

to actin, whereas fast myosins such as muscle myosin-2 and some class-1 myosin members have a low duty ratio (< 0.1). The members of the respective groups are assumed to use different ways to couple conformational changes at the nucleotide binding regions to changes that occur at the actin binding sites. Conserved active-site elements termed switch-1 and switch-2 play a major role in the coupling mechanism; however, whether and to which extent small variations in the sequence of switch-1 and switch-2 affect the duty ratio of a given myosin, thus determining its ability for processive movement, fast contractility or tension bearing remains unresolved. Based on structural considerations and confirmed by mutational analyses, we identified key residues in the nucleotide binding pocket that are responsible for making ADP dissociation kinetics dependent on the concentration of free magnesium ions. The exchange of a single amino acid in switch-2 affected the motor properties of all myosins tested, but also transformed low duty ratio motors into high duty ratio and vice versa. In addition, x-ray structural analyses and molecular modeling allowed us to relate the observed changes to altered coupling between the active sites and actin binding regions. These results, together with our cell biological studies demonstrating for the first time that magnesium ions have a regulatory role on motor protein function in vivo reveal that, switch-2 can act as magnesium sensor critically determining the mechanochemical properties of myosins.

22-Subg

From Single Molecule Fluctuation to Muscle Contraction: A Brownian Model of A.F. Huxley's Hypotheses

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Adaptive force generation of muscle in response to external stimuli is the result of thermally fluctuating, cyclical interactions between myosin and actin, which together form the actomyosin complex. Normally, these fluctuations are modelled using transition rate functions that are based on muscle fiber behaviour, in a phenomenological fashion (Huxley, 1957; Huxley & Simmons, 1971). However, such a basis reduces the predictive power of these models. As an alternative, we propose a model which uses direct single molecule observations of actomyosin fluctuations (Kitamura, et al. Nature 1999, BIOPHYSICS, 2005; Iwaki et al. Nat. Chem Biol. 2009). We precisely estimate the actomyosin potential bias and use diffusion theory to obtain a Brownian ratchet model that reproduces the complete cross-bridge cycle. The model is validated by simulating several macroscopic experimental conditions, while its interpretation is compatible with two different force-generating scenarios (Lorenzo & Yanagida, PLoS One, 2012).

23-Subg

Watching Individual DNA Helicases and Motor Proteins Behaving and Misbehaving

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We can now watch individual proteins acting on single molecules of DNA. Using these approaches, we have been imaging the translocation of motor proteins, such as the ssDNA translocases and DNA helicases, RecBCD, RecQ, and Sgs1, and the dsDNA translocases and chromatin-remodeling enzymes, Rad54 and Tid1 proteins.

The RecBCD enzyme comprises two motor subunits: RecB, a 3' → 5' SF1-helicase, and RecD, a 5' → 3' SF1-helicase. Although the mean unwinding rate of individual RecBCD enzyme molecules is close to ensemble measurements, the rates of individual enzyme molecules vary widely. Individual RecBCD molecules unwind DNA at constant rates, suggesting static, not dynamic, disorder. This apparent conformational heterogeneity is static on the experimental time scale of DNA unwinding. We discovered that transiently halting a single enzyme-DNA complex changes the rates of the RecBCD molecule. We will demonstrate that the behavior of individual RecBCD enzymes manifests a basic tenet of the ergodic hypothesis, suggesting that ligand binding kinetically traps a single conformer that is capable of redistributing to all other states within the population of molecules at equilibrium.

Using TIRF microscopy, the helicase activity of RecQ was visualized on single molecules of DNA using a fluorescent ssDNA sensor. By monitoring the formation and progression of individual unwinding forks, we observe that both the frequency of initiation and rate of unwinding are highly dependent on RecQ concentration. We establish that unwinding forks can initiate internally by melting into dsDNA and can proceed in both directions. The findings suggest that initiation requires a RecQ dimer, and continued unwinding involves the repeated cooperative action of multiple monomers at the DNA unwinding fork. We propose a distinctive model wherein RecQ melts dsDNA internally to initiate unwinding, and subsequently unwinds DNA as a dynamic assembly of proteins cooperating at the fork.

24-Subg

Kinesin-14: A League of their Own

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Kinesin-14 represents a subfamily of kinesins that are nonprocessive, promote microtubule (MT) minus-end-directed force generation, and contain C-terminal motor domains that are dimerized through an N-terminal coiled-coil. Unlike the well-known N-terminal motor domain kinesins that use an asymmetric hand-over-hand mechanism for MT plus-end-directed processive stepping, Kinesin-14s use a MT minus-end-directed powerstroke to generate force to crosslink and slide one MT relative to another. While most Kinesin-14s are homodimers like *Drosophila* Ncd, *S. cerevisiae* Kar3Vik1 is a heterodimer. The C-terminal domain of Vik1 exhibits the structural fold of a kinesin motor domain, binds MTs independent of Kar3, yet lacks a nucleotide-binding site. Furthermore, Kar3Vik1 binds across adjacent MT protofilaments, a non-canonical MT binding configuration. The results indicate that Kar3Vik1 collides with the MT through Vik1, promoting MT binding by Kar3. The tight binding of Kar3 destabilizes the Vik1 interaction with the MT, positioning Kar3Vik1 for the start of the powerstroke. Rapid ATP binding to Kar3 is associated with the rotation of the coiled-coil stalk, and post-powerstroke ATP hydrolysis is independent of Vik1 providing additional evidence that Vik1 rotates with the coiled-coil during the powerstroke. Detachment of Kar3Vik1 from the MT completes the cycle and allows the motor to return to its initial conformation. Supported by NIH GM54141.

