Towards DNA-AddAB Interactions In Magnetic Tweezers

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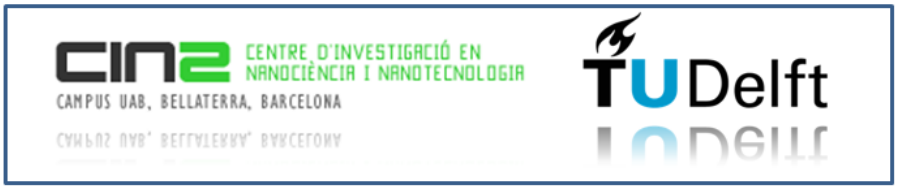


Abstract

Proteins belonging to the AddAB family of molecular motors are thought to be the functional analogues of RecBCD-like enzymes. These type of enzymes are responsible for the initiation of double strand break repair. In this report we discuss the results of a series of *in vitro* experiments carried out on Bacillus Subtilis′ AddAB proteins.

We demonstrated the ability of wild-type AddAB (wt-AddAB) and a biotynilated AddAB (BIO-AddAB) to degrade double strand DNA molecules. As a control experiment for future experiments in a magnetic tweezers setup, we also showed that BIO-AddAB is able to bind to streptavidin coated magnetic beads, thereby retaining its activity to process double strand DNA molecules.

Next to these *in vitro* experiments, we tested our magnetic tweezers setup, by carrying out force extension measurements on dsDNA molecules. By fitting the obtained data to the theoretical worm-like chain model, fit parameters for the persistence length P of double strand DNA and the length L of the molecule were obtained. In our exemplified experiment these values were P= 49.4nm and L= 7.37μm. These values show good agreement with the theoretical persistence length (P= 50nm) and our dsDNA molecule length (L $\approx$ 7.45 μm). We therefore conclude that the magnetic tweezers setup functions adequately.

Contents

Chapter 1

Introduction

1.1 DNA

Deoxyribonucleic acid (DNA) is the molecule in which the genetic information is stored. DNA is a nucleic acid, formed by two long chains of nucleotides twisted around each other in a double helical geometry. Nucleotides are the building blocks of DNA and consist of a nitrogenous base, linked to a sugar ring ($2'$-deoxyribose) to which a phosphate group is attached, as depicted in figure 1.1(a). DNA comprises four different nucleotides, each characterized by a different nitrogenous base: adenine (A), thymine (T), guanine (G) and cytosine (C). To form nucleic acids, nucleotides are linked to each other by a phosphodiester bond between the $3'$ and the $5'$ carbon positions of neighboring sugar rings, yielding the formation of large polymers. The orientation of a polynucleotide is defined by the number of the carbon atom not linked to another nucleotide of the polymer. This polymeric chain, involving the linkage of the nucleotides, is known as the primary structure of DNA.

The secondary structure of DNA is the above mentioned double helical geometry of the molecule. Two polynucleotide strands wind around each other forming a double helix, as shown in figure 1.1(b). Both helices are right-handed and run in an anti parallel direction with respect to each other, meaning that the orientation of their polymeric chains is opposed. The backbone of the molecule is formed by the sugar-phosphate chains, thereby minimizing the repulsion between the negatively charged phosphate groups. The two strands are held together by hydrogen bonds between complementary bases, forming so-called base

