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Complex Temperature Dependent Equilibria Dictate DNA Polymerase Exchange Processes during Synthesis

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Most organisms encode for multiple DNA polymerases with similar substrate affinities, but vastly different fidelities. Proper genomic maintenance by the high fidelity (PolB1) and lesion bypass polymerases (PolY) from *Sulfolobus solfataricus* involves a complex solution equilibria of protein complexes and specific recognition of appropriate DNA substrates. Using isothermal titration calorimetry (ITC) and temperature dependent electrophoretic mobility shift assays (tEMSAs), we have found differences in oligomeric assemblies of Dpo1 and Dpo4 on DNA that include unusually strong temperature dependence changes in heat capacity (ΔC_p), which switch from positive to negative values as temperature increases over a 60°C range. The thermodynamic data suggests that binding of PolB1 to DNA is favored over PolY by changes in solution multiequilibria (monomer-oligomer) with temperature that influence ΔC_p values. We have also followed polymerase exchange events between PolB1 and PolY and themselves and with the sliding clamp, PCNA, during active DNA synthesis using ensemble kinetic and fluorescence resonance energy transfer (FRET) assays. Surprisingly, the assembled PolB1 holoenzyme complex synthesizes DNA distributively and with low processivity, unlike most other well-characterized DNA polymerase holoenzyme complexes. Exchange between polymerases is temperature and concentration dependent process that is orchestrated by several contacts with PCNA. Simultaneous binding of PolB1 and PolY1 to PCNA allows for dynamic exchange of polymerase active sites during replication and lesion bypass synthesis. Taken together, our results distinguish between specific thermodynamic parameters including temperature dependent coupled equilibria and structural complementary; kinetic processes; and protein contacts that direct binding for uninterrupted but dynamic DNA replication and repair processes at high temperatures.

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Mechanistic Studies of DNA-Protein Interactions in Bacteriophage T4 DNA Replication Complexes at Single-Base Resolution

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Combining biophysical measurements on the function and control of T4 bacteriophage replication complexes with detailed structural information can throw light onto the mechanism of action of these 'macromolecular machines'. In this study we use the low energy circular dichroism and fluorescent properties of site-specifically introduced fluorescent base analogues to monitor the binding and interactions of gene 32 protein (gp32), the ssDNA binding protein of the T4 DNA replication complex, and gene 59 protein (gp59), the helicase loader protein involved in loading the T4 primosome complex onto replication forks. We show that gp32 binds preferentially to the 3'-end of a short ssDNA oligomer and that only two or three nucleotide bases at the 3' end are directly perturbed by the binding interaction, although the binding site size in the polynucleotide binding mode is 7 nts. Loss of exciton coupling in the CD spectrum and an increase in fluorescence intensity shows that gp32 binding causes extension of the sugar-phosphate backbone of the oligonucleotide. Mechanisms involved in gp32 binding to its ssDNA targets in both the isolated and the cooperative binding modes will be described. In addition our CD and fluorescence experiments with base analogue probes show the gp59 binding to a forked DNA construct perturbs the bases at the junction and suggests that once gp41 is loaded the perturbation extends deep into the double helix. Mechanisms whereby these proteins work in tandem in setting up various functional interactions with the other components of the DNA replication complex will be discussed.

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A Novel FRET-Based Structure of DNA Polymerase Complexed with Kinked Gapped-DNA

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DNA polymerase I Klenow Fragment (Pol) adopts multiple conformations during high-fidelity DNA replication and repair, many of which have not been resolved by conventional techniques because of their transient and dynamic nature. Early in the DNA repair process, Pol must recognise and bind a one-nucleotide-gapped DNA substrate. Both the structure of this complex and the recognition mechanism by which it is formed are currently unknown. After demonstrating the sub-angstrom accuracy of our distance measurements using duplex DNA standards, we present a single-molecule FRET-restrained structural model of the DNA-Pol complex and show that binding of the gapped-DNA is consistent with a conformational-selection mechanism.

Experiments on donor-acceptor-labelled gapped-DNA alone showed it adopted a low-FRET (unkinked) and a higher-FRET (kinked) conformation. Increasing concentrations of Pol stabilised the higher-FRET conformer, whilst also leading to the appearance of a third state, attributed to binding of a second Pol. We selected the DNA-Pol binary species from this dynamic equilibrium for structural characterization, obtaining >30 DNA-DNA and DNA-Pol distances, sufficient to restrain a unique molecular model of the complex (a structure which has not been resolved by NMR or crystallography). In this structure, the DNA exhibits a ~90 degree bend which is likely important for both the search for the site of polymerization and for subsequent catalysis.

To further investigate the Pol-DNA binding mechanism we developed a protein induced fluorescence enhancement (PIFE)-FRET coupled assay, which reported simultaneously on the DNA conformation and the presence of the Pol. This assay showed that all bound DNAs were kinked. Taken together, these data are consistent with a conformational-selection mechanism, in which the gapped-DNA substrate is recognised in its kinked state. This mechanism contrasts with experiments on the nucleotide binding, which we show to proceed via an induced-fit mechanism.

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Time-Resolved Plasmid Counting by Way of Transcription Factor Sequestration

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In contrast to the chromosome, genes expressed from plasmids exist in high copy number which changes continuously during the cell cycle. There are many models for how the control mechanisms associated with plasmid copy number dictate the cell-cycle dependent plasmid copy number, which have unique predictions for the dynamics of replication. We demonstrate a non-invasive method for dynamical quantification of plasmid copy number over the course of the cell cycle using the level of transcription from a reporter gene which shares a transcription factor with the plasmid. Using this method, we explore the cell-cycle dependent nature of plasmid replication for different origins of replication. Our method allows for discrimination of single-cell characteristics such as the single-cell division time and morphology and may permit discrimination of different replication mechanisms.

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Concentration-Dependent Exchange of Replication Protein a on Single-Stranded DNA Revealed by Single-Molecule Imaging

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Replication protein A (RPA) is a ubiquitous eukaryotic single-stranded DNA (ssDNA) binding protein necessary for all aspects of DNA metabolism involving an ssDNA intermediate, including DNA replication, repair, recombination, DNA damage response and checkpoint activation, and telomere maintenance. The primary role of RPA is to protect the ssDNA until it can be delivered to downstream enzymes. Therefore, RPA must bind very tightly to ssDNA, yet also be easily displaced from ssDNA to allow other proteins to gain access to the substrate. Here we use total internal reflection fluorescence microscopy and nanofabricated DNA curtains to visualize the behavior of Saccharomyces cerevisiae RPA on individual strands of ssDNA in real-time. Our results show that RPA remains bound to ssDNA for long periods of time when free protein is absent from solution. In contrast, RPA rapidly dissociates from ssDNA when free RPA is present in solution allowing rapid exchange between the free and bound states. In addition, the S. cerevisiae DNA recombinase Rad51 and E. coli single-stranded binding protein (SSB) also promote removal of RPA from ssDNA. Our results reveal an unanticipated exchange between bound and free RPA suggesting a binding mechanism that can confer exceptionally slow off rates, yet also enables rapid displacement through a direct exchange mechanism that is reliant upon the presence of free ssDNA-binding proteins in solution. We propose that the dissociation of RPA from ssDNA involves a partially dissociated intermediate, which exposes a small section of ssDNA allowing other proteins access to the DNA. The ability of RPA to directly ''hand-off'' ssDNA to other proteins before completely dissociating into solution would ensure that ssDNA intermediates remain continuously bound and protected while at the same time allowing downstream DNA processing enzymes to readily access the ssDNA.