proteins with a battery of ligands or a plethora of buffer conditions. In all cases, an environmentally sensitive fluorescent dye (SYPRO orange) is used to monitor melting temperature shifts under various conditions. HTTS has also been used to characterize protein-protein interactions, but its general applicability to protein complexes remains to be tested. Here, we use the complex formed by the N-terminal fragments of Protocadherin-15 (pcdh15) and Cadherin-23 (cdh23), essential for inner-ear mechanotransduction, to test modified HTTS assays that evaluate protein-protein binding affinities. By mixing cdh23 and pcdh15 at different ratios, we observed increased thermal stability of pcdh15 caused by shifts in chemical equilibrium towards the complex. In addition, protein fragments carrying missense mutations known to cause deafness and reduce binding affinity lack these shifts. These results suggest that HTTS can be used to qualitatively evaluate cadherin protein-protein interactions. We further validated our HTTS results using surface plasmon resonance-based affinity measurements. Surprisingly, we found that in some cases thermal stability shifts correlate well with koff rather than Kd. Our modified protocols might be applicable to any heterodimeric protein complex with affinities ranging from ~ 1 nanoM to 100 microM.

1920-Pos Board B64

An iLBP Family Member Domain Swapped Dimer is Evidence for a Highly Ordered Folding Intermediate

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Human Cellular Retinol Binding Protein II (hCRBP II), a member of the intracellular Lipid binding protein (iLBP) family, is a monomeric protein responsible for the intracellular transport of retinol and retinal. HCRBP II and other members of this family have been shown to be remarkably stable to mutations. Previous studies on other proteins of this family have shown that they fold into monomeric species. However we report, for the first time, that wild-type (WT) hCRBP II gives rise to an extensive domain swapped dimer (involving almost 50% of the protein sequence) during bacterial expression. In addition, there is no evidence of interconversion between monomer and dimer at room temperature, even after weeks of incubation, suggesting that two non-interconverting folds can result from the same amino acid sequence. Though wild-type hCRBP II forms the dimer, the propensity for dimerization can be substantially increased via mutation at Tyr60. Structural studies of wild-type and several mutant dimers suggest that an ‘‘open monomer’’ folding intermediate gives rise to both monomer and dimer, and their ratio depends on the relative orientation of the two halves of the protein in the open monomer intermediate. Therefore, Nature may build in an interaction (a Tyr60-Glu72 hydrogen bond) in a folding intermediate to prevent dimerization to yield the physiologically relevant monomeric protein, begging the question of whether other members of the iLBP family form physiologically relevant dimer species.

1921-Pos Board B65

Increase in Domain Swapping of the DNA-Binding Domain of Human FoxP1 is Related to a Decrease in Monomer Folding Stability

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Domain swapping is a folding-upon-binding phenomenon that allows proteins to form dimers by exchanging segments of their structure with another equivalent subunit. Recently, solved structures of the DNA-binding domain of the P subfamily of forkhead box transcription factors, FoxP, showed that these proteins can also form swapped dimers. Biologically, mutations of the DNA-binding domain of these proteins are linked to diverse diseases in humans. Moreover, FoxP1 and FoxP2 can reach monomer-dimer equilibrium in solution after hours of incubation, suggesting a low kinetic barrier separating both species in contrast to other domain-swapping proteins where this process takes days. Using wild type FoxP1 (WT) as a model of domain swapping, we analyzed the temperature and protein concentration effects on dimer dissociation, obtaining the free energy change and enthalpy of the process, indicating that the dimerization is an enthalpy-driven process. To understand how FoxP1 could swap, we performed equilibrium unfolding experiments with the dimer, showing that it follows a three-state mechanism, where the first transition corresponds to dimer dissociation and occurs with little loss of secondary structure. To further corroborate the relevance of hinge region and helix H3 in the domain swapping process of FoxP1, we engineered a previously described hinge monomeric mutant of FoxP1 (A539P) and we constructed a mutant in helix H3 (R553H) related to human health diseases. The dimerization propensity of R553H is higher than WT, suggesting that helix 3 and hinge region are relevant in the dimerization. Folding stability comparison of A539P and R553H monomers with WT show that the order of stability is A539P>WT>R553H, concluding that the ability of FoxP1 to domain swap rapidly can be partially explained by its low monomer stability. Funding: FONDECYT 1130510, 11140601.

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Coiled-Coil Probes Identified the Unfolding Pathway of Yeast Phosphoglycerate Kinase

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Large multi-domain proteins which have complicated reacting interfaces and dominate the proteomes of life remain mysterious regarding to their folding behaviors and mechanisms. Comprehensive studies towards the understanding of the fundamentals of protein folding mechanisms tend to bias small single-domain monomeric proteins, possibly due to their simplicity and reversible refolding capabilities. Yet the understanding of protein folding behaviors acquired from studying small single-domain proteins are not adequate to generate an accurate extrapolation for predicting the folding mechanisms of proteins that contain more than one domain. Here we present the characterization and interpretation of the unfolding pathway of a model large multi-domain protein yeast phosphoglycerate kinase (PGK) using single-molecule force spectroscopy (SMFS) and insertion of the antiparallel coiled-coil (CC) polypeptide probe we developed previously. When placed into different loop regions inside of yeast PGK, the CC probe affected different portion of the unfolding profile. Statistical comparison between the force-extension (FE) curves of multiple CC inserted yeast PGK's and the wildtype yeast PGK provided insights into figuring out the effects of conformational changes in domains and across the interfaces between domains on the unfolding pathway of the entire yeast PGK protein. Together with coarse-grain simulation modeling of the unfolding pathway, the sequence of the unfolding events occurred along the unfolding pathway of yeast PGK is precisely determined.

1923-Pos Board B67

Folding Analyses of the Major Folding Intermediate of Prouroguanylin using Deletion Mutants

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Uroguanylin serves as an endogenous ligand for guanylyl cyclase C, and consists of 16 amino acid residues and two disulfide bonds. Uroguanylin is secreted in the form of the precursor protein prouroguanylin, in vivo, and the pro-peptide region of prouroguanylin plays the role of an intra-molecular chaperone in the formation of the native conformation of the mature peptide, uroguanylin [1,2]. Our previous studies dealing with the disulfide-coupled folding pathway of prouroguanylin suggested that a folding intermediate (isomer 2) with one and two mis-bridged disulfide bonds in the pro-peptide and mature region is formed and is then was converted to the native form by a disulfide exchange reaction. Structural analyses of isomer 2 and the native form of prouroguanylin indicated that isomer 2 possesses a slightly larger of α-helical structure content than the native form. These results indicate that isomer 2 is a major folding intermediate in the formation of prouroguanylin and the disulfide conversion, which may be induced by β-sheet formation between the N-terminal and the C-terminal region of the pro-peptide and the mature region, respectively, from isomer 2 to the native form results in a decrease in the α-helical structure content of the molecule. Therefore, a structural analysis of isomer 2 is important in terms of understanding the disulfide-coupled folding of prouroguanylin. In this study, to investigate the folding mechanism of prouroguanylin, a series of C-terminal deletion mutants of isomer 2 was prepared and their secondary structures estimated by means of CD spectroscopy. The results indicated that the C-terminal Cys77-Ala81 region is required for the kinetic or thermodynamic stability of isomer 2. The folding mechanism of prouroguanylin via isomer 2 will be discussed in this presentation.

1924-Pos Board B68

Interdomain Contacts and RNA Polymerase Control Native State Interconversion of the Transformer Protein RfaH on a Dual-Funneled Landscape

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