High fidelity DNA replication and repair: new structures and mechanisms using cryogenic electron microscopy

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Discussion

All animals, plants and fungi share a common ancestor, and though they have differentiated over billions of years of evolution, they all possess the essential machineries needed for cells to grow and divide. At the heart of this is DNA replication, the process that creates two identical copies the genetic material every time a cell divides. The fidelity of DNA replication is fundamental to prevent accumulation of mutations in the genome that can cause problems for long-term survival [1]. This result is achieved thanks to the replication and mismatch repair enzymes, that synthetize the daughter DNA strands and remove erroneously incorporated mismatches, respectively [2,3]. In this research we initially zoomed in on the molecular mechanisms involved in DNA mismatch repair, using a combination of structural biology, biochemical and biophysical methods. Next, we demonstrated how bacterial DNA polymerases can be exploited for the development of novel antibiotic to improve the treatment of bacterial infectious diseases. Finally, we described the development of a new plunge freezing technique that aims to improve the reliability of cryo-EM sample preparation together with the possibility to perform time resolved cryo-EM experiments.

DNA mismatch repair

First, we addressed a fundamental question in DNA mismatch repair, which is how ATP binding and hydrolysis drive the conformational changes in MutS that are needed to initiate the different steps of the mismatch repair cascade. For this we have determined four cryo-EM structures of *E. coli* MutS during sequential stages of the ATP hydrolysis cycle: bound to ADP, ATP, AMPPNP (a non-hydrolysable ATP analogue) and ADP-vanadate (a transition state analogue). The four structures presented here together with DNA binding and FRET experiments, provide a detailed understanding of the MutS ATPase cycle, and how this cycle is modulated by different signals on the DNA during the mismatch repair pathway. In absence of DNA the MutS dimer opens and closes upon ATP binding and hydrolysis. The closure of the dimer is blocked on homoduplex DNA and can only continue when MutS encounters a mismatch. Indeed, Mismatch binding induces small

rearrangement of the MutS domains that acts as a licensing step enabling the closing of the dimer into the sliding clamp state [4]. In the clamp state, the ATPase active sites are compatible with ATP hydrolysis, which would reset MutS to the open state. However, the MutS dimer is held close from the interaction with the DNA, allowing the recruitment of the MutL protein to continue the repair reaction. When MutS encounters ssDNA generated by helicases and exonucleases during the excision of the daughter strand, the interaction with the DNA is lost, and MutS is recycled for a new round of DNA binding and ATP hydrolysis.

Next, we focused on the final stages of the DNA mismatch repair pathway, which are the resection and subsequent resynthesis of the mismatch containing strand. In these steps the MutL protein, that is recruited onto the DNA by MutS [5,6], activates the helicase to unwind the DNA while an exonuclease excises the daughter strand. During this process a large section (up to 1 kilo base) of the newly synthesized strand is removed [7,8], which subsequently needs to be resynthesized by a DNA polymerase [7]. When resection and resynthesis take place in the same direction (5' to 3'), DNA synthesis can initiate while the strand containing the mismatch is being removed. In contrast, when resection occurs with a 3' to 5' direction, the action of the polymerase is opposite to that of the helicase and therefore needs to be prevented. The structure of the MutL-DNA complex reported in this thesis indicates that MutL could play a pivotal role in this regulation by binding to the 3' end of the resected DNA strand therefore blocking access of Pol I and Pol III to the DNA. As MutL also promotes the unwinding by recruiting the helicase UvrD, it acts as a 'traffic cop' on the DNA that blocks DNA access to one type of proteins, DNA polymerases, while promoting the action of another protein, the UvrD helicase.

Future research may shed more light on how the binding of MutL to the 3' resected DNA ends influences the resection process during DNA mismatch repair. It is well established that MutL enhances the processivity of UvrD to unwind the DNA [9, 10] , but how MutL can switch from the "polymerase protection mode" to the "helicase activation mode" is still unclear. One possibility could be that the binding of UvrD through the C-terminal MutL domains favours the dissociation of the N-terminal domains from the DNA and consequent initiation of the UvrD helicase activity. In addition to this, the binding of MutL to the 3' resected DNA end could also obstruct the release of MutS molecules from the ssDNA that ultimately must happen to cease the activation of all mismatch repair enzymes. The advent of modern cryo-EM that enables the structure determination of multiple structural states of a single or more proteins will be essential to decipher the complex functions of MutS, MutL and UvrD during the final steps of the mismatch repair process.

