force-extension curves qualitatively match those from recent AFM pulling experiments on CTPR by Chiba, et al. We will extend these studies to include larger numbers of repeats and measure ensembles of pulling trajectories to capture the statistics of the unfolding events.

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Dodging The Crisis Of Folding Proteins With Knots

Joanna I. Sulkowska[1], Piotr Sulkowski[2], Jose Onuchic[1].
[1]The Center for Theoretical Biological Physics, San Diego, CA, USA, [2]Physikalisches Institut der Universitat Bonn and BCTP, Bonn, Germany.

We prove that proteins with nontrivial topology, containing knots and slipknots, have the ability to fold to their native states without any additional external forces invoked. Our studies are based on a simple coarse-grained model with interactions modeled only by the native contacts. We demonstrate that folding of knotted proteins YibK and YbeA proceeds through an intermediate configuration with a slipknot. Analysis of topological barriers and backtracking associated to these trajectories reveals to which extent various native contacts are responsible for a folding process. From this we conclude how to modify their strength to get more realistic model, with a higher ratio of properly folded structures. We also discuss how folding properties of knotted proteins are affected when additional chains are attached to one or both termini, and when protein does not have knot topology.

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Continuous Dissolution Of Structure During The Unfolding Of A Small Protein

Santosh Kumar Jha[1], Deepak Dhar[2], G. Krishnamoorthy[3], Jayant B. Udgaonkar[1].
[1]National Centre for Biological Sciences (TIFR), Bangalore, India, Bangalore, India, [2]Department of Theoretical Physics, Tata Institute of Fundamental Research, Mumbai, India, Mumbai, India, [3]Department of Chemical Sciences, Tata Institute of Fundamental Research, Mumbai, India, Mumbai, India.

An unresolved question in the field of protein folding is whether a protein unfolds in a two-state (N↔U) cooperative manner with only two species being populated during the entire unfolding reaction or in a non-cooperative fashion with a continuum of intermediate forms being populated. To make a definitive distinction between the two has been a difficult challenge, because of the difficulty in identifying and quantifying populations of different species present together during the unfolding reaction.

Time-resolved fluorescence resonance energy transfer (TR-FRET) method can differentiate and measure selectively the populations of N, U and I forms, if present together. In this method, energy transfer efficiency is estimated by collecting the decays of fluorescence intensity of the donor fluorophore in the presence or absence of an acceptor. When such fluorescence intensity decays are analyzed by the maximum entropy method (MEM), distributions of fluorescence lifetimes are obtained, which can be used to generate a distribution of distances between the donor and acceptor.

In this study, a multi-site, TR-FRET methodology coupled to MEM analysis has been used, for the first time, to study the time evolution of the probability distributions of four intra-molecular distances in the small plant protein monellin, as it unfolds starting from the native state. Surprisingly, one distance is seen to increase completely in a gradual manner, while the increase in the other three distances appears to have both discrete and gradual components. Hence, the protein is found to sample many intermediate conformations, characterized by different intra-molecular distances, before unfolding completely. The observed data can be explained by a simple physical model based upon swelling of a homopolymer chain undergoing diffusive dynamics according to the Rouse model.

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Experimental Studies on Protein Folding in the Presence of the Hsp70 Chaperone System

Margarita Santiago, Ashok Sekhar, Silvia Cavagnero.
University of Wisconsin-Madison, Madison, WI, USA.

The Hsp70 chaperone system (including DnaK, DnaJ and GrpE in bacteria) plays a vital role in preventing aggregation and assisting protein folding. While a lot of the chaperone biochemistry has already been worked out, very little is know about the interaction of DnaK and DnaJ with substrates. So far, such interaction had only been probed with small peptide or protein substrates incapable of independent/efficient refolding. This paucity of information has precluded studies on the competition between inter-molecular binding and intra-molecular folding. Such competition is important for cell viability because the accessibility of DnaK to substrate binding is modulated by the intrinsic rates of substrate folding/unfolding and by the substrate's thermodynamic stability. We developed a computational kinetic model to predict the interaction of protein substrates exhibiting two-state folding behavior with DnaK, DnaJ and GrpE. We found that, under physiological conditions, only proteins that fold slowly and/or have moderate thermodynamic stability are predicted to bind chaperones during their folding cycle.

