369-Pos Board B138
Structure Analysis of Ultramarine Fluorescent Protein Sirius
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The fluorescence protein is the essential tool for live cell imaging and the color variants from blue to red has been developed for multi color imaging. Compared with active development of red variants, there were only a few variants giving violet to blue fluorescence. Therefore we created a mutant which substituted the Trp66 for Phe (mSECFP-W66F) on the chromophore of cyan fluorescent protein mSECFP derived from Aequorea GFP. Furthermore, through the mutagenesis we developed ultramarine fluorescence protein Sirius which express the enough fluorescence for live cell imaging and have the shortest wave length among the existing fluorescence proteins. In this study, we performed X-ray crystallographic analysis of Sirius and series of mutants to reveal the factor for the shortest wavelength, resistance to photobleaching, pH insensitivity and increasing of the fluorescence quantum yield through the development of the Sirius. In the determined 3D structure of Sirius, the side chain of the Gln65 of chromophore pushed it out perpendicularly to the plane containing 6 membered-ring and 5 membered-ring of the chromophore. It buried the space of the chromophore neighborhood. In addition, from the structure of the mSECFP-Y66F having extremely low fluorescence quantum yield, it became clear that its chromophoric 6 membered-ring was deleted like EGFP-Y66F known as a colorless variant. We'll also discuss about the factor to increase of the fluorescence quantum yield by comparing structure of mutants.

370-Pos Board B139
Backbone Conformational Fluctuations as Determinants of pK_a Values of Surface Residues in Proteins
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The pK_a values of surface ionizable residues in proteins are usually similar to the pK_a values of ionizable residues in water. This is still difficult to reproduce using structure-based electrostatics calculations with static structures, which tend to exaggerate the shifts in pK_a values because the magnitude of Coulomb effects is exaggerated and the balance between Coulomb and dehydration effects is not reproduced correctly. These problems can be minimized by using artificially high protein dielectric constants, but sometimes the discrepancy persists even when the protein interior is treated with the dielectric constant of water. This suggests that pK_a values are affected by the conformational dynamics of the protein, which are not reflected in the crystal structure. Molecular dynamics or Monte Carlo simulations can be used to attempt to reproduce these dynamic effects implicitly, but there are no useful data for testing this approach directly. To examine the role of fluctuations of the backbone in determining pK_a values, NMR spectroscopy was used to measure the pK_a values of all 20 Asp and Glu residues in variants of staphylococcal nuclease with Gly substituted at select locations. These substitutions were intended to enhance backbone fluctuations without affecting the overall protein structure. Some Gly substitutions detectably shifted the pK_a values of some carboxylic groups. Crystal structures of these variants showed no significant changes relative to the structure of the reference protein. Calculations with continuum methods using these crystal structures do not reproduce the measured shifts in pK_a values. NMR spectroscopy studies suggest Gly substitutions affect the dynamics of the protein. Efforts are underway to better characterize these changes. Our results suggest that the high apparent polarizability of proteins might be due to subtle structural fluctuations that are difficult to reproduce computationally.

371-Pos Board B140
NMR Characterization of Adenylyl Cyclase Toxin-Calmodulin Complexes
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Bordetella pertussis, etiological agent of whooping cough, secrets a calmodulin (CaM) - activated adenylate cyclase toxin (CyaA) virulence factor which assists in respiratory colonization. The worldwide increase in pertussis cases emphasizes the necessity of understanding CyaA's role in disease progression. Intact CaM consists of tethered N- and C-terminal domains both of which can separately bind to and activate CyaA. Moreover, interaction with intact calmodulin substantially increases the binding affinity between CyaA and CaM by unknown structural mechanisms. A crystal structure determined in the absence of N-terminal CaM elucidates the molecular mechanisms by which C-terminal CaM activates CyaA. While it is reported that the second N-terminal calcium-binding site in CaM is likely involved in stabilizing interactions with the catalytic pocket of CyaA, no high-resolution structure detailing this protein-protein interface is currently available. In this study, nuclear magnetic resonance (NMR) chemical shift mapping and paramagnetic relaxation enhancement (PRE) techniques have been used to probe molecular interactions between intact CaM and CyaA. Chemical shift perturbations induced in labeled CaM by CyaA binding were localized primarily to helices IV and V, VI and VII in the N- and C-terminal domains of CaM, respectively. Binding of CyaA resulted in the structural modification of intact CaM as evidence by the protection of specific resonances in helices IV, V, and VI of labeled CaM from spin labeled induced broadening. These data suggest that conformational modulation in both the N- and C-terminal domains of CaM upon CyaA binding contributes to the maximal activation of the toxin.

