Protein Nucleic Acid Interactions II

2130-Pos Board B100

Probing DNA Unwinding By Single Helicases

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DNA helicases are multi-functional motor proteins which translocate along DNA using the energy released from ATP hydrolysis. Translocation is coupled to diverse enzymatic activities, such as duplex unwinding, strand degradation and protein displacement. Resolving such complex processes requires our ability to detect rare, or short-lived intermediates and observe their dynamics. To this end, we have developed a generic fluorescence-based assay, which enables monitoring of DNA unwinding by single helicases in vitro. Biotinylated double-stranded DNA fragments are specifically immobilised on poly-ethylene glycol-coated surfaces through biotin-neutravidin interaction. Total internal reflection fluorescence (TIRF) microscopy is used to achieve high signal-to-noise ratio. To probe DNA unwinding, we use a fluorescently labelled mutant of the E.Coli single-stranded DNA-binding protein (SSB), which specifically binds single-stranded DNA. DNA unwinding events mediated by single helicases are observed as fluorescent spots of increasing intensity, as increasing numbers of SSB molecules bind to the ssDNA product of the helicase. Using an objective-based TIRF microscope, these events are recorded at video rate. The increase in fluorescence intensity directly correlates to the rate at which single helicases unwind DNA. Here, we show that the assay is a powerful tool for probing DNA unwinding by various helicases as a function of DNA sequence, ATP and salt concentration. In comparison with bulk measurements, our single molecule data demonstrate that DNA unwinding involves multiple phases which are masked during ensemble averaging. Hence, this assay adds to the single molecule toolbox available for studying DNA processing enzymes.

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Helicase Activity As Monitored By Dual Colour Fluorescence Correlation Spectroscopy

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Helicases are responsible for the unwinding of DNA. These proteins play a key role in the maintenance of the genomic integrity, their defect in humans lead to several disorders including Bloom syndrome (cancer predisposition) and Werner syndrome (premature ageing). The E. coli RecQ helicase activity was studied by Fluorescence Cross Correlation Spectroscopy under single turnover and Michaelis-Menten conditions. The influence of the DNA substrate size on the unwinding activity was clearly different depending on the condition, excess of enzyme or DNA substrate. Moreover, addition of single-stranded DNA of varying size, complementary to one of the DNA substrate strand, displays both stimulating and inhibitory effects depending on the concentration range with efficiencies depending on the size. Our results indicate distinct mechanisms of action of RecQ helicase depending on the enzyme:DNA ratio used.

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Single-Molecule Fluorescence Resonance Energy Transfer Studies of Hjm/Hel308 DNA Helicase in Mesophilic Archaeon, *Methanosarcina acetivorans*

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One class of superfamily-2 (SF2) helicases, the RecQ family, plays crucial roles in DNA repair and homologous recombination in order to maintain the genome integrity. Archaeal RecQ-like DNA helicases, such as Hjm/Hel308, have been biochemically characterized in hyperthermophilic and thermophilic archaea. The studies suggest that Hjm/Hel308 helicases target stalled replication forks and have specificity for unwinding lagging strands. In this study, we cloned and purified Hjm/Hel308 homologue (MacHjm) from a mesophilic archaeon, *Methanosarcina acetivorans*. Single molecule fluorescence resonance energy transfer (smFRET) assay was used to study the single-stranded DNA (ssDNA) binding ability and behavior, the double-stranded DNA (dsDNA) unwinding kinetics, and Holliday junction migration activity induced by MacHjm. By this method, we determined that four MacHjm molecules were able to bind to 17-nucleotide ssDNA with each MacHjm occupying three to four nucleotides of the DNA. In addition, we were able to observe the binding and unwinding activity of MacHjm on DNA in real time. The MacHjm was observed to bind to ssDNA and translocate on ssDNA in 3′ to 5′ in ATP dependent manner. Within three seconds MacHjm was able to complete the unwinding of 18 base-pair dsDNA. The results from our smFRET studies on MacHjm provide important insights into DNA unwinding, stalled replication fork processing, and Holliday junction migration mechanisms for SF2 helicase.

