration of DOPG from 30 mol% to 10 mol% lowering Mg²⁺ bridging. Under this condition, Min protein binding to the membrane will also be reduced.[10b] Unexpectedly, the Min oscillations now promoted robust vertical bud formation (Figure 4b–d; Figure S8b, Movie S6). During bud formation, the contact area of the flat vesicle with SLB decreased (13% for Figure 4d). To better understand the factors driving bud formation, we separately reduced protein and Mg²⁺ concentration at 30 mol% DOPG. It turned out that deleting Mg²⁺ from the buffer effectively promoted bud formation (Figure 4e) while reducing the concentrations of Min proteins did not (Min wave patterns still formed; Figure S9). Moreover, replenishing Mg²⁺ reduced already formed buds (Figure S10). Therefore, robust bud formation can be obtained by deleting Mg²⁺ from the buffer or reducing DOPG concentration.

Budding may be induced by a number of possible mechanisms, for example, a slight area difference between the two monolayer leaflets of the membrane, a change in the vesicle area-to-volume ratio and the interplay of bending rigidity and line tension of phase separated domains.[20] MinD-ATP binding can induce an area difference between the two monolayer leaflets and change the flat vesicle area-to-volume ratio. Moreover, MinD-ATP binding has been shown to induce a remarkable increase in membrane viscosity and the formation of acidic phospholipid-enriched domains in a mixed acidic-zwitterionic membrane.[21] Surface viscous flows have been shown to provide a dominating energy dissipation mechanism for budding.[21] Therefore, at reduced
membrane adhesion, these factors may act in concert to promote robust bud formation. Because of the relatively low ATPase activity of MinD in the absence of MinE and the consequently slow detachment of MinD,[23] the budding effect of MinD-ATP in the absence of MinE will disappear when lipid mobility equilibrates changes in membrane curvature (Figure 4b).[24] When MinE is added, however, Min oscillations can generate spatially and temporally inhomogeneous protein distributions on the flat vesicles,[5] which may actively promote continuous bud formation.[24]

A Versatile Model Membrane System to Study the Direct Coupling of Biochemical Energy Dissipation to Membrane Transformation

Compared with standard model membrane systems, the here introduced flat vesicle stacks on supported membranes have small internal volumes, resulting in large surface-to-volume ratios and facilitating the transportation and exchange of inside buffer[25] to enhance the dynamic responses. Moreover, the flat vesicles connect with membrane reservoirs, and the tension changes due to area expansion can therefore be accommodated. Importantly, flat vesicles stacks retain properties of free-standing membranes, such as the higher lateral lipid mobility and access of binders to both leaflets. At the same time, the presence of a supported membrane beneath the stacks promotes membrane adhesion between the SLBs and the flat vesicles, which is required to prevent elastic retraction and enables progressive membrane spreading. These features enable the straightforward coupling between biochemical reactions and mechanical work performed on the membrane, resulting in out-of-equilibrium large-scale membrane transformation. As the reaction-diffusion (RD) models that are paramount in the study of pattern formation do not traditionally include the possibility of a feedback between biochemical reactions and membrane elastic deformations,[26] this model membrane assay is an ideal starting point for studying the chemo-mechanical coupling of Min protein self-organization, and thus provides a unique tool to stimulate further experimental and theoretical studies on this interesting protein system.

Conclusion

We have introduced a new model membrane assay particularly suited to investigate large-scale membrane remodeling processes. With this, we could demonstrate that the ATP-driven self-organization dynamics of the E.coli MinDE protein machinery does not only induce dynamic concentration gradients on membranes, as required in vivo for positioning the division machinery, but can also lead to dramatic transformations of free-standing membranes. Although the forces required to pull tubules or flat vesicles from our soft membrane stacks are likely much below the ones that have previously been measured on molecular motors pulling tubes from giant vesicles,[13b,27] there is a clear indication that energy dissipation in the form of ATP hydrolysis by MinD drives this non-equilibrium process forward and performs mechanical work (for tubule extension of 1 μm, we estimated the work of more than 200 k_BT performed by Min oscillations). The fact that the membrane spreading and pulling occurs reversibly—unless the proximity of the supported bilayer and the presence of surface interactions arrests a once-expanded structure in place before the next membrane binding cycle—indicates that the retraction forces by the elastic membrane or membrane-protein aggregates are considerable.

Moreover, with lower concentration of DOPG or Mg^{2+}, Min oscillations promoted robust bud formation from the planar flat vesicles. In the absence of other more complex bud-forming protein machineries, Min oscillations provided the non-equilibrium cue to support these continuous membrane transformations through mechano-chemical coupling.[27] Since the pancake shapes of our flat vesicle membranes are abundant in living cells, such as lamellipodia or pseudopods of moving cells[28] and flattened ER cisternae and Golgi,[29] this model membrane assay holds great promise to generally explore advanced protein functions[30] in the context of synthetic and artificial cells.
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Conflict of interest

The authors declare no conflict of interest.

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