**1994-Plat**
**Force Dependent Unbinding Kinetics of Actin Crosslinking Proteins Using a Four-Bead Optical Tweezers Assay**
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While the interplay between actin filaments and actin crosslinking proteins has been extensively characterized in bulk studies, only very few experiments address this interaction on the level of single molecules. Hence, the longstanding question whether unfolding of crosslinking proteins is relevant to the mechanical response of the actin cytoskeleton remains to be elucidated.
To this end, we developed a four-bead optical tweezers assay which allows probing individual actin-crosslinker-actin bonds. Two actin filaments decorated with crosslinking proteins are suspended crosswise between two trapped beads each, forming a double dumbbell geometry. A feedback control keeps formed bonds under constant force thus enabling us to measure the force dependence of lifetime distributions. As the probed filaments are freely suspended in solution, the presented experimental approach mimics the situation in actin networks very well. We used this assay to characterize the well-known actin crosslinking proteins α-Actinin und Filamin. Our results indicate that unfolding of these proteins in a physiological context seems very unlikely as the bond between filament and crosslinking protein ruptures first.

**1995-Plat**
**The Actin-binding Site of Adducin Is Regulated by Intramolecular Interactions that Occur Within a Natively Unfolded Domain**
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Adducin is an actin-binding protein with convergent functions in cell motility, including binding of calmodulin, capping of the barbed end of actin filaments, and recruitment of spectrin to the fast growing end of actin filaments. This protein consists of an unfolded C-terminal tail domain containing a positively charged phosphorylation site domain (PSD) that binds to actin, an alpha helical neck domain responsible for oligomerization, and an N-terminal globular head domain with unknown function. We recently established that MARCKS, a protein with a homologous PSD, forms intramolecular interactions that regulate the accessibility of this domain to its binding partners. Our data establish that the PSD of adducin is similarly regulated. Salt bridge formation between the PSD and a negatively charged region within the tail domain stabilize this interaction. The result is a unique configuration in which the tail is looped over upon itself without canonical structural elements so as to sterically regulate binding events at the PSD. Moreover, our data show that oligomerization of adducin results in activation of the PSD. Oligomerization is shown to be mediated either by self-association (occurring in the alpha helical neck domain) or by association with spectrin. Thus, these results explain at a structural level earlier observations showing that spectrin-adducin interactions affect actin-binding function. Finally, a polymorphism of adducin that has been associated with adverse cardiovascular outcomes in humans is shown to affect the actin-binding function of adducin by altering adducin oligomerization, and, therefore, by changing the availability of the PSD.

**1996-Plat**
**Towards a Molecular Understanding of Actin Bundle Stability and Mechanics**
**Mark Bathe**.
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Fascin and fimbrin are highly conserved actin-binding proteins used by cells to crosslink filamentous actin into compact ordered bundles. Despite the importance of these ubiquitous actin-binding proteins to cytoskeletal function, the molecular basis for their crosslinking and mechanical properties remains unknown. Here, we present a multi-scale approach aimed at reconciling the conserved sequence and structure of fimbrin and fascin with the unique properties that they endow to actin bundles. Equilibrium and nonequilibrium measurements indicate differential mediation of bundle mechanical properties, as well as the capacity for remodeling under sustained load. Atomistic simulations of fimbrin and fascin reveal distinct mechanisms of conformational flexibility that may have consequences on their cooperative actin-binding and mechanical properties in situ. Integrated structure-based modeling combined with experimental assays of bundle mechanics provides a promising approach to reconciling the conserved structure and sequence of actin crosslinking proteins with emergent actin bundle stability and mechanical properties.