Subgroup: Nanoscale Biophysics

25-Subg

Single Molecule Enzymology

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We use single molecule force-clamp techniques to study the activity and chemical mechanisms of oxidases and oxidoreductase enzymes. In this approach the length of an extending protein is measured while the pulling force is actively kept constant with a feedback loop. Using the force-clamp technique we have investigated the force-dependency of protein folding, unfolding and of chemical reactions. For example, using various types of force pulses we can drive a single substrate protein to well defined extended states and monitor the reduction of its disulfide bonds by thioredoxin enzymes, with sub-Angstrom resolution (1). By varying the pulling force on the substrate we can also identify different chemical mechanisms of reduction. Using maximum likelihood techniques we have resuscitated ancient thioredoxin enzymes, going back billions of years. We have studied the activity of our resuscitated enzymes using our single molecule techniques in an effort to observe the evolution of the chemical mechanisms of reduction over time (2). Finally, we have used these single molecule techniques to study the activity of oxidases such as PDI and DsbA, and of glutaredoxin (3). Our results demonstrate that single molecule force-spectroscopy techniques provide an entirely novel and powerful approach to study enzymes, at an unprecedented resolution. These experiments show that force-clamp AFM probes dynamic rearrangements within an enzyme's active site, which cannot be resolved by any other current structural biological technique. We anticipate that these studies will be extended to a wide range of other enzymes.

1. Wiita et al, (2007) Probing the chemistry of thioredoxin catalysis with force. *Nature*, 450:124-7.

2. Perez-Jimenez, et al, (2011). Single-molecule paleoenzymology probes the chemistry of resurrected enzymes. *Nat Struct Mol Biol*, 18(5): 592-596.

3. Kosuri, P et al, (2012). Protein folding drives disulfide formation. *Cell*, in press

26-Subg

Dynamics of the Ribosomal Subunit Interface during tRNA Translocation at Near-Atomic Resolution

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During protein synthesis, movement of the transfer RNAs during translocation involves large structural motions of both the ribosome and the tRNAs. In particular, large scale collective inter-subunit rotations occur. Despite this rotation, the two ribosomal subunits remain bound during translocation via contact patches on the surface of the subunits. Combining crystal and cryo-EM structures with all-atom explicit solvent molecular dynamics simulations, we have characterised intermediate states and conformational motions of spontaneous tRNA translocation at near-atomic resolution. Systematic analysis of the dynamics of all inter-subunit contact patches suggest mechanisms by which the subunits communicate, and how the two subunits maintain their fine-tuned

affinity despite drastic conformational changes. The results are important for understanding the fundamental principles and underlying forces that generate movement within molecular machines.

27-Subg

New Insight into Lipid-Protein Membrane Organization and its Functionality with Super-Resolution STED Microscopy

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Stimulated Emission Depletion (STED) far-field microscopy allows the study of living cells with nanoscale resolution, otherwise impeded by the limited spatial resolution of conventional microscopes. Besides the recording of images, the combination of STED with single-molecule sensitive spectroscopic tools such as Fluorescence Correlation Spectroscopy (FCS) discloses complex dynamical processes hidden to the conventional observations. For example, STED-FCS offers novel insights into important cellular processes, such as lipid-lipid, lipid-protein interactions or the formation of so-called "lipid-rafts" in the cellular plasma membrane, and their role in cellular functionality. Improved insights are realized by the implementation of gated detection or by recording STED-FCS data during scanning.

28-Subg

Proteins as Mechano-Chemical Signalling Switches

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Since the physical and biochemical properties of extracellular matrix provide critical cues to bacteria and cells, from mechanoregulated bacterial adhesion to angiogenesis, and finally to the differentiation of stem cells, it is of major importance to gain mechanistic insights into how mechanical stretching of extracellular matrix molecules can alter various cell functions. While investigating these three distinct physiological processes, common motifs are emerging how bacteria and cells take advantage of mechanical forces to regulate the function of proteins by stretching them out of their equilibrium structures. In this context, new assays and techniques were developed that allow probing how the stretching of proteins alters their structure-function relationships. Taken together, new insights into various underpinning mechanotransduction events are emerging how mechanical cues are translated into biochemical signals that ultimately regulate bacterial adhesion and various cellular processes.

