represent the rate limiting step in the catalytic (topological) cycle. By substituting diaminopurine (DAP) deoxyribonucleotides for dATP in PCR reactions, stiffer DNA fragments have been produced and used as substrates for topoisomerase II-mediated relaxation of plectonemes introduced in single molecules using magnetic tweezers. The overall rate of relaxation of plectonemes by recombinant human topoisomerase II alpha decreased on the stiffer DNA. In addition the ability of recombinant *E. coli* gyrase to wrap DNA also decreased for DAP-substituted DNA in which every base pair has three hydrogen bonds. These dynamic measurements of DNA bending and wrapping by type II topoisomerases are consistent with the hypothesis that DNA flexibility affects the rate determining step for type II topoisomerase activity.

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Characterization of HIV-1 Reverse Transcriptase 3TC Specificity By Conformationally Sensitive Fluorescence Reveals New Insights Into the Kinetic Basis of Inhibitor Discrimination

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HIV-1 Reverse Transcriptase (HIV-RT) is the target of nine Nucleoside Reverse Transcriptase Inhibitors (NRTI's) currently approved by the FDA. Polymerase specificity is best quantified by analysis of the concentration dependence of the rate using single turnover rapid Quench-Flow methods which provide a rate of polymerization (k_{pol}) and an apparent dissociation constant (K_d) such that $k_{pol}/K_d = k_{cat}/K_m$. Analysis of nucleoside analog RT inhibitors (NRTIs) has led to the surprising conclusion that most appear to bind more tightly than normal nucleotides. For example, 3TC-triphosphate binds 10-fold tighter than the correct nucleotide (dCTP). Using a conformationally sensitive fluorophore attached to the fingers domain of the enzyme, we show that nucleotide binding is a two step process involving weak nucleotide ground state binding, followed by a conformational change from an ''open'' to ''closed'' state. These steps together define the true K_d for nucleotide binding at equilibrium. Examining the kinetics of 3TC incorporation, we show that contrary to previously reported findings, the dCTP analog binds 8-fold more weakly to the enzyme than the correct nucleotide. Further, we show that the enzyme's conformational change to the ''closed'' state is capable of sensing dCTP versus 3TC and results in an increased or decreased binding affinity, respectively. The result is a specificity constant (k_{cat}/K_m) of $9.7\mu M^{-1}s^{-1}$ for dCTP and $0.7\mu M^{-1}s^{-1}$ for 3TC. The specificity constant for dCTP is determined solely by the rate of nucleotide binding ($k_{cat}/K_m = K_1k_2$ in the two-step sequence), whereas the slower chemical reaction (k_3) for 3TC incorporation allows the binding and isomerization to reach equilibrium so that $k_{cat}/K_m = k_{pol}/K_1K_2$. This work provides mechanistic basis for discrimination of 3TC, and corrects how K_m, K_d, and $K_{d,app}$must be assigned for NRTIs.

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Coliphage 186 Genetic Switch: A Single Molecule Study

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It is increasingly clear that in most cases genes are regulated by wrapping or looping of DNA on large, cooperatively assembled protein complexes. In most eukaryotic organisms, 150 bp of DNA are wrapped around histone octamers (nucleosomes). Furthermore, interaction between proteins bound at distant sites on the DNA may cause looping out of the intervening DNA and have regulatory significance. The mechanism by which these DNA- protein nanostructures are formed is not clear. The interaction between the bacteriophage repressor 186CI (a disc-shaped heptamer) and its DNA is an ideal model system to study DNA wrapping and looping and to reveal fundamental principles of long-range interactions and regulation by nucleoprotein complexes. Here we report on AFM work aimed at elucidating the 186CI-DNA interaction. We analyzed the structure of the protein DNA complexes revealed by the AFM images and we propose a mechanism that leads to repression of the lytic genes in 186 and regulation of the repressor expression via DNA wrapping around a protein heptamer and protein repositioning along the DNA.

