3198-Plat
Syntaxin Clustering in Membranes
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Syntaxin-1A and SNAP25 form the receptor component of the SNARE complex, which has been shown to be the minimal machinery for membrane fusion. In vivo studies have revealed that syntaxin-1A exists in cholesterol-dependent clusters that are distinct from lipid rafts. Additionally, SNARE-mediated membrane fusion has been shown to be stimulated by regulatory lipids, such as phosphatidylinositol 4,5-bisphosphate (PI-4,5-P2). An appreciation of the lipid-protein interactions which define syntaxin clustering dynamics is essential to understand the membrane role in organization of SNARE-mediated membrane fusion.
We determined that syntaxin exists in cholesterol-dependent clusters, from which it may be released by as little as 1-5 mole percent PI-4,5-P2 by in vitro fluorescence assays. Lipid-protein fluorescence resonance energy transfer reveals that the phosphoinositide interaction is direct and mediated by electrostatics. To investigate the dynamics of clustering, single-molecule fluorescence quenching microscopy was developed. The observation of syntaxins in single vesicles allows the step-wise statistical analysis of discrete syntaxin-syntaxin interactions, and determination of their dependence on concentration and membrane composition. These in vitro results help explain the mechanisms of dynamic clustering of syntaxin in cell membranes, and the activation of fusion by PI-4,5-P2. Moreover, they suggest a working model for cell membrane regulation of syntaxin clustering.

3199-Plat
A Fast, Single-Vesicle Fusion Assay Mimics Physiological SNARE Requirements
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SNARE proteins play a central role in nearly all intracellular fusion reactions; fusion is thermodynamically driven by formation of trans-SNARE complexes (SNAREpins) through pairing of vesicle-associated v-SNAREs with complementary t-SNAREs on target membranes. However, the number of SNARE complexes required for fusion is unknown and there is controversy about whether additional proteins maybe required to account for the speed with which fusion can occur in cells. Small unilamellar vesicles containing the synaptic/exocytic v-SNAREs VAMP/synaptobrevin fuse rapidly with planar, supported bilayers containing the synaptic/exocytic t-SNARE syntaxin-SNAP25, with single fusion events occurring in ~10 ms to seconds. However, in previous reports the SNAP25 subunit of the t-SNARE was not required, or an artificial peptide was needed, raising questions about the physiological relevance of these results. We now include a lipid-linked polymer chain in both bilayers whose size and density mimic that of bulk membrane proteins that need to be cleared before fusion in physiological settings. The result is that SNAP25 dependence is restored but rapid fusion (mean delay after docking of 130 ms) remains. A dramatic drop in the overall fusion rate occurs as the number of v-SNAREs per vesicle is reduced below a threshold of 5-10 externally-oriented v-SNAREs per vesicle, directly establishing this as the minimum number required for rapid fusion. The distribution of delay times for fusion following docking by the first SNAREpin appears to be limited by the time required for additional t-SNAREs to diffuse to the docking site, and implies that 5-10 t-SNAREs must be recruited to achieve fusion, closely matching the v-SNARE requirement.

3200-Plat
Atomic-Resolution Simulations Yield New Insight into Vesicle Fusion and Fusion Protein Mechanisms
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Membrane fusion is critical to both cellular vesicle trafficking and infection by enveloped viruses. While the fusion protein assemblies that catalyze fusion are readily identifiable, the specific activities of the different proteins involved and nature of the membrane changes they induce remains unknown. Here, we report many atomic-resolution molecular dynamics simulations of both fusion by a pair of vesicles and assemblies of influenza fusion peptides in planar bilayers. The mechanism of fusion in our simulations is roughly consistent with the stalk hypothesis for fusion, but we observe several new features that help explain the mechanism of fusion proteins. Our high-resolution simulations yield new structural intermediates that differ substantially from continuum models of fusion and give specific structural details for the membrane-altering effects of fusion proteins. These results may yield a common mechanistic pathway for structurally diverse classes of fusion proteins.

3201-Plat
Docking is the Rate-Limiting Step in Reconstituted Neuronal SNARE-Mediated Liposome Fusion
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Over the past ten years, great efforts have been made to reconstitute SNARE-mediated vesicle fusion in vitro by monitoring the degree of lipid mixing between populations of vesicles in solution. The reconstitution approach is powerful because the vesicles have well-defined protein and lipid components and the effects of adding or subtracting a specific component can be assessed directly. In accord with previous work, we find that proteoliposomes composed of full-length SNAREs alone are capable of docking and fusing very slowly in solution. We have developed a FRET-based, single-vesicle fusion assay in which tethered v-SNARE vesicles containing synaptobrevin-II interact with solution-phase t-SNARE vesicles containing syntaxin-1A + SNAP-25. This enables separation of individual docking and fusion events. Our data clearly indicate that the rate-limiting step in this reaction is the docking step. Adding a peptide comprised of synaptobrevin-II(57-92) into the reaction significantly increases the efficiency of fusion by increasing the efficiency of docking. Once docking is achieved, fusion is very rapid, occurring on a time scale of ~0.3 s or less. The data strongly suggest that the synaptobrevin binding site in the t-SNARE complex is badly occluded in the absence of the syb(57-92) peptide. It seems possible that many added components that accelerate fusion in bulk assays act primarily to mitigate t-SNARE entanglements that prevent SNARE complex formation and enhance docking efficiency, not to accelerate the fusion step itself.