Developments of new antibiotics that target bacterial DNA polymerases

Chapter three describes an example of how DNA polymerases can be targeted for the development of novel antibiotics against *Mycobacterium tuberculosis* (Mtb). Mtb is one of the leading causes of death globally from an infectious disease [11], and the spread of drug-resistant strains has made the need to develop new antibiotics very urgent [12]. DNA polymerases have emerged as an attractive target for antibacterial drug discovery, due to the structural differences between prokaryotic and eukaryotic enzymes [13]. In the recent years, a series of inhibitors of the bacterial replicative polymerases PolC and DnaE have been reported, and one of them has recently entered clinical trials [14].

In this research we have demonstrated that nargenicin, a natural product that targets the replicative DNA polymerase of *Staphylococcus aureus*, is a bactericidal genotoxin that induces a DNA damage response and inhibits growth in *Mycobacterium tuberculosis*. We have shown that nargenicin mediates its bactericidal activity against *M. tuberculosis* through interaction with DnaE1 in a manner that depends upon the presence of the DNA substrate. In this interaction, the nargenicin molecule wedges itself between DnaE1 and the terminal base pair of the DNA and occupies the place of both the incoming nucleotide and the templating base. By analysing the physiological consequences of Mtb exposure to nargenicin, we show that the arrest in bacillary replication resulting from the nargenicin-DnaE1 interaction triggers induction of a DNA damage response coupled with an arrest in cell division and an apparent weakening of the mycobacterial cell envelope.

In addition to reaffirming the value of natural products as a source of novel anti TB agents, this work has provided the rationale for focusing target-led drug discovery efforts on promising new drug targets such as the DNA polymerases. Moreover, this work has confirmed how cryo-EM can be utilised to obtain high resolution structural information of inhibitors bound to their target protein. Current technological developments that enable high-throughput cryo-EM data collection and structure determination of multiple inhibitor-target complexes in a single data collection session, will revolutionize the way we performed structure-based drug design and will speed up the overall drug development process.

An air-blades based plunge freezer for improved cryo-EM grid preparation

Chapter four describes a new instrument for the preparation of cryo-EM grids, that can be used in cryo-EM laboratories to improve the efficiency of grid preparation. Considering the reliability and performance of modern electron microscopes and detectors, the determination of high-resolution cryo-EM structures often depends on the quality of the sample. Although multiple structures have been solved using standard ways of preparing grids, it is often required a time-consuming optimization process to find the perfect condition to vitrify the protein of interest [15,16]. This is because every protein has different biophysical properties and there is not, at the moment, a single condition that can always generate high quality grids. One of the main issue is the interaction between proteins and

the air-water interfaces, are demonstrated to be a surfaces of adsorption for proteins in solution, which in many cases can result in protein denaturation or preferred orientation [17,18,19,20,21,22]. Experiments performed with plunge freezers that can be as fast as 10-200 ms have shown that by reducing the time of residence of proteins in the thin layers of buffer, less denaturation and preferred orientational distribution occurs [23,18,24].

The instrument described in this thesis termed “Puffalot” is based on a new technique which is to use a stream of pressurized gas to remove the excess of buffer from cryo-EM grids. This allows a fast generation of thin layers of protein solutions on the grid in the fast time range of 100-200 ms. In addition to this, a fast and automated injection system is also present that allows the simultaneous deposition of two solutions on the grid surface. The double injection system and fast vitrification capabilities of the Puffalot were used to perform time resolved cryo-EM experiments with the mismatch repair protein MutS, that rapidly changes conformation in presence of ATP. As a future perspective, this approach will make it possible to perform a structural characterization on the entire mismatch repair cascade by trapping all the intermediate conformations that exist temporarily during the repair reaction.

This approach will be equally applicable to other DNA repair pathways, such as double strand break repair, nucleotide and base excision repair, and transcription coupled repair, as well as many other molecular pathways involved in diverse processes such as DNA replication, transcription and translation, intracellular transport, and signalling cascades. Such a 'molecular movie' database will generate a more comprehensive understanding of the many molecular machines that are essential for all life on earth.

References

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