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Protein-Induced DNA Unwinding is An Intrinsic Feature of Certain Sequence-Specific DNA-Binding Proteins

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Protein-DNA interactions play a key role in many fundamental biological processes, such as DNA replication, transcription, and recombination. Very often, the DNA-binding proteins induce structural changes of the target DNA-binding sequences. These DNA structural changes include DNA bending, twisting, and wrapping. Although protein-induced DNA-bending has been widely studied, other protein-induced DNA structural changes have not been fully explored due to the lack of a feasible experimental approach. In this study, we developed a new experimental strategy to probe the protein-induced DNA-unwinding. Our first step is to make a circular plasmid DNA template containing multiple tandem copy of a DNA-binding site of a sequence-specific DNA-binding protein, such as λ O protein and LacI. This plasmid also contains one nicking-enzyme recognition site, such as Nb.Bsm I. Next, we nicked this plasmid using the nicking enzyme, Nb.Bsm I, and then titrated the nicked plasmid by increasing the concentration of the DNA-binding protein. The DNA template was ligated by T4 DNA ligase. The linking number (Lk) of the ligated DNA products was determined by one or two-dimensional agarose gel electrophoresis in the presence of chloroquine. The linking number changes (ΔLK) was calculated using equation $\Delta LK = Lk - Lk^{\circ}$, where Lk and Lk° are the Boltzmann centers of the topoisomers band in the presence of the DNA-binding protein and in its absence, respectively. If we define the apparent protein-induced DNA unwinding angle (α_{obs}) equals to $360 \times \Delta LK$, i.e. $\alpha_{obs} = 360 \times \Delta LK$, then the protein-induced DNA-unwinding angle (α) and the DNA-binding constant (K) can be simultaneously determined. Using this method, we have determined the DNA-unwinding angle and the DNA-binding constant for the following sequence-specific DNA-binding protein: λO, LacI, GalR, AraC, and CRP. Our results indicate that protein-induced DNA-unwinding is a unique feature for these sequence-specific DNA-binding proteins.

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Kinetic Mechanism of Duplex rRNA Unwinding by the DEAD-box Protein, DbpA

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DEAD-box RNA helicases are enzymes that couple cycles of ATP binding, hydrolysis and product release to the unwinding of duplex RNA. We have previously determined the rate and equilibrium constants defining the ATPase cycle of DbpA, a DEAD-box protein from *E. coli* that is specifically activated by rRNA. In this study, we have measured rRNA duplex unwinding using a real-time fluorescence assay. Efficient and rapid unwinding of an 8 base pair duplex RNA requires chemical cleavage of ATP. Strand displacement coincides with product release step. Collectively, our analysis allows us to determine how the unwinding kinetic intermediates are coupled to specific ATPase cycle transitions. Because DEAD-box proteins are highly conserved among prokaryotes and eukaryotes, these results will be applicable to eukaryotic DEAD box RNA helicases involved in fundamental aspects of RNA metabolism in the cell.

2135-Pos Board B105

Studying RecBCD Helicase Translocation along Chi-DNA Using Tethered Particle Motion with a Stretching Force

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E. coli RecBCD helicase unwinds blunt-end duplex DNA to repair damaged DNA molecules in the homologous recombination pathway. Previous single-molecule experiments show that RecBCD recognizes an 8 nt DNA sequence, chi, and lowers its unwinding rate afterwards under saturating ATP condition. We have developed a single-molecule Force Tethered Particle Motion (FTPM) method, which is modified from the conventional TPM method, and applied it to study RecBCD motion in details. In the FTPM experiment, a stretching force is applied to the DNA-bead complex, and suppressed bead's Brownian motion, resulting in an improved spatial resolution at long DNA substrates. Based on the equipartition theorem, the mean square displacement (MSD) of the bead Brownian motion measured by FTPM correlates linearly to DNA extension length with a predicted slope, circumventing the difficulties, such as non-

