Assembly dynamics of supramolecular protein-DNA complexes studied by single-molecule fluorescence microscopy
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Chapter 1

Introduction

Sarah Stratmann
1.1 DNA metabolism

“14% N, 5.8% P, 1.8% S.” This result of the chemical analysis of purified nuclei from leucocytes by Friedrich Miescher in 1871 (1) heralded a new era of research in physiological chemistry. It let the author speculate about a so far unknown chemical moiety that he named ‘nuclein,’ richer in phosphor and nitrogen than any other known protein. As it turned out, Miescher succeeded for the first time to extract DNA from cell nuclei. Following Miescher’s work, Albrecht Kossel decoded the chemical groups of nucleic acid, the five bases adenine, thymine, guanine, cytosine, and uracil. Remarkably, Kossel realized that proteinaceous mass was co-purified with the nucleic acids from nuclei, indicating a more complex and perhaps biologically relevant structure. He suggested a novel class of nucleic-acid associating proteins that he named histones (2). He postulated that these basic proteins, rich in lysine and arginine, might have transformed from ordinary proteins to interact with nucleic acids in ‘chromosomes’ (later called chromosomes) through ionic interactions (3).

Today we know that DNA-interacting proteins play roles in every aspect of DNA metabolism: A machinery of enzymes replicates DNA, specialized enzymes constantly repair errors in the bases to prevent genomic mutations, structural proteins package DNA into three-dimensional structures, and nucleases degrade DNA in the process of cell death. Besides all these enzymes involved in processing DNA, a large number of proteins and enzymes are involved in transcription and translation and the vast regulatory network associated with it.

This thesis deals with two topics in DNA metabolism, investigated using biochemical as well as single-molecule approaches: Assembly of DNA-replication complexes in E. coli, and the sensing of pathogenic DNA in mammalian systems. These two processes represent illustrative examples of the maintenance and processing of genomic material and the regulation required to control these processes. DNA replication is a highly regulated process in all organisms. In prokaryotes, nutrient supply represents a major factor triggering replication, whereas in eukaryotes, cell-cycle factors determine whether the cell-division phase is to be entered. The identification of pathogenic DNA also presents a tightly regulated mechanism in the context of propagation of genomic material, albeit a negative one. Eukaryotic cells have developed specific sensors that detect DNA and other forms of nucleic acid belonging to invading pathogens and that prevent their replication in the context of an inflammatory response. In the following sections I will provide a brief background summary on both topics and define the various research questions as a context for the work I present in this thesis.

1.2 DNA replication

Organisms have developed complex strategies to regulate the timing of replication initiation, to ensure that the replication of genomic material is timed correctly in the context of the cell-division cycle. In eukaryotes, multiple enzymes are involved in initiating the formation of a large number of replication complexes on various positions on the chromosomal DNA. The key proteins in this process are subject to a variety of cell-cycle dependent activation processes, such as phosphorylation by cyclin-dependent kinases leading to protein ubiquiti-
nation and degradation or export from the nucleus (4). In prokaryotes, regulatory systems of replication initiation have been shown to involve fewer regulatory components and are regulated more by the environment than an internal cell cycle. In rich media, for example, cells can grow with overlapping replication cycles, enabling cell-division times shorter than one round of genome replication. Here, rigid and global regulation of initiation allows simultaneous firing on partially replicated chromosomes (5).

Replication of the circular *E. coli* chromosome is initiated at a single origin of replication, a 245-bp specific sequence called oriC. Key regulator of DNA replication is the AAA+-family ATPase DnaA that binds to the five 9-bp long DnaA boxes within oriC. Oligomerization of ATP-bound DnaA along DNA induces positive supercoiling and local melting of the three nearby located AT-rich 13-bp long sequences called DUE (DNA unwinding element). This local unwinding then allows binding of two complexes that each consist of the replicative helicase DnaB and its loader protein DnaC (6-9). The next steps of replication depend on the concerted action of several key players at the replication fork. The ring-structured replicative helicase DnaB encircles single-stranded DNA and couples the energy released from nucleotide hydrolysis to directional movement and unwinding of the parental duplex. DNA polymerase III holoenzyme, a complex of eight different subunit proteins, synthesizes the two daughter DNA strands, starting from RNA primers produced by the primase DnaG (a detailed description of the bacterial replisome is given in Chapter 2).