**1997-Plat**
**Caldesmon and Tropomyosin Synergistically Regulate Actin Dynamics**
**Renjian Huang**, Chih-Lueh A. Wang.
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Our preliminary experiments suggest that actin undergoes a conformational transition during polymerization. Both caldesmon (CaD) and nonmuscle tropomyosin (Tm) arrest actin filaments at an intermediate state if added initially, but stabilize ''matured'' filaments when added after the transition. We have tested this hypothesis by making use of the calmodulin (CaM)-dependent dissociation of a CaD fragment, H32K, from F-actin. When CaM was added to H32K-arrested, pyrene-labeled F-actin in the presence of Ca2+, the pyrene-actin emission increased, reflecting the maturation of actin filaments upon dissociation of H32K. When free Ca2+ was removed by EGTA and H32K became re-associated with F-actin, we observed an accelerated increase in pyrene fluorescence. These results are consistent with the hypothesized conformational transition and a differential effect of CaD on the two states of actin filaments. The combined effect of CaD and Tm on the actin conformational transition was also tested during polymerization. H32K and Tm5a were first incubated with actin before polymerization was initiated. Shortly after polymerization started, CaM was added to dissociate H32K. Since Tm5a alone was sufficient to inhibit the maturation process, the transition corresponding to the conformational change was not observed, and the pyrene-actin emission jumped to the level of F-actin•Tm5a. At a later time when CaD fragment re-associated with the addition of EGTA, we saw an instant decrease, instead of a further increase, in pyrene fluorescence to the level of actin with both H32K and Tm5a, indicating that F-actin was still kept at the intermediate state by Tm5a. Since the actin cytoskeleton at the cell leading edge is extremely dynamic, it is expected that actin filaments there are kept at a less stable configuration. CaD and Tm5a may therefore function to maintain such a configuration by binding to nascent actin filaments as they are assembled.

**1998-Plat**
**Tropomyosin Phosphorylation Has Filament-Level And Crossbridge-Level Effects On Actin-Myosin Interactions**
**Vijay S. Rao**, Amy M. Clobes, William H. Guilford.
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The α-isoform of tropomyosin (Tm) can be phosphorylated near the C-terminus, and dephosphorylation of Tm has been shown to decrease actin-myosin ATPase rates (Heeley et al., 1989). Additionally, Tm polymerizes with adjacent Tm molecules in a head-to-tail manner. Phosphorylation of Tm might therefore influence the strength of these interactions, and thus regulate the degree of cooperative activation by myosin binding. We tested Tm phosphorylation effects by measuring the force and velocity of actin-myosin interactions at the level of single actin filaments using a combination of actin-Tm binding assays, *in vitro* motility assays, and a novel, high throughput laser trap assay to measure isometric force. In these assays we used purified heavy meromyosin (HMM) and actin filaments reconstituted with either natively phosphorylated or dephosphorylated Tm. Dephosphorylation of Tm did not result in significant changes in actin-Tm binding or unloaded sliding velocities. Isometric force measurements showed that thin filaments were cooperatively activated by myosin, but only when Tm was phosphorylated. When Tm was dephosphorylated, the reconstituted filaments behaved like bare actin filaments. These results suggest transmission of cooperative activation beyond one thin filament regulatory unit when Tm is phosphorylated. Moreover, Tm phosphorylation increased isometric force production ~50% compared to bare actin filaments at intermediate HMM surface densities. In combination with sliding velocity data, this result suggests that Tm phosphorylation may have effects at the level of a single crossbridge that cannot be explained by steric hindrance. We hypothesize that Tm phosphorylation accelerates the rate of crossbridge attachment. Single molecule kinetic and force spectroscopy experiments are underway to gain insight on this possibility.

## Platform AO: Protein Folding & Stability I

**1999-Plat**
**Solvation Free Energy of and Solvent Mediated Force on Proteins**
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In the theoretical study of protein folding it is important to have a fast and accurate method for predicting the free energy landscape of a protein in different geometrical configurations. One very important contribution to the free energy landscape is the olvation free energy, which originates from the interaction between the protein and the solvent.
We propose a theory for calculating the solvation free energy of a protein in a given geometrical configuration based on morphological thermodynamics. In morphological thermodynamics one separates the solvation free energy into four geometrical or morphological terms. These four terms are

proportional to the protein's volume, the solvent accessible surface area, the integrated (over the surface area) mean curvature and the integrated (over the surface area) Gaussian curvature. The coefficients proportional to these geometrical measures are geometry independent thermodynamic coefficients, which characterize the interaction between the solvent and the protein. Since the thermodynamic coefficients are independent of the geometry, they can be calculated in a simple test geometry.
The separation of the solvation free energy into four geometrical measures and corresponding geometry-independent thermodynamic coefficients has important consequences: (1) It allows for fast and accurate calculation of the solvation free energy of a protein in a given geometrical configuration, which is important when comparing different structures of a protein. (2) It allows for fast and accurate calculation of the force on the protein in a given configuration mediated by the solvent, which is important in a simulation of the folding process of the protein.