3202-Plat
Single Molecule Content Mixing Analysis of SNARE-Mediated Membrane Fusion
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A single liposome fusion assay has been developed in our lab to unambiguously detect different stages of fusion including docking, hemi and full fusion via fluorescence resonance energy transfer. [1, 2] While this assay provides the extent of lipid mixing, the presence of the fusion pore opening cannot be detected. In order to further push the technique and to reveal the minimal machinery of SNARE-mediated membrane fusion, we have developed a single molecule assay based on content mixing of a large DNA probe. It has been confirmed that yeast SNARE proteins are enough to produce large and stable fusion pores for content mixing. For the neuronal case, our results show that SNARE complexes are not enough to expand fusion pores, and the fusion regulator protein synaptotagmin is necessary for the completion of content mixing via pore expansion.
References
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Platform AY: Molecular Mechanics & Force Spectroscopy

3203-Plat
Domain Insertion Effectively Regulates the Mechanical Unfolding Hierarchy of Elastomeric Proteins: Toward Engineering Multifunctional Elastomeric Proteins
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The architecture of elastomeric proteins controls fine-tuned nanomechanical properties of this class of proteins. Most elastomeric proteins are tandem modular in structure, consisting of many individually folded domains of varying stability. Upon stretching, these elements unfold sequentially following a strict hierarchical pattern determined by their mechanical stability, where the weakest element unfolds first and the strongest unfolds last. Although such a hierarchical architecture is well-suited for biological functions of elastomeric proteins, it may become incompatible with incorporating proteins of desirable functionality in order to construct multifunctional artificial elastomeric proteins, as many of these desired proteins are not evolved for mechanical purpose.

Thus, exposure to a high stretching force will result in unraveling of these proteins and lead to a loss of their functionality. To overcome this challenge, we combine protein engineering with single molecule force spectroscopy to demonstrate that domain insertion is an effective strategy to control the mechanical unfolding hierarchy of multi-domain proteins and effectively protect mechanically labile domains. As a proof-of-principle experiment, we spliced a mechanically labile T4 lysozyme (T4L) into a flexible loop of a mechanically stronger host domain GL5 to create a domain insertion protein. Using single molecule force spectroscopy, we showed that the mechanically labile T4L domain unfolds only after the mechanically stronger host domain GL5 has unfolded. Such a reverse mechanical unfolding hierarchy effectively protects the mechanically labile T4L domain from applied stretching force and significantly increased the lifetime of T4L. The approach demonstrated here opens the possibility to incorporate labile proteins into elastomeric proteins for engineering novel multifunctional elastomeric proteins.

3204-Plat
Paleoenzymology at the Single-Molecule Level: Probing the Chemistry of Resurrected Enzymes with Force-Clamp Spectroscopy
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A journey back in time is possible at the molecular level by resurrecting proteins from extinct organisms. In the last two decades several methods based on statistical theory have been developed to computationally reconstruct ancestral protein sequences. Laboratory resurrection of these ancestral proteins provides an excellent opportunity to explore aspects of ancient life that cannot be inferred from fossil records. Here we report the resurrection of ancestral thioredoxin enzymes (Trx) from the Precambrian era, dating back between 1.5 to 4 billion years (Gyr). Using single molecule force-clamp spectroscopy we demonstrate that all ancestral enzymes efficiently reduce disulfide bonds. From the force-dependency of the rate of reduction of an engineered substrate, we conclude that the Precambrian enzymes have similar chemical mechanisms of reduction that the extant enzymes. By contrast, the resurrected enzymes show thermal stabilities 20 to 30 °C higher than those of modern E. coli and human Trx as revealed by Differential Scanning Calorimetry (DSC). This high thermostability illustrate that ancient organisms lived in hot environments that have progressively cooled. Trx enzymes adapted to these environmental temperatures with similar chemical mechanisms than those observed in extant Trxs. Our work demonstrates that the combination of single molecule force spectroscopy together with the resurrection of ancestral proteins is a powerful new approach to study molecular evolution and the sequence-chemistry relationship in enzymes.