372-Pos Board B141
Oligomeric State of a Pannexin Channel
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Pannexins are a recently-discovered family of integral membrane proteins in vertebrates that form non-selective homomeric or heteromeric pores in the plasma membrane. These channels seem important in ATP signaling in the central nervous system and the immune response, as well as in ischemic and apoptotic cell death, yet their mechanism remains unknown. One important question is how pannexin subunits come together to form the large channel. While the membrane topology suggests that pannexins resemble hexameric connexins, there has been no bona fide evidence supporting such structural analogy between the two distinct large-pore channels with no sequence similarity. To address how many subunits constitute pannexin channels, we first identified a pannexin orthologue that forms a monodisperse oligomer upon solubilization in a relatively mild detergent, such as dodecyl maltoside, using the fluorescence detection size exclusion chromatography strategy. Interestingly, we discovered that most of the 28 arbitrarily collected pannexin channels from multiple organisms tend to aggregate or dissociate into monomers upon solubilization in detergents, making the selected stable pannexin channel an ideal paradigm for biochemical characterization. We subsequently purified this stable pannexin channel using a baculovirus-insect cell expression system. In this poster, we present the oligomeric state of the pannexin channel measured through multi-angle light scattering and crosslinking experiments.

373-Pos Board B142
One Drop Analysis and Automated System for CD Measurement
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Circular Dichroism (CD) has become an indispensable tool for studying biological molecules such as proteins. CD measurement requires a relatively small amount of sample at specific concentrations compared to other analytical methods. However, in the case of very rare and precious samples, researchers cannot use the conventional cells that normally require 200-300 mL. To reduce sample volume as much as possible, JASCO has designed 'a micro sampling disk' for CD spectra measurement using just one drop. Micro sampling disk requires no more than 10 mL of sample to measure CD spectra.
CD is easier and requires less time to measure and analyze data compared with nuclear magnetic resonance (NMR) or X-ray crystallography. Accordingly, CD is often used for screening multiple samples, such as lead or seed compounds in drug discovery. To automate CD measurements of a large number of samples, JASCO has designed the High-throughput Circular Dichroism (HTCD) system. Exclusive auto-measurement software executes CD spectra measurements and saves the results as a data set according to a defined sampling sequence. Dedicated analysis software can be used to analyze secondary structure estimation (SSE).

374-Pos Board B143
Amyloid Formation by Human Growth Hormone
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The tendency of proteins to form amyloid is proposed to be inherent that can either perform native functions in a host such as providing mechanical strength (e.g.: spider silk), storage (e.g.: hormone storage), or can result in fatal effects like the plaques formed in amylodosis in human. Recently it was reported that, hormones are stored in secretory granules as amyloid- like fibrils (Maji et al., 2009). But the conditions necessary for amyloid formation varies for different hormones. Here, growth hormone (GH) is taken to study the amyloid formation and its role in hormone storage within the secretory granule of anterior pituitary. Analysis of GH protein sequence using TANGO and WALTZ algorithms showed that it has amyloidogenic potential. Further the conditions for GH aggregation were studied in presence and absence of various solvents, denaturants, glycosaminoglycans, salts and cations. It was found that GH in presence

of equimolar concentration of Zn^{2+} ions formed amyloid like aggregates as observed by secondary structural changes by CD and ability to bind amyloid specific dyes such as congo red and thioflavin T. These aggregates showed fibril-like morphology observed under TEM. The role of Zn^{2+} ions in GH aggregation was further probed by NMR analysis.The involvement of Zn^{2+} in amyloid formation is confirmed as incubation of GH in presence of Zn^{2+} ions and EDTA didn't result in any amyloid formation. Additionally GH fibrils formed with Zn2+ ions destabilizes when incubated with EDTA and results in release of monomers. This data suggests possible mechanism of GH storage and release in somatotrophs of anterior pituitary which could further help in understanding GH deficiency caused due to faults in storage of GH in secretory granules.

375-Pos Board B144

Structural Studies of Betaine Homocysteine Methyl Transferase (BHMT) and a Dimeric Mutant by Conventional and 2DCOS Moving Lapse IR Spectroscopy

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Betaine homocysteine S-methyltransferase (BHMT, EC 2.1.1.5) is one of the two enzymes known to methylate homocysteine (Hcy) to generate methionine in the liver. The increase in plasma levels of Hcy (homocysteine) has been established as an independent risk factor for cardiovascular and Alzheimer diseases. BHMT uses betaine as the methyl donor to synthesize methionine, allowing recovery of one of the methyl groups used in choline synthesis by transmethylation. Changes in BHMT activity have been detected under several dietary conditions, during development and in pathologies such as cirrhosis and hepatocellular carcinoma. Rat liver BHMT is a 407-aminoacid cytosolic protein that is more than 90% identical at the amino acid level with its human and pig counterparts. The enzyme contains zinc co-ordinated to three conserved cysteine residues and assembles as a homotetramer. BHMT possesses seven tryptophan residues per subunit located along the sequence including the dimerization arm. Also they are implied in the dimerization arm. We have used a conventional and 2DCOS infrared approach to make a structural study of the differences between BHMT and the dimeric W325F mutant. In these studies, temperature has been used as perturbation. We have also used to extract information from the 2DCOS maps a ''moving lapse'' approach which uses a narrow frame of 2D spectra along perturbation in order to highlight local variations in structure instead of attending global changes. The mutant has a different amide I shape with a decrease in the area percentages of α-helix and an increase in the band at 1624 cm-1 attributed to extended structures. The moving lapse approach shows the sequence of changes and the structural difference between BHMT and the mutant.