**2000-Plat**
**Dissociation and Unfolding of Insulin Dimers**
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Insulin monomers bind one another through the folding of an interchain β sheet. How does binding mediate protein folding? The energetics and rate of this fast folding process are difficult to translate into the mechanistic details that underlie classical paradigms such as conformational selection and fold-on-contact. We study the conformational dynamics at the dimer interface upon binding and dissociation using two-dimensional infrared spectroscopy (2D IR). 2D IR reveals coupling among α helix and β sheet vibrations for secondary structural sensitivity with picosecond time resolution that can resolve all relevant structural changes. Cross-peak features provide monomer and dimer 2D IR signatures that yield the binding constant and its solvent and temperature dependence. These spectra are interpreted in detail using molecular dynamics simulations of insulin dimers, disordered and compact monomers to quantify the disordered monomer ensemble. Transient dissociation and unfolding are rapidly initiated using a nanosecond temperature-jump. Conformational changes occurring on the fastest resolvable nanosecond timescales are observed for the disordering of the interchain β sheet insulin dimers.

**2001-Plat**
**Evidence For Metastable States Of Lysozyme Revealed By High Pressure FTIR Spectroscopy**
**Laszlo Smeller**.
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Metastable protein conformations play an important role in the folding process because such partially disordered states can be gates for the misfolding pathway, leading sometimes to pathological structures, like fibrous aggregates. High pressure is a very useful tool in the study of metastable states, since application of the pressure is fully reversible, contrary to chemical agents. FTIR spectroscopy allows us to follow simultaneously the secondary structure, the packing (tertiary structure) and the aggregation of the protein using the amide I, amide II and the 1616 cm-1 bands respectively. We performed a systematic study on the temperature-pressure phase diagram of lysozyme and found a two-step unfolding profile both in the pressure and temperature directions. Hydrogen/deuterium exchange results show evidence for the molten globule formation at 57°C @130 MPa and 580 MPa @30°C, which are considerable lower values than those of the complete unfolding (e.g. 75°C@130MPa).
Refolding of the protein after pressure unfolding is a slow process, with a time constant in the range of hours. Partially refolded structures present in this time range have different aggregation propensity. The kinetics of the aggregation has a biexponential character with a time constants of 1060 and 8600 s at 40C ambient pressure. Analyzing the time dependence of the amide I band shape we found that the strengthening of the intermolecular hydrogen bond network was accompanied by decrease of the folded secondary structure content. Moderate pressure of 300 MPa was found to be able to dissociate the aggregates, while the secondary structure is not yet unfolded. This fact together with the small temperature effect on the aggregation kinetics suggests that the rate of the aggregation of the metastable conformations is determined by the high activation volume rather than the high activation energy.

**2002-Plat**
**Single Molecule FRET On Alpha Synuclein Membrane-bound Conformational States**
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Alpha-Synuclein (αS) is the primary component of the Lewy body plaques that are characteristic of Parkinson's disease (PD). Large insoluble αS aggregates compose Lewy bodies, but smaller soluble αS oligomers are implicated as the cytotoxic species in PD. Though αS is natively unstructured in solution, it forms a N-terminal alpha helix upon binding to lipid membranes. Extensive evidence also shows that αS gains structure upon forming oligomeric species. In order to learn more about the transition of monomeric αS to toxic oligomeric species and to identify critical conformational states along this pathway, we use single molecule Förster resonance energy transfer (smFRET) and fluorescence correlation spectroscopy (FCS) to characterize the monomeric conformational states of αS. Our evidence shows that αS populates at least two distinct, monomeric conformational states, as a function of curvature, on lipid membranes or lipid mimetics. This could mean αS forms distinct conformations based on whether it binds synaptic vesicles or other cellular membranes. Perhaps one of these conformations is more susceptible to conversion to toxic species, and so this finding may enhance our understanding of how toxic oligomers are formed in PD.