linearality and low resolution of long DNA substrates in conventional TPM experiments. The FTPM method offers the best resolution (56 bp at 433 bp long DNA) in the presence of only a small stretching force (0.20 pN). We have used the FTPM method to investigate the RecBCD helicase motion along 4.1 kb long chi-containing duplex DNA molecules, and observed that translocation rate of RecBCD changes after chi sequence under limited ATP concentrations. This suggests that chi recognition by RecBCD does not require saturating ATP conditions, contrary to what have been previously reported.

2136-Pos Board B106
Kinetic Mechanism for Single stranded DNA binding and Translocation by *S. cerevisiae* Isw2
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The chromatin remodeling complex Isw2 from *S. cerevisiae* (yIsw2) mobilizes nucleosomes through an ATP-dependent reaction that is coupled to the translocation of the enzyme along intranucleosomal DNA. In this study we demonstrate that yIsw2 is capable of translocating along single-stranded DNA in a reaction that is coupled to ATP hydrolysis. We find that single-stranded DNA translocation by yIsw2 occurs through a series of repeating uniform steps with an overall macroscopic processivity of $P = (0.92 \pm 0.01)$; this processivity corresponds to an average translocation distance of (24 ± 4) nucleotides before dissociation. This processivity corresponds well to the processivity of nucleosome sliding by yIsw2 thus arguing that single-stranded DNA translocation may be fundamental to the double-stranded DNA translocation required for effective nucleosome mobilization by the enzyme. Furthermore, we find that a slow initiation process, following DNA binding, is required to make yIsw2 competent for DNA translocation. We also provide both evidence that this slow initiation process likely corresponds to the second step of a two-step DNA binding mechanism by yIsw2 and a quantitative description of the kinetics of this DNA binding mechanism.

2137-Pos Board B107
Single-molecule Measurements Of DnaB
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The replicative helicase of E. coli, DnaB, is a ring-shaped hexameric motor protein capable of unwinding double-stranded DNA (dsDNA) at a fork. It is thought to do this through ''steric occlusion,'' in which DnaB encircles and translocates along one single strand of DNA (ssDNA), forcing the other single strand (the occluded strand) to pass outside the ring. Using magnetic tweezers, we have performed single-molecule measurements of the unwinding activity of DnaB in which the dsDNA is destabilized by force applied to either the occluded or encircled strand. Based on measurements of the velocity of the motor as a function of force applied to the occluded strand, we conclude that DnaB does not unwind dsDNA with a ''passive'' mechanism, i.e. it does not simply rely on thermal fluctuations to open proximal basepairs before stepping. We also present preliminary data of DnaB activity with force applied to the encircled strand, which probes DnaB's possible mechanisms for binding and translocation along ssDNA. Finally, we will report on the effects of the helicase loader DnaC and the primase DnaG on DnaB activity.

2138-Pos Board B108
Structural Transitions Of a Helicase-Partial Duplex DNA Complex during ATP Hydrolysis Cycle
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Helicases are ATP-dependent enzymes that use the structural changes induced by ATP binding, hydrolysis and release to unwind double stranded nucleic acids. We have studied the structural transitions of partial duplex DNA bound *E-coli* Rep helicase monomer by in vitro single molecule Förster resonance energy transfer (FRET) methodology. Constrained triangulation procedures were applied globally on FRET measurements from eight Rep mutants, donor labeled at different residues, and three DNA substrates, acceptor labeled at different nucleotides on duplex, to study these conformational states. A total of 96 different measurements were performed and used in the triangulation analysis. Such over-sampling reduces the likelihood of a single site with unusual photophysical properties to negatively impact the results. Our results show that binding of ATPγS to Rep induces a large conformational change which is then reversed in two approximately equal steps during ATP dephosphorylation (ATP to ADP.Pi transition) and ADP release. We do not observe a significant conformational change upon phosphate release (ADP.Pi to ADP transition). The large conformational change upon ATPγS binding is consistent with the rotation of the Rep domains in a direction that brings them closer to the duplex. In addition, we show that Rep has a preference to bind to ssDNA/dsDNA junction compared to the other sites along the ssDNA. Finally, we show that Rep remains in the closed conformation during all ATP hydrolysis intermediates when bound to the vicinity of ssDNA/dsDNA junction. Our studies not only reveal the structural transitions of Rep helicase-partial duplex DNA complex during ATP hydrolysis cycle but also demonstrate the potential of triangulation analysis as a versatile single molecule technique for probing structural information in physiological conditions.