3205-Plat
The Unfolding Behavior of RNase H Under Force
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We have used optical tweezers to revisit the energy landscape of E. coli RNase H under mechanical force. This protein's equilibrium energetics and folding pathway have been studied in bulk and in single-molecule mechanical denaturation experiments, which showed the presence of a collapsed folding intermediate that is on-pathway to the native state (1).
Taking advantage of improvements in our optical tweezers instrumentation, we have now revealed the existence of a short-lived high-energy unfolding intermediate. Our data suggest that this intermediate is obligatory in the mechanical unfolding pathway for this protein. This unfolding intermediate appears to be a local, partial denaturation of the C-terminus of the protein's structure. We explore the energetics of this transient state, and characterize how it defines the unfolding kinetics of RNase H.
1) Cecconi, Shank, Bustamante, Marqusee. Science 2005

3206-Plat
Analysis of Reversible Two-State Systems Under Force
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The dissociation of inter- and intra-molecular bonds by force is a process that occurs regularly in biological machinery and many cellular events. Forcing such transitions in a controlled environment has also emerged as a modern practice in the laboratory for studies of the physical principles of bond lifetimes and protein unfolding. It is commonly assumed that force-driven dissociation is irreversible, which leads to the analysis of first-passage statistics and results in simple analytical results for the distribution and moments of the transition force. However, the irreversible model is a first-order approximation which is only valid very far from equilibrium, or under specific irreversible circumstances. Furthermore, the irreversible model has led many to conclude that force spectra that deviate from linearity unequivocally represent multiple energy barriers along the intermolecular reaction coordinate.
We show that irreversible first-passage analysis, which fails for two-state systems, can be replaced by analyzing the conditional single-passage time between the two states. We find simple solutions for the forward and time-reversed distributions of the transition force, and the isothermal work, which analytically satisfy the fluctuation theorem. We also define how stochastic force trajectories should be measured when multiple forward-reverse events occur. By accounting for reversibility, we show that both the distribution and the first moment of the rupture force significantly differ from the irreversible model and clearly connect with the equilibrium regime. We find that the resulting spectrum of rupture forces is not monotonic with log of the loading rate, but follows at least two major regimes - a linear-response and a dynamic response - with the linear regime tending to the equilibrium free energy change. We validate our analytical results with simulations and experimental data on bond rupture and protein unfolding.

3207-Plat
Multi-dimensionality of Proteins' Free-Energy Landscapes Revealed by Mechanical Probes
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The study of mechanical unfolding, through the combined efforts of atomic force microscopy and simulation, is yielding fresh insights into the free-energy landscapes of proteins. One-dimensional models of the free-energy landscape have been widely used to analyze experiments. We show that as the two ends of a protein are pulled apart at a speed tending to zero, the measured mechanical strength of filamin plateaus at about 30 pN instead of going towards zero. Simulations reproduce this phenomenon and indicate that it can be explained by a switch between parallel pathways. Insightful analysis of mechanical unfolding kinetics needs to account for the multi-dimensionality of the free-energy landscapes of proteins, which are crucial for understanding the behavior of proteins under the small forces experienced in vivo.

3208-Plat
Noise Induced Regulation of DNA Loops
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Protein-mediated DNA loop formation is a ubiquitous means of regulating gene transcription. Loop formation in ds-DNA is driven by tiny forces on the order of fN arising from thermal fluctuations within the intracellular environment. Surprisingly, these forces are much smaller than the typical piconewton forces that arise from various intracellular processes, such as the procession of molecular motors or DNA-cytoskeletal attachments. This has led to theoretical predictions, and a recent experimental confirmation by our lab, that forces as small as a few hundred femtonewtons can severely reduce the rate of loop formation. We further explore the utility of using tension as a regulatory mechanism by asking how this mechanism is effected by noise. From empirical data that we have collected on loop formation rate as a function of substrate tension, we develop an effective potential that reproduces the loop transition rates given by the mean-first passage time of escape from the potential. We next incorporate this effective potential into a stochastic model of DNA subjected to an applied, fluctuating force.
The theory predicts a strong enhancement in the rate of loop formation under increasing levels of noise and, when normalized to the noise free rate, displays a universal behavior relatively independent of the mean force. This suggests that applying a varying level of tension to the DNA may be a robust method for regulating transcription. We then compare the theory with an experiment performed in our lab where we have subjected DNA, capable of forming LacI mediated loops, to a fluctuating force by means of axial optical tweezers.

3209-Plat
Single Molecule Force Spectroscopy of Peptide Aptamers
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Aptamers have broad applications in sensing, diagnostic, therapy and in the design of novel materials via molecular assembly. Peptide aptamers engineered to