**2003-Plat**
**Postdoctoral Research Scholar**
**Harekrushna Sahoo**.
University of Massachusetts, Amherst, MA, USA.
**Exploring Folding Intermediates of a β-Clam Protein by FRET Analysis**
Harekrushna Sahoo[1], Anne Gershenson[2] and Lila M. Gierasch[1,3].
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A complete understanding of a protein-folding landscape requires detailed characterization of intermediates that are populated during a folding reaction. We are exploring the folding intermediates sampled by a 136-amino acid β-barrel protein, cellular retinoic acid-binding protein I (CRABP I). This model protein is made up of two five-stranded orthogonal β-sheets wrapped around a central ligand-binding cavity. Previous work has shown that the folding of CRABP I involves well-defined stages: An early intermediate forms in ca. 300 μs by hydrophobic collapse, next (~100 ms) an intermediate is populated that has native topology including the ligand-binding cavity, and lastly, in ca. 1 s, interstrand hydrogen bonds form and native packing of side chains develops. The nature of the intermediates is relatively poorly understood, including structural details, compactness, and the size of the intermediate ensembles. To address these questions, we have designed CRABP I mutants with solvent-accessible Cys residues (M1C, S55C, N64C, K106C, and D103C) suitable for attachment of thiol-reactive fluorophores, as well as transglutaminase (TGase) tags at their C-termini for enzymatically mediated labeling with a second fluorophore. The Cys residues have been labeled with HyLite488 or BODIPY-FL (donor), and the TGase tags have been labeled with tetramethylrhodamine (acceptor). As an additional strategy to deduce the nature of the folding intermediates, we are varying solvent conditions using salts that differentially affect species stabilized by hydrophobic, electrostatic, or hydrogen bonding interactions. Taken together, ensemble and single-molecule FRET studies of doubly-labeled variants are providing a increasingly detailed picture of the CRABP I folding landscape. *[Supported by NIH grant OD000945]*

**2004-Plat**
**Measurement of Single Molecule Folding/unfolding Trajectories**
**Hoi Sung Chung**, John M. Louis, William A. Eaton.
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We have measured folding/unfolding trajectories of single protein G (B1 domain) molecules, a simple two-state folder, by simultaneously measuring the fluorescence intensity, lifetime, and spectrum at various concentrations of denaturant. Protein molecules were labeled by a fluorescence resonance energy transfer (FRET) pair, Alexa Fluor 488 and Alexa Fluor 594 and were immobilized on a glass surface coated with polyethyleneglycol via streptavidin-biotin linkage. The vast majority of molecules (~ 85%) exhibits simple two-state trajectories, with either high or low values of the FRET efficiency, corresponding to the folded and unfolded states, respectively, with unresolvable jumps between them. About 10% of the trajectories show transitions in the unfolded state that can be attributed to a ~20 nm spectral shift of the donor, as revealed by measurements of their emission spectra. The mean FRET efficiency of immobilized molecules matches the value measured in free diffusion experiments. There is a distribution of these values beyond the width expected from shot noise, which can, however, be quantitatively accounted for by the distribution of acceptor lifetimes. In spite of these complications from photophysics, rate coefficients obtained from the exponential distribution of residence times in either the folded or unfolded state yield relaxation times that agree within a factor of 2 with those measured on the dye-labeled protein by stopped flow kinetics. In addition, no correlation is observed between the donor and acceptor intensity in the unfolded state from microseconds to seconds suggesting that structural averaging between unfolded conformations occurs on the nanosecond timescale, as expected from previous measurements by B. Schuler and coworkers (*PNAS*:104,2655,2007). All these results indicate that we have successfully