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Interaction Of The Parkin UBL Domain With SH3-containing Proteins Involved In Synaptic Vesicle Endocytosis: Structure And Role In Protein Ubiquitination
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Mutations in the ubiquitin ligase Parkin have been associated with the development of autosomal recessive juvenile Parkinsonism (ARJP). Mutations have been found in the RING domains, but also in the N-terminal ubiquitin-like (UBL) domain. In an effort to identify protein substrates implicated in ARJP, we identified Endophilin-A as a ligand of the rat Parkin-UBL domain. Endophilin-A is a BAR-domain containing protein that induces membrane curvature and is implicated in synaptic vesicle endocytosis and recycling. Parkin-UBL interacts directly with the Endophilin-A C-terminal SH3 domain with an affinity of 10-15 uM. The interaction is specific as ubiquitin, Nedd8 and the Plic1-UBL domain do not interact with the SH3 domain. The Parkin-UBL domain only interacts with a subset of SH3 domains. We determined the crystal structure of rat Endophilin-A1 SH3 domain at 1.4 Angstrom resolution. Using NMR spectroscopy, we mapped the protein-protein interaction surfaces on both the rat UBL and SH3 domains. Using our SH3 structure and the crystal structure of murine Parkin-UBL, we calculated a docking model of the UBL:SH3 complex using NMR chemical shift perturbations and residual dipolar couplings. The UBL surface consists of the hydrophobic patch located around Ile44 as well as the basic C-terminal tail of the UBL domain, which is major specificity determinant. Using single-site mutagenesis, we identified key specificity and affinity determinants in the SH3 domain that explains that specificity of Parkin-UBL towards different SH3 domains. The SH3 surface involved in binding the UBL domain is centered around an invariant proline previously shown to be involved in proline-rich domain (PRD) binding. Parkin-UBL effectively competes with synaptojanin PRD for binding Endophilin-A1 SH3 domain. Finally, we show that the UBL:SH3 interaction is required for Endophilin-A ubiquitination by Parkin.

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Mechanisms Underlying the Binding Diversity of Dynein Light Chain
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Dynein light chain LC8 was first discovered as an essential component of the microtubule-based molecular motor dynein where it associates directly with dynein intermediate chain IC. A large fraction of LC8, however, is not associated with dynein, suggesting alternate functions for LC8 independent of its function in dynein. LC8 interacts with non-dynein proteins in diverse systems, including neuronal nitric oxide synthase (nNOS), the proapoptotic Bcl2 family protein Bim, Swallow (Swa), and Nup159 of the nuclear-pore complex. Based on the diversity of these interactions, and the fact that all known LC8 partners share a common binding groove on LC8 with dynein IC, we have proposed that the wide array of binding partners reflects the role of LC8 as an essential hub protein. In this capacity, LC8 functions not simply as a dynein cargo adaptor, as widely viewed, but as a promoter of dimerization of its monomeric, partially disordered binding partners. Here we address the mechanisms underlying LC8 binding diversity using crystallographic, solution NMR and thermodynamic characterization of several LC8/peptide complexes. Peptide binding is associated with a shift in quaternary structure that expands the hydrophobic binding surface available to the ligand, in addition to shifts in tertiary structure and ordering of LC8 around the binding groove. NMR peptide titrations show evidence of allosteric interaction between the two binding sites. Backbone 15N relaxation shows significant conformational heterogeneity in the LC8 dimer which is retained in LC8/IC but is lost in LC8/Swa, suggesting that the degree of ordering is ligand dependent. The reduced complexity of motion in LC8/Swa correlates with the less favorable entropy of binding of LC8 to Swa relative to IC. We propose that these properties observed for LC8 may be general to other dimeric hub proteins.