Experimental protein folding kinetics followed by stopped-flow in the presence and absence of the Hsp70 chaperone system shows good agreement with the predictions by the computational model. Furthermore, gel filtration and reverse phase chromatography data further support the stopped-flow results by providing evidence for DnaK-DnaJ-substrate interactions at equilibrium. This result is in agreement with the predictions of the computational model. In summary, the combination of experiments and computational predictions developed in this work is a powerful tool to help unveiling the relations between protein folding and chaperone binding.

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Folding Mechanism Of The Z Mutant Of Human Antitrypsin Studied By H/D Exchange

Tanusree Sengupta, Patrick L. Wintrode.
Case Western Reserve University, Cleveland, OH, USA.

The serpins are a unique family of serine protease inhibitors which possess an inherent ability to fold to their active metastable state and avoid thermodynamically more stable conformation. This metastability is crucial for their biological function as they undergo large conformational changes to the stable relaxed (R) state during protease inhibition. Though dramatic conformational mobility of serpins is required for their activity, this also renders them vulnerable to misfolding and polymerization. Misfolding and polymerization are observed in some genetic variants of plasma serpins such as antitrypsin, antichymotrysin and antithrombin III leading to a diverse range of diseases including cirrhosis, dementia, thrombosis, angioadema and emphysema. Studies on the Z variant of human α1 antitrypsin (α1-AT) suggested that extremely retarded folding traps the molecule in an intermediate conformation which has a high tendency to form polymeric aggregates. However, there is no specific report on the nature of the folding intermediate. In the present study we have tried to follow the refolding pathway of the Z variant of α1-AT by hydrogen/deuterium exchange mass spectrometry. Hydrogen/ deuterium exchange mass spectrometry is a powerful method in analyzing the folding/ unfolding of a protein in a region specific manner. We observed different refolding kinetics for different parts of the protein. For most of the regions only 25-30% refolding was observed even after 20 hours. Maximum refolding of 60% was observed for residues 120-142 containing helix E. These studies will shed new light on the nature of the polymerization prone folding intermediate of the Z variant.

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Urea H-bonds to the peptide group, but Gdm does not

Woon K. Lim[1], Jörg Rösgen[2], Walter Englander[1].
[1]University of Pennsylvania, Philadelphia, PA, USA, [2]University of Texas Medical Branch, Galveston, TX, USA.

Great attention has been devoted to studying protein conformational stability for about one century. Denaturants have played a prominent role in these investigations for decades, but it is still unclear what the exact mechanism of denaturant action on proteins is. On a more general level, denaturants have been found to preferentially interact with proteins, particularly with the peptide backbone. A prominent model proposes that urea accumulates at the backbone through H-bonding. Guanidine has been proposed to additionally engage in planar stacking interactions.

We test these models using hydrogen-deuterium exchange (HX). When denaturant H-bonds to a peptide group, HX is blocked. Thus, denaturant-dependent slowing of HX is a direct measure of peptide-denaturant H-bonding. We find that urea strongly H-bonds to the peptide group, evidenced by a conspicuous drop in HX rate. Guanidine, in contrast, barely reduces the HX rate. Thus guanidine is likely to denature proteins by an entirely different mechanism than urea. While the urea H-bonding is sufficient to account for its experimental preferential interaction with peptides, guanidine probably accumulates at the peptide group through stacking interactions.

420-Pos Board B299

The Effect of High Concentration Salt on the Structure, Stability, and Aggregation of RecA

William R. Cannon, Gina MacDonald.
James Madison University, Harrisonburg, VA, USA.

The *Escherichia coli* protein, RecA, is critical for maintaining genetic integrity. RecA catalyzes DNA pairing and strand exchange reactions that are utilized in

DNA recombination and DNA repair. Buffer and salt conditions influence the aggregation and activity of RecA. In low salt conditions, RecA is a DNA-dependent ATPase. However, prior research demonstrated that high salt concentrations allow RecA to hydrolyze ATP in the absence of DNA and at levels comparable to those obtained in the presence of DNA [Pugh, B. F. and Cox, M. M. (1988) *Journal of Biological Chemistry 263*, 76-83]. We have used circular dichroism (CD) and fluorescence spectroscopies to better understand the salt-induced effects on RecA structure and function. CD and fluorescence studies were performed in order to monitor the thermally induced unfolding of RecA in the presence of a variety of salts. We found that different salts had unique effects on RecA unfolding transitions and stability. Unfolding studies performed under salt conditions known to activate RecA's ATPase activity showed unique, thermally stable RecA structures. A comparison of the influences of different ions on RecA unfolding will be presented. These studies may help to elucidate how different ions influence RecA activity, structure, aggregation, and stability.