Intriguingly, these replisomal machineries cannot be purified as intact complexes - in contrast to for example the macromolecular ribosome (10). The paradoxal situation of a complex that supports highly processive replication of millions of basepairs while being held together with weak interaction has been subject of recent research studies (11-13). These studies have shown that multi-site interactions within the replication complex seem to allow dynamic exchange of DNA polymerases and polymerase holoenzymes while ensuring robustness of the complex. With the classical picture of a stable replisome recently being challenged, the question arises whether the replicative helicase DnaB remains stably integrated in the replisome (14, 15) or whether it also dynamically exchanges protein components with those present in

![Figure 1: DNA replication in *E. coli*. A) At the oriC sequence, two replication complexes are established that progress bidirectionally along the chromosome, until they are stopped at the termination (Ter) sites. B) The initiator protein DnaA oligomerizes at the oriC, unwinding the AT-rich domains. Two complexes that each contain a DnaB helicase and DnaC helicase loader assemble onto the single-stranded region and trigger the formation of a pair of replisomes.](image-url)
solution. In this thesis, we challenged the stability of DnaB during loading and replication fork progression.

1.3 DNA sensing by the auto-immune system

Regulation of DNA replication initiation not only impacts the cellular division cycle, but also presents an important factor in combating invading pathogens and preventing parasitic replication. This process lies at the interface of DNA metabolism and cellular immunity. In both bacteria and eukaryotes, immune systems have been discovered that either act adaptively against invading pathogens and build an immunological memory against the respective organism, or that are encoded in the innate immune system, providing a direct and broader response.

In bacteria, the CRISPR/Cas system has been recently identified to serve as an efficient adaptive immune strategy that memorizes past invasions of bacteriophages (16, 17). An innate defense mechanism is provided by the restriction-modification system, relying on the expression of restriction enzymes that cleave specific sequences of non-methylated DNA (18). To protect the self-DNA, DNA methylase enzymes methylate the corresponding sequences within the genome immediately after replication.

Vertebrates possess highly complex adaptive and innate immune systems to respond to pathogenic threats. Whereas adaptive immunity is based on clonal gene rearrangements to express antigen-specific receptors, the innate immune system has been recognized as more general (19). Innate immune reactions present the first line of defense, with their activation upon pathogenic infection and cell invasion, and seem to be a prerequisite for activation of the adaptive immune system (19-21). Several classes of sensors, named Pattern-Recognition Receptors (PRRs), have been identified that detect structural elements of pathogens, so-called Pathogen-Associated Molecular Patterns (PAMPs), and that trigger signaling cascades for the expression of pro-inflammatory molecules. Usually, PAMPs are structural elements such as nucleic acids, surface glycoproteins, lipoproteins and membrane components of pathogens, each recognized by specialized PRRs (19). Pathogenic membrane structures such as glycoproteins are recognized by the immune system as foreign, since these structures normally do not exist inside the eukaryotic host cell. But how does a cell distinguish foreign DNA from its own genomic material? Different scenarios are possible: 1) an exclusively cytoplasmic localization of the PRR, 2) detection of specific motifs of pathogenic or damaged DNA such as unmethylated CpG islands, or 3) detection of structural properties of foreign DNA such as the lack of tight nucleosomal packing (20, 22). The strict discrimination between foreign and self-DNA is a substantial requirement for the proper activation of inflammatory reactions. As a consequence, auto-immune disorders often result from misregulation in the process of foreign-DNA detection or downstream processes (23, 24).

A number of DNA-recognizing PRRs have been reported to be confined to the cytoplasm, like AIM-2 (25), or to exclusively detect pathogen-specific DNA motifs such as CpG islands, like TLR-9 (26, 27). Amongst the different human PRRs that detect DNA, the IFI16 protein is unusual in that it is present within the cytoplasm as well as the nuclear region (28-30).
Pathogen detection by PAMP sensing. Pattern recognition receptors such as Toll-Like-Receptors (TLR's), AIM2, and IFI16 have as a key task the detection of certain foreign structures. IFI16 is present in the nucleus, where it senses pathogen DNA and triggers export of the DNA to the cytoplasm. STING and ASC/caspase-1 are assumed to interact with the IFI16-DNA complex and start the inflammatory response. First shown to be engaged in a p53-mediated apoptotic pathway (31, 32), IFI16 is now mostly thought to be involved in immune mechanisms against invading pathogens, such as Kaposi's sarcoma-related herpes virus (KSHV), HIV, listeria or salmonella (28, 33-35). IFI16 belongs to the interferon (IFN)-inducible p200-protein family, with two 200-amino acid long domains (HIN-A and HIN-B) at the C-terminus that interact sequence-independently with the sugar-backbone of the DNA (36), and an N-terminal Pyrin domain. It has been demonstrated that the tandem-arranged HIN domains each bind independently to dsDNA, whereas the Pyrin domain promotes protein-protein interactions (37). An N-terminal nuclear localization sequence enables transport to the nucleus, with lysine acetylations playing a regulatory role in the cellular localization (38).