Subgroup: Intrinsically Disordered Proteins

29-Subg

Structural Studies on the Activation and Substrate Binding of a Conditionally Disordered Acid-Activated Chaperone

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HdeA is a 9.7kDa chaperone that is inactive at neutral pH but becomes activated and disordered by shifts to low pH. The chaperone is required for bacterial pathogens to resist the acid-mediated protein aggregation that would otherwise occur in the human gut. We sought to identify which of HdeA's acid-titratable residues were the key players in its activation. Using constant pH molecular dynamics calculations and site-specific mutagenesis, we identified several residues involved in HdeA activation and have isolated variants that are destabilized and constitutively active at even neutral pH. These mutants help us to understand how pH-driven changes in HdeA flexibility drive activation.

One of the pressing problems in chaperone biology is how chaperones interact with a multitude of client proteins to facilitate their folding. HdeA's size and accessibility to NMR also makes it ideal for monitoring the structural changes that take place in this chaperone upon activation and upon client binding. Our predicted NMR structure of HdeA at neutral pH is a folded protein and very similar the crystal structures previously solved for the HdeA dimer. Upon shift to low pH however, HdeA simultaneously becomes active as a chaperone and acquires a largely disordered conformation. We have obtained structural information that gives us insights into HdeA's activation process and client binding. The use of small NMR accessible client proteins raises the exciting possibility of monitoring the structural changes that take place within the client upon interaction with the chaperone and in doing so gaining insight into the fundamental question of what chaperones do to their clients to facilitate folding.

30-Subg

Alpha-Synuclein, an Intrinsically Unstructured Protein. How Interesting Can It Be?

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N-terminal acetylation of alpha-synuclein (aS), a 140-residue protein implicated in the etiology of Parkinson's disease, is common in mammals. The impact of this modification on the protein's structure and dynamics in free solution and on its membrane binding properties has been evaluated by both NMR and CD spectroscopy. While in contrast to literature reports, no tetrameric form of acetylated aS could be isolated, N-terminal acetylation resulted in ca 15% transient population of alpha-helical structure for its first six residues. The ¹H, ¹⁵N, and ¹³C chemical shifts for residues 13-140 remain unaffected by acetylation. Nevertheless, a substantial increase in affinity of aS for negatively charged lipid membranes is observed, likely to be of strong functional significance. A new method for residue-specific NMR probing of lipid binding is demonstrated for aS and assigns a new putative function to this enigmatic protein. Although free aS in the absence of lipids has backbone chemical shifts that are exceptionally close to random coil values, considerable positional variation for the distribution of its backbone torsion angles and the time scale of local reorientation can be deduced from its 3JHH and NOE data.

31-Subg

Intrinsic Protein Disorder in the Regulation of Large Molecular Machines

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We here report the molecular overlap of the linkage of three essential protein complexes that coordinate the formation of the mitotic spindle. These proteins are dynein, a large motor complex that moves machinery inside cells, and two of its regulators: a protein complex called dynactin, that is a dynein activator, and a protein called NudE whose depletion in mice produces a small brain and mental retardation. What is intriguing about the dynein/dynactin/NudE interplay is that dynactin and NudE bind to a common segment of dynein that is intrinsically disordered but with distinct binding modes. Elucidating details of these distinct modes explains how one regulator is selected over the other even when both are present in the same cellular compartment. Using NMR spectroscopy and isothermal titration calorimetry we show that intrinsic disorder in a specific segment of dynein intermediate chain promotes local modifications like phosphorylation and splicing, promotes rapid equilibration of ensemble components in solution samples of complexes with moderate to weak binding affinities, and creates a bi-segmental binding site such that residue-level modification in and near one segment have minimal effect on the structure of the second segment thereby aiding in segregating of functions between two consecutive segments. These results underscore the role of disorder in the versatility of dynein binding to different regulators, and have far reaching impact not only on our understanding of processes essential for formation and orientation of the spindle, but also offer a novel role for protein disorder in controlling cellular processes, and highlight the advantages of NMR spectroscopy in elucidating atomic level characterization of extremely complex dynamic cellular assemblies.

32-Subg

Probing the Polymeric Properties of Unfolded and Disordered Proteins with Single-Molecule Spectroscopy

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Single-molecule spectroscopy provides new opportunities for investigating the structure and dynamics of unfolded and intrinsically disordered proteins (IDPs). The combination of single-molecule Förster resonance energy transfer (FRET) with nanosecond correlation spectroscopy, microfluidic mixing, and related methods can be used to probe their distance distributions and reconfiguration dynamics on a wide range of time scales, and even in heterogeneous environments. In view of the large structural heterogeneity of these systems, a description in terms of polymer physical principles is often a useful way of conceptualizing their behavior. I will provide examples ranging from the influence of amino acid composition and temperature on the structure and dynamics of unfolded proteins and IDPs to the effects of crowding and molecular chaperones.

33-Subg

A Long Disordered Linker in Nuclear Transport of Membrane Proteins

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Nuclear Pore Complexes (NPCs) embedded in the nuclear envelope allow selective transport of macromolecules between the cytosol and nucleoplasm. Transport factors shuttle cargo through the NPCs by interacting with the disordered proteins that encode the binding sites for the transport factors, the