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Folding/Unfolding of Glycolipid Transfer Protein: Molten Globule-Like Intermediates?

Ravi Kanth Kamlekar[1], Roopa Kenoth[1], Helen M. Pike[1], Franklyn G. Prendergast[2], Sergei Yu. Venyaminov[2], **Rhoderick E. Brown[1]**.
[1]University of Minnesota, Austin, MN, USA, [2]Mayo Clinic College of Medicine, Rochester, MN, USA.

Glycolipid transfer proteins (GLTPs) are small, soluble, single-chain proteins (~24 kDa) that selectively accelerate the intermembrane transfer of glycolipids in vitro. The GLTP-fold is unique among lipid-binding proteins. However, little is known about GLTP stability and folding/-unfolding. During isolation of heterologously expressed GLTP, FPLC size exclusion chromatography showed peaks corresponding to monomer, multimer, and a third peak of intermediate elution volume. Unexpectedly, native gel electrophoresis showed that the intermediate protein peak migrated as monomer rather than dimer, raising the possibility of a molten globule-like state. Intrinsic GLTP tryptophan fluorescence showed a blue-shifted (~2nm) emission wavelength maximum (λmax), indicating an altered tryptophan environment compared to monomer. ANS binding resulted in a large blue shift (~20nm) in λmax and dramatically enhanced emission intensity (~120%). Far-UV-CD showed retention of ordered secondary structure (>95%), but substantially reduced cooperativity during thermally-induced melting. Near-UV-CD analysis of induced optical activity of GLTP Trp/Tyr residues was insufficient to establish tertiary folding changes. To further evaluate GLTP unfolding intermediates, the effect of urea was studied. Trp emission changes suggested a two-step unfolding pathway involving intermediate formation at 4M urea and characterized by blue-shifted Trp emission. Additional urea induced further unfolding marked by red-shifted Trp emission. Far-UV-CD analyses of the 4M urea-induced intermediate indicated reduced ordered secondary structure and cooperative melting at lower temperature compared to native GLTP, but the near-UV-CD signal did not provide definitive insights into tertiary folding status. ANS binding showed 1nm blue shift and 60% increase in fluorescence intensity compared to untreated GLTP. Conditions are identified under which GLTP may exist in molten globule-like and other partially unfolded states. Studies on the significance of these intermediates with respect to function are underway. [Support: NIH/NIGMS GM45928 & GM34847, NIH/NCI CA121493, The Hormel & Mayo Foundations]

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Elucidating The Specificity Determinants Responsible For ClpX-Adaptor Interaction

Tahmeena Chowdhury, Peter Chien, Robert T. Sauer, Tania A. Baker.
MIT, Cambridge, MA, USA.

The ClpXP proteolytic machinery in bacteria consists of the AAA+ ATPase protein ClpX and the serine peptidase ClpP. ClpX recognizes, unfolds, and translocates substrates into the protease chamber where hydrolysis of the polypeptide occurs. To control substrate-specificity, the cell uses adaptor proteins, such as SspB, to modulate substrate selection. The N-terminal domain of ClpX is the adaptor-docking site and also directly recognizes some substrates. However, very little is known about the sequence specificity of this important ClpX N-domain protein-binding site.

Recently, a homolog of *Escherichia coli* SspB was identified in *Caulobacter crescentus*. Despite structural and functional homology, there is limited sequence similarity between the two SspB adaptors. To learn more about ClpX-adaptor interaction specificity, we carried out a systemic mutational analysis of *C.crescentus* SspB to identify the residues responsible for its interaction with ClpX. Functional assays monitoring adaptor-stimulated degradation of a model fluorescent substrate (GFP with a degradation signal) were used to assess SspB variants. In addition, monitoring direct-binding between the ClpX N-domain and variant fluoresceinated-SspB peptides by fluorescence anisotropy provided a quantitative assay for the interaction. Results reveal that a minimal C-terminal region (residues: ^{152}KIVSLDQFRKK^{162}) of SspB is responsible for docking with ClpX, and at least 5 specific residues (I^{153}, V^{154}, L^{156}, R^{160}, K^{162}) within this region play key roles in the interaction. Additional residues (F^{159}, K^{161}) may also be contributing albeit more subtly. This binding region is much longer and shares little sequence homology with the *E.coli* SspB ClpX-binding region (^{161}LRVVK^{165}). However, *C.crescentus* SspB binds *E. coli* ClpX and can clearly deliver substrates to it. This cross-species interaction demonstrates the sequence-versatility of the ClpX N-domain interactions in a functionally relevant manner and highlights the challenge in identifying ''consensus sequences'' for AAA+ protease interaction signals.

