used to quantify the stoichiometry of protein association and to determine the number of proteins in a complex. In protein hetero-dimerization studies, it is highly desirable to be able to control protein co-expression fractions. The expression of each protein should be selected in order to maximize the likelihood of protein association. Yet protein expression is the output factor from a lipid-mediated transfection process in which only the plasmid DNA (input parameter) can be explicitly controlled. In this work we develop and test a working model which relates the protein expression fraction of two proteins to the plasmid DNA mixing fraction. Experimentally, we express two differently colored fluorescent proteins in CV-1 cells and split their emitted signal into two different detection channels based on color. The intensity fraction from the two channels is used to determine the relative amount of expressed protein in the cell. The experiments show that the DNA mixing fraction determines the average protein expression fraction. We extend this work by examining the cell-to-cell variation in protein expression and constructing a simple model which relates the expression variation to the average number of active plasmids. This study provides insight into the fundamentals of lipid-mediated transfection and demonstrates that we can control protein co-expression fractions through selection of plasmid DNA mixing fractions. This work is supported by NIH grant R01GM064589.

Molecular Mechanics & Force Spectroscopy II

3908-Pos

DNA Stretching Kinetics and Entropic Bottlenecks

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The use of a force clamp protocol during single molecule measurements of the DNA over-stretching transition by means of optical tweezers allow to experimentally access the kinetic of both DNA elongation and relaxation. The data are interpreted by means of a phenomenological two state model which allows to measure the cooperativity of the process. The comparison between experiments performed at different temperatures highlights the entropic nature of the free energy barrier that separates the compact and extended states of DNA. Insights on the structure of the intermediate state are provided.

3909-Pos

Multiple Binding Modes of Actinomycin D Reveal the Basis for its Potent HIV-1 and Cancer Activity

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Actinomycin D (ActD) is a well studied anti-cancer agent that is used as a prototype for developing new generations of drugs. However, the biophysical basis of its activity is still unclear. Because ActD is known to intercalate double stranded DNA (dsDNA), it was assumed to block replication by stabilizing dsDNA in front of the replication fork. However, recent studies have shown that ActD binds with even higher affinity to imperfect duplexes and some sequences of single stranded DNA (ssDNA). These features suggest that ActD may alternatively destabilize complementary dsDNA. In this work we use optical tweezers to stretch and relax single dsDNA molecules in the presence of varying ActD concentrations. We observe that ActD binds with highest affinity to two separate DNA strands that are connected by ActD. This binding mode is ~1000-fold stronger than ActD's intercalation into dsDNA. We are able to characterize at least two classes of ActD-ssDNA binding sites that differ in dissociation times (~10% of sites with ~1000 sec off time, and the rest with ~10 sec off time). The much weaker ActD binding to dsDNA relative to ssDNA leads to duplex destabilization, in contrast to conventional intercalation. At saturation, the ActD-dsDNA complex becomes indistinguishable from the saturated ActD-ssDNA. These results suggest that two separate, anti-parallel DNA strands constitute the highest affinity natural substrate for ActD binding, with K_d ~10-100 nM and a relatively slow off rate. This finding supports the hypothesis that the primary characteristic of ActD that contributes to its biological activity is its ability to inhibit cellular replication by stabilizing DNA bubbles during RNA transcription, thereby stalling the transcription process.

3910-Pos

Atomistic Simulation of Estrogen Receptor-Coactivator Peptide Complexes to Identify Specific Binding Sites

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Estrogen Receptors (ER) can lead to gene transcriptions that are found responsible for certain type of cancers including 70% of breast cancers. However, the process of gene transcription is preceded by the binding of an estrogenic ligand to the ER and then binding of this liganded ER with an activator protein which mediates the signal to the DNA. There are antiestrogenic compounds that can bind to the ER and block the binding of any coactivator protein to the liganded ER. However, no single antiestrogenic compound is found to work in all tissues - that means it cannot always block the binding of the coactivator protein. A successful drug molecule need to inhibit the liganded ER in all tissues. This needs a detail atomic level understanding of the interaction pattern of the liganded ER with coactivator protein. We investigate liganded ER interaction with small peptides to identify specific binding sites. Details of our investigation will be reported at the meeting. Acknowledgements: Authors acknowledge financial support from the National Institutes of Health (grant number 5P20MD002725-03-0002), through the NCMHD -RIMI program.