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Structural Basis Of RIG-I Auto-inhibition And RNA-induced Activation
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RIG-I (retinoic-acid-inducible-gene I) plays a critical role in regulating innate immune response against RNA viruses. RIG-I binds to 5′ triphosphate RNA of viral origin and activates downstream signaling pathways resulting in the induction of type I interferons. RIG-I contains two caspase-recruitment activation domains (CARD) in its N-terminus, a DExD/H helicase domain and a C-terminal repressor domain (RD). The function of RIG-I is regulated by auto-inhibition. While CARD domains activate downstream signaling, RD inhibits the activity of CARD domains in the absence of RNA. To investigate the RNA dependent structural transitions that activate RIG-I, we determined the structures of free and 5′ triphosphate RNA bound RIG-I using negative stain electron microscopy (EM) and single particle reconstruction. Preliminary biochemical studies show that free RIG-I, which is a monomer, undergoes dimerization upon binding to RNA. Analysis of the EM structure of RIG-I:RNA complex shows that dimerization is mediated by bivalent and parallel interactions between the two ends of adjacent RIG-I monomers. Structural modeling suggests that the dimer interfaces involve inter-molecular, homotypic, CARD-CARD and RD-RD interactions. In contrast to RNA bound RIG-I, which adopts an extended 'open' conformation, free RIG-I adopts a compact 'closed' conformation mediated by extensive intra-molecular interactions. Structural modeling indicates that the auto-inhibition of RIG-I is potentially mediated by a direct intra-molecular interaction between CARD and RD. Indeed, biochemical analysis demonstrates that isolated RD and CARD domain regions of RIG-I interact in the absence of RNA. We propose that the binding of 5′ triphosphate RNA to RIG-I disrupts the intra-molecular interactions that mediate auto-inhibition, and promote inter-molecular interactions that result in dimerization and subsequent activation. These results provide novel structural insights into the mechanism of RIG-I auto-inhibition and RNA-dependent activation.

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Cross-Correlation of Fluorescence-Quenching and Infrared Absorption in the Study of Protein Ligand Binding Sites
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We have developed a generalized multidimensional spectoscopic approach to investigate long range conformational coupling in proteins. It employs the integration of fluorescence emission and infrared absorption data recorded simultaneously from the same protein sample that undergoes conformational transitions in response to an external perturbation. Long range coupling in the signal transfer through rhodopsin has recently been identified by Fluorescence-IR-cross-correlation. Using 2D-cross-correlation techniques, the kinetic asynchronicity of the emission from natural or artificial site-specific fluorophores relative to the secondary structure-sensitive IR-absorption bands can be determined. Thereby, IR absorptions can be identified in a model-free and unbiased way that can be assigned to secondary-strutural elements that become specifically stabilized by ligand interactions. Here, we demonstrate in a cytoskeletal protein the correlation of the loss of ligand-dependent static quenching of intrinsic tryptophan emission during thermal unfolding with the loss of structure monitored by FTIR spectroscopy. The high signal to noise ratio in 2D-correlation and the ''synchronicity tagging'' of the IR bands through their correlation with an independent monitor of ligand dissociation allows detecting ligand protein interactions with an accuracy that is not achieved by FTIR-spectroscopy alone. In addition, topological information can be obtained from the emission wavelength of the tryptophans that become gradually unquenched during temperature-induced ligand dissociation. Fluorescence-IR-cross-correlation spectroscopy thus extends the IR-based conformational analysis by the inclusion of site-specific information on local physical parameters (polarity, electrostatics, etc.) specifically affecting the emission of fluorophores. We show how this approach provides structural information on flavonoid binding to actin, a cytoskeletal and nuclear protein that has recently been shown to respond to the binding of these natural compounds by flavonoid-specific conformational changes.

Platform AM: Emerging Single Molecule Techniques I

1983-Plat
Single-Molecule Fluorescence Imaging with Sub-nanometer Resolution
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Objective: The emerging field of fluorescence nano-scopy promises to revolutionize biomedical research, and remarkable progress has been made in the measurement of nano-meter distances. Although diffraction of visible light limits the far-field optical resolution to ~200nm, the center position of spatially resolved fluorescent molecules or nano-particles can be located to much higher precision. Notably, in high resolution microwave and optical spectroscopy there are numerous examples where the line-center is determined with a precision of less than 0.000001 of the line-width. In contrast, the brightest single fluorescent emitters can be detected with a Signal-to-Noise-Ratio of ~100, limiting the localization precision to 0.01 (~1.5nm) of the microscope Point-Spread-Function (PSF) width. Moreover the error in co-localizing two or more single emitters is notably worse, remaining greater than 0.03-0.05 (5-10nm) of the PSF width.
Results: We achieve two-color single-molecule imaging with 0.5nm absolute localization and registration accuracy as well as demonstrate 0.7nm absolute