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Deciphering the Mechanism of RNA helicase eIF4A in Translation Initiation
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Eukaryotic initiation factor eIF4A is a prototype protein of the DEAD box family of RNA helicases, and is part of the translation initiation complex eIF4F [1].eIF4A binds to the 5′ cap of mRNA and unwinds structures in the 5′-untranslated regions of mRNAs in ATP dependent manner. Although eIF4A has been studied extensively by classical bulk biochemical methods, a direct, unambiguous measurement of its helicase activity and its processivity has not been reported. Here, we use single molecule fluorescence assays to visualize its binding to RNA and melting secondary structures in RNA. Specifically, FRET efficiency dynamics is used to explore the binding location of eIF4A and its unwinding function. Our single molecule studies show that eIF4A has higher binding affinity towards the duplex site. We seek to elucidate any elementary steps and kinetic mechanisms involved with eIF4A unwinding of RNA. Furthermore, we selectively target eIF4A activity with small-molecule inhibitors acting in opposite manners[2]. The dynamics of stimulation and inhibition of eIF4A activity by *pateamine* and *hippuristanol* are measured at the single molecule level. The results will provide insight into the eIF4A's helicase activity and will distinguish between passive versus active unwinding mechanism. Our long-term goal in this project is to decipher the role of the initiation complex eIF4F in ribosomal recruitment, and develop methods to control this process. Then, we will probe eIF4A activity in the presence of other initiation (co)factors.
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2140-Pos Board B110
PcrA Helicase ATPase Mechanism: RepD Modulation During Unwinding
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Helicases catalyse the unwinding of double stranded DNA or RNA for a variety of functions through various mechanisms. The monomeric, bacterial helicase PcrA is well characterized: it translocates ssDNA with discrete steps of one base per ATP. The ATPase activity is enhanced by DNA and the cycle includes a rate limiting cleavage step, followed by rapid phosphate release.
PcrA and the plasmid encoded replication initiator protein, RepD, act together during replication of some plasmids, containing antibiotic resistance. RepD is a dimer and binds to a specific origin sequence (*ori*) containing inverted complementary repeat (ICR) elements. ICRIII provides affinity and plasmid specificity, whereas ICRII is conserved amongst *ori* family members and contains the nick site for initiation. RepD binds to ICRIII and then nicks at a specific site in ICRII exposing a single-stranded region. PcrA helicase then binds to the ssDNA and begins unwinding. RepD has been shown to increase PcrA helicase activity; in the absence of RepD PcrA is a poor helicase. Measurements have shown that unwinding occurs at a reduced rate but the coupling ratio is unaltered.
We have analyzed the mechanism of the PcrA ATPase cycle with ssDNA and extended this to include probing how DNA junctions and RepD affect this cycle. The individual rate constants were determined to see how RepD modulates the ATPase rate. This included utilizing the fluorescent ATP analogue mantATP to monitor fluorescence intensity in rapid-reaction, kinetic experiments, allowing the initial binding and release kinetics to be explored. The analogue was further used for analysis of the hydrolysis step using quenched-flow measurements. Additionally, the fluorescent phosphate binding protein (MDCC-PBP) independently measures the phosphate release step.

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Single-Molecule Studies Of ATP-Dependent Restriction Enzymes
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