3911-Pos

Sources of Heterogeneity in the Forced Unfolding Pathway of Streptokinase Beta Revealed through High-Temperature Steered MD Simulations

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Force-pulling experiments on the unfolding of mechanical and non-mechanical protein domains have greatly increased our understanding of the structural stability of proteins. Because these experiments are done on the single molecule level, they also enable experimentalists to observe differences in the unfolding behavior of individual molecules. However, it is difficult to determine the source of unfolding heterogeneity through experiments alone. We present here evidence from experiments and simulations that the ß domain of Streptokinase, a non-mechanical protein, unfolds under force via three distinct pathways. High temperature SMD simulations were used to determine the source of the velocity-dependent heterogeneity observed in AFM force puling experiments. We show that hydrophobic interactions in the core of the protein underlie the differences observed in experiments and contribute significantly to the structural stability of the protein under force. Using an expansion of the Jarzynski equality[12], we calculate free energy surfaces to describe the energetics of the different pathways.

[1] C. Jarzynski, PRL **78**, 2690-2693 (1997)
[2] D. Minh, J. Phys. Chem. B. **111**, 4137-4140 (2007)

3912-Pos

Exploring the Function of Titin Kinase by Mechanical Single-Molecule Pump-And-Probe

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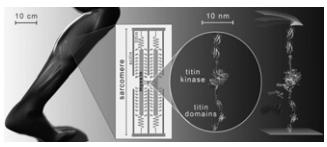
Protein function like catalytic activity or molecular recognition is tightly coupled to conformation and its dynamics. Since protein conformation can be controlled by forces, diverse mechanisms evolved allowing biological systems to respond to mechanical strain. Recently, it was shown in combination with single-molecule force spectroscopy, MD-simulations and enzymatics that titin kinase acts as a force sensor regulating muscle gene expression and protein turnover [1]. However, there is no experimental access to investigate which force-induced conformation during the activation pathway is competent for ATP-binding. Here we develop a new AFM-based single-molecule pump-and-probe protocol to mechanically prepare a predefined conformation and to read out afterwards whether ATP bound. We show that ATP only binds to the conformation prepared after barrier two. Therefore, titin kinase exhibits a dual mechanical autoinhibition, which cannot be overcome by thermal fluctuations but by physiological forces acting on the M-band structure of the muscle sarcomere. This single-molecule approach might also become useful for the investigation of other conformation controlled processes such as hidden binding pockets, catch bonds or motor proteins.



[1] Puchner, E.M. et al. Mechanoenzymatics of titin kinase.Proc Natl Acad Sci U S A, 2008. 105(36): p. 13385-90.

3913-Pos

Single Molecule Study of the Motion of Matrix Metalloprotease MMP1 on Type I Collagen Fiber Shows Proteolysis Driven Hindered Biased Diffusion

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Diffusion plays an important role in many biological processes. Using single molecule fluorescence techniques, we have studied the diffusive motion of

matrix metalloprotease MMP1 on Type I collagen fiber from rat tail. Collagen is the most abundant protein in humans. Fibril forming Type I collagen is the main component of the extracellular matrix (ECM) that supports and defines most tissues. Degradation of collagen in the ECM by matrix metalloproteases (MMPs) is an important process in tissue remodeling. By tracking single fluorescently labeled MMPs moving on a collagen substrate, we were able to characterize the diffusive motion with high temporal resolution. These measurements suggest that proteolytic cleavage of the collagen substrate by Wild Type MMP1 (WT MMP1) both biases and hinders the diffusive motion of MMP1 on the collagen fiber. Both bias and hindrance are temperature dependent for WT MMP1. The diffusion was neither hindered nor biased for a point-mutant MMP1 and for MMP9, both of which are incapable of cleaving native Type I collagen from rat tail. To separate the effects of hindrance and bias, we specifically created hindrance by incubating collagen with WT MMP1 prior to measuring the motion of a catalytically inactive mutant MMP1. The resulting nonlinear diffusion of mutant MMP1 was characteristic of diffusion in hindered space. These results provide insight into the process of collagen degradation.

3914-Pos

High Frequency Asynchronous Rotation of Magnetic Microspheres and Biophysical Applications - Higher Sensitivity Regime for Magnetic Bead Biosensors

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Asynchronous rotation is an emerging platform technique with applications ranging from micro mixing to femtoliter viscometry and biophysical sensors (Applied Physics Letters 91, 224105 (2007)). Asynchronous rotation occurs when a driving magnetic field exceeds a critical frequency, above which the driving field is rotating faster than the driven body. The critical frequency depends on the viscosity of the fluid, size of the driven system, and the strength and quality of the driving field. The dynamics of a magnetic bead rotating at high frequencies were studied using a simple setup, consisting of a bright field microscope that was used to focus a 5 mW laser onto the particle of interest. The rotational frequency of the particle was measured by analyzing the intensity modulation of the laser beam, focused through the particle. Previously reported asynchronous rotation frequencies of magnetic beads range from 100 mHz to 12 Hz. Here, we report a system with an order of magnitude higher asynchronous rotation frequency, in water. The mixing efficiency and sensor sensitivity depend on the rotational frequency of the microsphere, among other factors. Higher rotational frequencies enable increased sensitivity of drag-based sensors, and also open up new capabilities for the method, such as novel force spectroscopy studies.