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Using Real-Time, Single-Molecule Experiments To Monitor RecA-Mediated Pairing and Strand Exchange Reactions in Various Nucleotide States

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RecA recombinases play a central role in homologous recombination pathway. Once they assemble on single-stranded (ss) DNA, the RecA/ssDNA filament mediates the pairing of homologous DNA sequence and strand exchange processes. We used tethered particle motion (TPM) experiments to investigate the details of E. coli RecA-mediated pairing and strand exchange steps at the single molecule level. TPM experiments measure the DNA tether length change according to the bead Brownian motion. In the ''incoming bead'' experiment, ssDNA molecules bound with sub-micron sized polystyrene beads were coated with RecA and then paired with homologous duplex DNA tethered on surface. Therefore, the appearance of the bead tether and its Brownian motion amplitude permit the direct observation of RecA-mediated pairing and strand exchange processes in real-time. In the ''leaving bead'' experiment, surface-bound hybrid duplex DNA molecules were tethered with polystyrene bead, and then reacted with RecA-coated complementary ssDNA. Disappearance of the tethered beads indicates the completion of strand exchange. It was found that pairing and strand exchange steps are more efficient under low pH=6.5 condition in which the strand exchange efficiency of 0.17 ± 0.02, is higher than that in pH=7.5 (0.11 ± 0.05). The pairing process occurs successfully in both ATP and its non-hydrolyzable analog, ATPγS state, but not in ADP state where the three-stranded intermediate are found to be unstable (halflife time=0.7s). Surprisingly, the strand exchange efficiency under ATP and ATPγS states are similar (0.19 ± 0.03 and 0.18 ± 0.01 for ATP and ATPγS respectively), suggesting ATP hydrolysis of RecA is not necessary to complete strand exchange step in our experiment. These single-molecule experiments provide new mechanistic details on the RecA-mediated processes.

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Intersubunit Regulation Between Nuclease and Helicase Domains of Recbcd Enzyme

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The *Escherichia coli*RecBCD helicase/nuclease initiates homologous recombinational repair of damaged blunt-end duplex DNA molecules. RecBCD, a multifunctional enzyme complex, contains two DNA motors as well as a nuclease domain to process duplex DNA and generate single-stranded DNA molecules. We used single-molecule tethered particle motion (TPM) experiments to investigate the regulation mechanism between the nuclease domain and two helicase domains of RecBCD enzyme using calcium ions, which specifically inhibit nuclease activity. In the absence of calcium ions, RecBCD translocation rate is found to slow down after recognizing chi sequence. However, in the presence of calcium ions, the rate change in individual RecBCD translocation is abolished, returning similar averaged translocation rate before (71 ± 20 bp/s) and post (81 ± 36 bp/s) chi-sequence, under 30μM ATP. Furthermore, large portion of individual RecBCD unwinding time courses (13 out of 32) revealed repetitive forward and backward translocation along individual DNA molecules. Compared with the experiments carried out without calcium ions, the processivity of RecBCD also decreases when the nuclease domain is inhibited. About 50 percent of translocating tethers (17 out of 32) stalled within 1.5 Kb DNA used in the presence of calcium ions. Together, these observations suggest that the nuclease domain, located in the RecB subunit, plays regulatory roles not only in RecBCD translocation properties but also in chi-regulated intersubunit interaction in this complex machine of the RecBCD enzyme.

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A Structural Model For RNA Remodeling By a Dimeric Dead Box Helicase

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DEAD box helicases couple ATP hydrolysis to RNA structural rearrangements. *T. thermophilus* Hera (heat resistant RNA-dependent ATPase) consists of a helicase core and a C-terminal extension. In single molecule FRET experiments we identified fragments of the 23S rRNA comprising hairpin 92 and RNase P RNA as substrates for Hera. RNA binding requires the C-terminal extension. Both substrates switch the helicase core to the closed conformation and stimulate the intrinsic ATPase activity of Hera. ATP-dependent unwinding of a short helix adjacent to hairpin 92 of 23S rRNA suggests a specific role for Hera in ribosome assembly, in analogy to the *E. coli*and *B. subtilis* helicases DbpA and YxiN. In addition, the specificity of Hera for RNase P RNA may be required for RNase P RNA folding or RNase P assembly.

Hera forms a stable dimer in solution, setting it apart form other helicases. Crystal structures show that the C-terminal extension is bipartite, forming a highly flexible dimerization motif with a novel fold and an additional RNA-binding module that adopts the fold of a degenerated RNA recognition motif (RRM). Comparison with RRM/RNA complexes suggests an RNA binding mode similar to that of the spliceosomal protein U1A. The structure-based model for the complete Hera dimer bound to RNA reveals a likely binding