Upon foreign-DNA detection, IFI16 activates an inflammatory response and restricts the replication and transcription of pathogenic DNA in infected cells. Orzalli and co-workers have shown that IFI16 mainly limits un- or underchromatinized viral DNA, when comparing the effects on transfected bare SV40 DNA with those seen in SV40 viral infection, with the viral DNA nucleosomally packed by host-cell histones (39). After detection of pathogenic DNA, the IFI16-DNA complex is exported to the cytosol and activates STING (stimulator of interferon genes) (40) and caspase-1 (41). Regulation mechanisms of IFI16-induced inflammatory responses are not understood in depth, however a cellular antagonist, the Pyrin-domain only protein (POP3), was shown to inhibit IFI16-mediated processes (42, 43). Together with in vitro analyses that proved that the Pyd domain is responsible for oligomerization, catalyzed by binding to DNA (37), inflammatory IFI16 action is assumed to rely on very specific aggregation on foreign DNA. How this detection and aggregation process is achieved, has been one of our main interests in this thesis.
1.4 Aim of this thesis

As seen from this brief overview, DNA replication, sensing, and control are key biological processes. In this thesis, using state-of-the-art fluorescence microscopy techniques and data analysis tools, we aim at unraveling main aspects of DNA metabolism on the single-molecule level. Real-time, non-invasive techniques such as single-molecule fluorescence imaging offer insights into transient biochemical steps in DNA interaction or enzymatic cycles and help us identify intermediates in DNA-protein complex assembly. We profit from classical biochemical techniques that enable the high-throughout generation of protein-DNA interaction maps (44, 45) and elucidate structural motifs of DNA sequence specificity by proteins by atomic resolution structures (46, 47), and bulk activity assays to resolve DNA metabolism by specialized enzymes. By observing at the single-molecule level assembly steps of the IFI16 inflammatory complex on DNA, as well as of the replicative DnaB helicase within the replisomal complex, we can quantify binding stabilities of single molecules versus protein complexes, characterize DNA-binding modes and search mechanisms along DNA.

In Chapter 2, we summarize biophysical developments that have contributed to our understanding of the fundamental mechanisms underlying DNA replication. We focus on technologies especially in fluorescence microscopy and their applications in single-molecule studies on different replication systems such as bacteriophage T7, *E. coli* and eukaryotic cells. We strengthen the impact of high-resolution techniques to build mechanistic models of multi-enzymatic and multiprotein reactions in detail, providing spatial and time information of intermediate reactions and protein interactions that cannot be achieved by ensemble averaging methods.

In Chapter 3, we implement single-molecule tools to characterize the dynamics of the bacterial helicase DnaB at the replication fork. We show that DnaB stably associates at artificial DNA forks and allows subsequent replisome assembly, thereby supporting efficiently replication initiation. Relative to the lifetimes of all other replisomal components and the discontinuity frequency during coupled replication, we show that DnaB remains stably incorporated within the replisome and thus may act as the central organizing hub within the replisome to provide its overall integrity.

In Chapter 4, we use a combined biochemical and biophysical approach to unravel the nucleic-acid detection mechanism of the human DNA sensor IFI16. We show that IFI16 scans along duplex DNA with a high one-dimensional diffusional velocity to search for other IFI16 molecules, eventually forming a multi-protein complex stably associated with DNA. This complex serves as signaling platform to trigger inflammatory pathways and is physiologically switched on upon pathogen invasion into the cellular nucleus. We show that highly specific sensing of foreign DNA relies on the increased reaction cross section that IFI16 can exploit on under-chromatinized genomes.

Chapter 5 comprises a methodological advance related to practical challenges encountered in observing DNA-protein interactions at the single-molecule level. One major hurdle in the research described in this thesis is the passivation of support surfaces to prevent nonspecific interactions between these surfaces and the protein-DNA complexes under study. In Chapter 5, we describe the development of a novel microfluidic chip design based on suspended
nanowires. We span gold nanowires across channels and apply a straightforward protocol for functionalization and biomolecule attachment. This configuration allows us to analyze protein-DNA associations and dynamics even at high protein number per DNA molecule. We use IFI16 diffusion and aggregation along DNA to demonstrate the advantages of the suspended nanowire configuration compared to established surface immobilization protocols in single molecule microscopy.

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