3915-Pos

Measurement of the Elastic Modulus of Individual Type I Collagen Fibrils

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The primary focus of this work was to measure the mechanical properties of hydrated individual type I collagen fibrils using atomic force microscopy (AFM) techniques. In particular, we are investigating anisotropy in the elastic modulus of these fibrils. Radial measurements were performed by compressing the fibril between the tip of an AFM cantilever and the flat substrate on which the fibril was supported. The elastic modulus was extracted from this data using a Hertzian analysis. To investigate the axial properties, a three-point bending technique was used. The fibril was suspended between adhesive supports and deflected centrally by the AFM tip. Here, the bending modulus was found by fitting the data to the equation describing the bending of an elastic beam affixed and supported at each end. Both the radial and the axial experiments were performed on fibrils assembled from pepsin digested collagen monomers, and the results were compared with similar measurements perform on intact, native fibrils. The outcomes are of interest to those investigating extracellular matrix mechanics, especially with application to cell differentiation and tissue engineering.

3916-Pos

How Do Osmolytes affect the Stability of Polycystin-1 PKD Domains?

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Autosomal dominant polycystic kidney disease is one of the most common life-threatening genetic diseases, and is a leading cause of renal failure. The majority of cases are caused by mutations in the PKD1 gene, which encodes for polycystin-1 (PC1). PC1 is a large membrane protein that has a long N-terminal extracellular region (about 3000 aa) with a multimodular structure including sixteen Ig-like PKD domains. PC1's extracellular domain may function as a mechanical antenna that senses mechanical cues such as shear flow converting them into signaling processes that control cell differentiation and growth. PC1 is expressed along the renal tubule, where it is exposed to a wide range of concentrations of urea (from 5mM in the proximal tubule to up to ~800mM in the collecting duct). Urea is known to destabilize proteins. Other osmolytes found in the kidney such as sarcosine, betaine and trimethylamine N-oxide (TMAO) are known to counteract urea's negative effects on proteins. Here we used nano-mechanical techniques to study the effects of osmolytes on the biophysical properties of PC1's PKD wild-type and mutant domains. Upon increasing the concentration of urea we observe a systematic decrease in the mechanical stability. We also found that the refolding rate constant is slow down by urea (as much as ~5-fold at 2M). Moreover, we found that stabilizing osmolytes can effectively counteract the effect of urea at a ratio of 1:1 (urea/sarcosine) or 1:0.5 (urea/TMAO). We recently reported that some pathogenic missense mutations can significantly destabilize PKD domains. Interestingly, we found that stabilizing osmolytes increase the mechanical strength of a mutant domain. Our studies have the potential to provide new therapeutic approaches (*e.g.* through the use of osmolytes or chemical chaperones) for rescuing destabilized and misfolded mutant PKD domains.

3917-Pos

Real Time Detection of Mechanical Stress in Specific Cytoskeletal Proteins

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A molecular force sensor cassette (stFRET) was incorporated into actinin, filamin, and spectrin in endothelial cells and into collagen-19 in C. elegans. In vitro double strand DNA stretching essay confirmed the force sensitivity of stFRET. stFRET detected constitutive stress in all the proteins. In endothelial cells, the stress in actinin, filamin and spectrin could be eliminated by releasing focal attachments from the substrate. Our data also indicated the highest resting strain in spectrin in three cytoskeleton protein tested. When the intact C. elegans worm was reversibly stretched using micromanipulators, stFRET reversibly sensed the force in collagen-19. stFRET is a general purpose dynamic sensor of mechanical stress in filamentous proteins that can be expressed in cells in vitro and in whole animals.

We have developed another force sensor, named sstFRET, in which we substituted the alpha helix linker with a spectrin repeat domain. Our in vitro DNA stretching assay showed that 20 pN force is sufficient to deform the spectrin repeat linker, leading to a dramatic change of FRET. By inserting sstFRET into alpha-actinin, we succeeded in monitoring the change in actinin constitutive stress before and after cells are osmotically challenged. In addition, we observe substantially lower force loads on actinin in bovine aortic endothelial cells (BAEC) than that in human embryo kidney (HEK) cells.

3918-Pos

Unfolding and Refolding Dynamics of Filamin a Protein under Constant Forces

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Filamin A is an important cytoskelton cross-linking protein containing 24 immunogloble domains. Force spectroscopy of Filamin A has been studied by AFM. We use a new technology to study the response of Fimalin A to stretching force. When we apply a constant force ~50 pN to unfold a construct that is composed of 1-8 domains, distinct and nearly equal unfolding steps are observed (Fig. 1). When decreasing force to ~5 pN, refolding events of several previously unfolded domains is observed (Fig. 2). At the same time, from the force extension curve of unfolded proteins, the persistence length of polypeptide is estimated to be ~0.6 nm.