DNA Replication, Recombination, and Repair

376-Pos Board B145

Studies of DNA Gyrase at the Single Molecule Level

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Gyrase, a bacterial type II topoisomerase, is the only topoisomerase capable of introducing negative supercoils into DNA. Negative supercoiling results from the coordinated movement of double stranded DNA and protein domains linked to ATP hydrolysis. Thus, it is important to combine structural data with dynamic information of protein and/or DNA movements to obtain a comprehensive picture of the mechanism of gyrase. Although the structures of several individual domains of different type II enzymes have been elucidated, there are still no structures of the intact gyrase heterotetramer or an intact gyrase/DNA complex precluding the correlation of conformational changes in the protein with movements of DNA. To alleviate this shortcoming, we are combining structural and single molecule studies to investigate the conformational changes that occur in gyrase as it alters DNA topology. To facilitate our research, we have optimized a procedure for purifying stable gyrase/DNA complexes in the amounts required for biophysical studies. This method results in homogeneous complexes that are captured in well-defined states along the catalytic cycle. We are also developing a novel single molecule technique combining magnetic tweezers and fluorescence microscopy to study the movements of both protein and DNA during the DNA supercoiling process. The combination of dynamic single molecule and structural information promises to provide a more comprehensive picture of the mechanism used by this molecular machine to alter DNA topology.

377-Pos Board B146

Real Time Visualization of hRPA Binding to Torsionally Controlled Double-Stranded DNA

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In eukaryotic cells, single-stranded DNA (ssDNA) is rapidly bound and stabilized by ssDNA-binding proteins (SSBs). This prevents ssDNA from binding back on itself into secondary structures. The main eukaryotic SSB is replication protein A (RPA), which is important for repair, replication and recombination. We study the dynamics of human RPA (hRPA) on topologically constrained DNA at the single-molecule level with magnetic tweezers. This assay allows us to apply varying torsional stress and stretching forces on the dsDNA, parameters that are known to influence the hRPA unwinding reaction. We are interested in uncovering positional preference, cooperativity and directionality of hRPA-DNA binding. To observe this directly, we visualize the position(s) of the hRPA along the DNA, using a combination of magnetic tweezers with fluorescence microscopy. With magnetic tweezers a dsDNA molecule is held between a glass surface and a magnetic bead by a pair of magnets. The stretching force and torsional stress is controlled by the distance and rotations of the magnets. The molecule is pulled sideways and the fluorescently labeled RPA is imaged with an inverted microscope. By applying negative supercoiling at high forces, bubbles of ssDNA open up. Multiple bubbles will allow RPA to bind to multiple sites, and such RPA-stabilized bubbles may coexist or slowly anneal into one larger bubble. Our first experiments show that more than one RPA-binding sites occurs when assembled on 20 kb dsDNA that is underwound. These RPA-bound spots appear to be stable in time. We will report the structure and dynamics of RPA-stabilized ssDNA bubbles at the meeting.

378-Pos Board B147

Single Molecule Observation of Direct Transfer of Escherichia Coli Single-Strand Binding Protein (SSB) between Single-Stranded DNA Molecules

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Echerichia coli Single-Strand Binding (SSB) protein is essential in DNA replication and repair processes owing to its ability to bind the intermediate single stranded DNA (ssDNA), thereby preventing unwanted reannealing or degradation. It was proposed that SSB is recycled by redistributing itself along long ssDNA, for example between two adjacent Okazaki fragments. The mechanism of this process was investigated by the Lohman group using ensemble kinetics methods and they proposed a ''direct transfer'' mechanism - forming a transient intermediate composed of SSB and two ssDNA prior to the transfer. We developed a single molecule assay to examine this direct transfer in detail through real-time observation of single molecule fluorescence resonance energy transfer (smFRET) signals. The introduction of competitor ssDNA oligonucleotides to an SSB protein bound to a surface immobilized ssDNA ultimately led to the dissociation of SSB from the surface immobilized DNA. The rate of SSB transfer is linearly dependent on the competitor DNA concentration up to 50 nM, suggesting that only one ssDNA molecule is involved in the rate-determining step of the transfer process. Prior to the full unwrapping of the original DNA bound to an SSB protein, fast FRET fluctuations with 100 ms time scale were observed with the distribution of the FRET signal shifted toward the lower value, indicating the structural destabilization of the ssDNA-SSB complex induced by multiple events of competitor ssDNA binding. The final dissociation event measured as a high to low FRET transition occurs rapidly. These findings are consistent with the kinetic model_short-lived, partially unwrapped, intermediate states were induced by the fast binding and unbinding of competitor oligos and only a small fraction of the intermediate state molecules can achieve complete transfer of SSB.

379-Pos Board B148

Structural Studies of Rolling Circle Replication Initiator Proteins

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pT181 family plasmids replicate by a rolling-circle mechanism. This is initiated by a plasmid-encoded Rep initiator protein, which has sequence-specific DNA nicking and religation activity. The replication origin is nicked by Rep, which binds covalently to one DNA strand via an active site tyrosine, initiating rolling circle replication and religating the strand at the end of the cycle. Rep proteins also associate with PcrA helicase to form a highly processive complex. We