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The Effect of Salts and Co-Solvents on the Cytochrome c Folding Pathway within a Sol-gel Glass

Eric S. Peterson, Michael A. Erbele, Brock B. Jas.
Concordia College, Moorhead, MN, USA.

The folding reaction of ferric cytochrome c (cyt c) was examined in the presence of several salts and co-solvents in both solution and within porous silica sol-gel glasses to characterize the conformational changes that cyt c can undergo if constrained to remain in a compact state. The sol-gel pores restrict protein motions to a volume slightly larger than the native state. Unfolding was induced by changing pH and addition of guanidine. The populations of four species characterized by their heme ligation were determined using UV/VIS absorption spectroscopy: the native HM state (His18/Met80), the partially folded HW (His18/water) and HH (His18/His33) intermediates, and the 5C (water) unfolded state. In solution, the native HM state unfolds primarily into the HH species, while in the gel the HW species is formed preferentially. This indicates that the steric constraints within the gel pores hinder some backbone motions. We and others have previously shown that the water solvating the protein within the gel pores is more ordered than bulk water, leading to a decreased hydrophobic effect. Here we present the effects of several salts and co-solvents on the folding pathway of cyt c. We find that addition of some, but not all, salts and co-solvents can alter both the folding kinetics and which conformations are accessed by the protein. The results are discussed in the context of molten globule folding models and the Hofmeister ranking of chaotropic agents.

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Prosegment Catalyzes Pepsin Folding to a Kinetically Trapped Native State

Derek R. Dee, Rickey Y. Yada.
University of Guelph, Guelph, ON, Canada.

Pepsin, an archetypical aspartic peptidase, initially contains an N-terminal, 44 residue prosegment (PS) that, upon folding, is removed to yield native pepsin (Np). Np is irreversibly denatured above pH 6, the basis for which was not understood. Our previous studies comparing Np and a denatured and subsequently refolded state (Rp) indicated that Rp was thermodynamically more stable than Np, having a higher T_m and being reversibly unfolded (Dee, D., et al., 2006, *Biochemistry 45*, 13982). Thus, it was suspected that Np is blocked from refolding by a large energy barrier, which is reduced by the action of the PS.

It was found that the exogenous addition of a synthetic 44 residue PS peptide catalyzes folding of Rp to the Np state. The PS displays foldase activity that follows Michaelis-Menten kinetics ($K_m = 3.1 \pm 2.0$ μM, $k_{cat} = 0.011 \pm 0.002$ sec^{-1}), binds with higher affinity to the product (Np, $K_i = 41 \pm 6$ nM) of the folding reaction than to the substrate (Rp, $K_d = 2.0 \pm 0.2$ μM), and increases the rate of folding by a factor of 10^5 compared to uncatalyzed folding. By comparing the rates of Np unfolding ($t_{1/2} = 9 \pm 1$ days) and uncatalyzed refolding of Rp to Np ($t_{1/2} = 64 \pm 6$ days), it is shown that Np is thermodynamically metastable relative to Rp, in agreement with previous calorimetry data (Dee, D. et al.).

The data support a model whereby the PS catalyzes folding before being removed, resulting in a kinetically trapped Np state that is stabilized by a large barrier to unfolding rather than by a lower free energy. Considering the high structural and functional similarities among the aspartic peptidases, PS-catalyzed folding and kinetic stabilization mechanisms may be quite common.

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Osmolytes Control Peptide Folding and Aggregation

Regina Politi, Shahar Sukenik, Daniel Harries.
Hebrew University, Jerusalem, Israel.

Nature has developed many strategies to ensure that protein folding occurs in vivo with efficiency and fidelity. Among the most widely employed