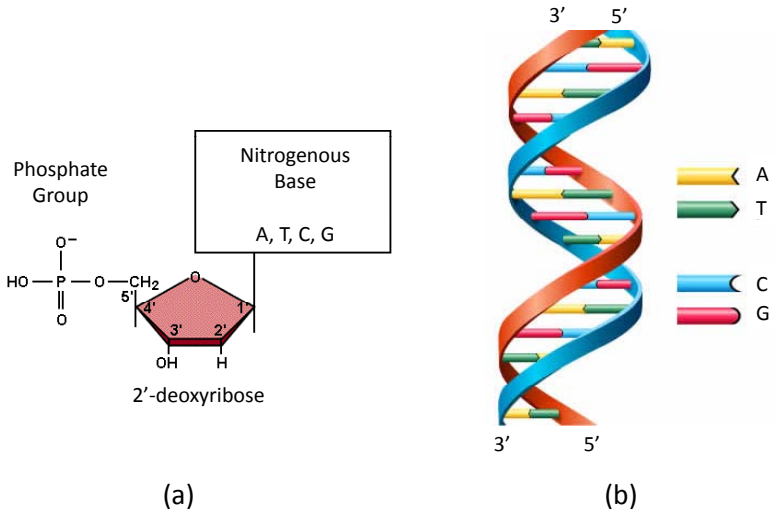


Figure 1.1: **(a)** *Schematic chemical representation of a nucleotide. A phosphate group is linked to the* $5'$ *carbon atom of* $2'$*-deoxyribose. A nitrogenous base, either Adenine (A), Thymine (T), Cytosine (C) or Guanine (G) is linked to the* $1'$ *carbon atom of the sugar ring.* **(b)** *Secondary structure of a DNA molecule, in which two nucleotide chains are twisted around each other, forming a double helical geometry. Complementary bases are linked to each other by hydrogen bonds.*

pairs: A is paired to T by two hydrogen bonds, while C is paired to G by three hydrogen bonds. This so-called complementary base pairing forms the core of the molecule and makes it possible for a single stranded DNA, to serve as a template for the synthesis of its complementary strand. This feature is essential in recombinational repair and replication of DNA.

1.2 Recombinational Repair

DNA damage is a serious thread for correct cell functioning. During a single day, up to 10^6 DNA lesions may occur, all caused by a large variety of endogenous and exogenous damaging sources. To counteract cell damage, cells possess different types of DNA repair mechanisms. One of these mechanisms is homologous recombinational repair: a method which promotes the exchange of polynucleotide strands between homologous DNA segments, finally leading to the reparation of double strand breaks (DSBs). DSBs can for instance be caused by the collapse of the replication fork, as a consequence of a single strand

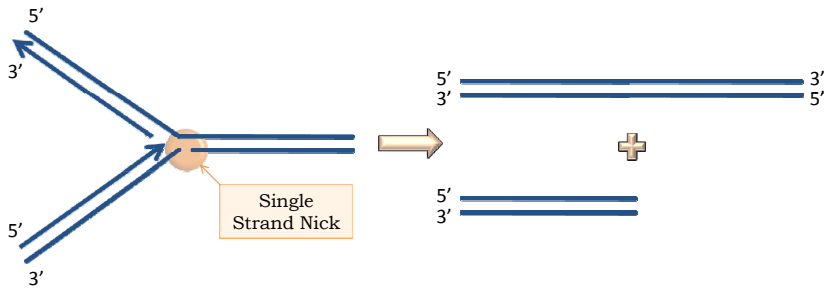


Figure 1.2: *Schematic representation of damage caused by the collapse of the replication fork. The presence of a nick in the leading strand, results in a double strand break.*

nick. A schematic representation of this process is depicted in figure 1.2.

Recombinational repair consists of four consecutive steps [1], as shown in figure 1.3. In this report we will focus on the first step of this process, in which the duplex DNA fragment is processed to obtain a 3′ single-strand overhang. The latter is a prerequisite for RecA loading, needed for the subsequent pairing and strand exchange with an homologous donor molecule, forming the second step of this DNA repair mechanism [2]. After the RecA nucleoprotein filament is formed, heteroduplex extension takes place, being the third step of the repair process. Finally, the last step involves the separation of the joined molecule complex.

1.3 DNA end processing by molecular motors

The first step of recombinational repair requires duplex DNA processing to obtain a 3′ single-strand overhang. This manipulation is done by molecular nanomachines such as helicases and nucleases. Helicase-nucleases are molecular motors, which can translocate along dsDNA, separate both strands and selectively degrade one of them.

The common mechanism of action of these enzymes is shown in figure 1.4. The molecular motor binds to a DNA end, translocates along the duplex separating both strands and producing random 'cleavage events' along the resulting single stranded DNA (exonuclease activity). After recognition of a specific nucleotide sequence - referred to as Chi - the exonuclease activity of the molecular motor is attenuated on one particular strand of the DNA molecule, finally pro-

ducing dsDNA fragments with 3′ ssDNA overhangs.

These motor proteins can be separated into two major classes of enzymes, depending on the type of bacteria considered: the RecBCD and the AddAB (or RexAB) family. The first group is found in Gram-negative bacteria, and are widely studied through Escherichia coli's RecBCD enzyme (for a review, see Kowalczykowski et al. 2000 [1]). The other group of enzymes is found in Gram-positive bacteria, although exceptions exist [3]. In Bacillus Subtilis - a Gram-positive bacteria - recombinational repair is initiated by an enzyme known as AddAB. The AddAB helicase-nuclease will be the main subject of this study.

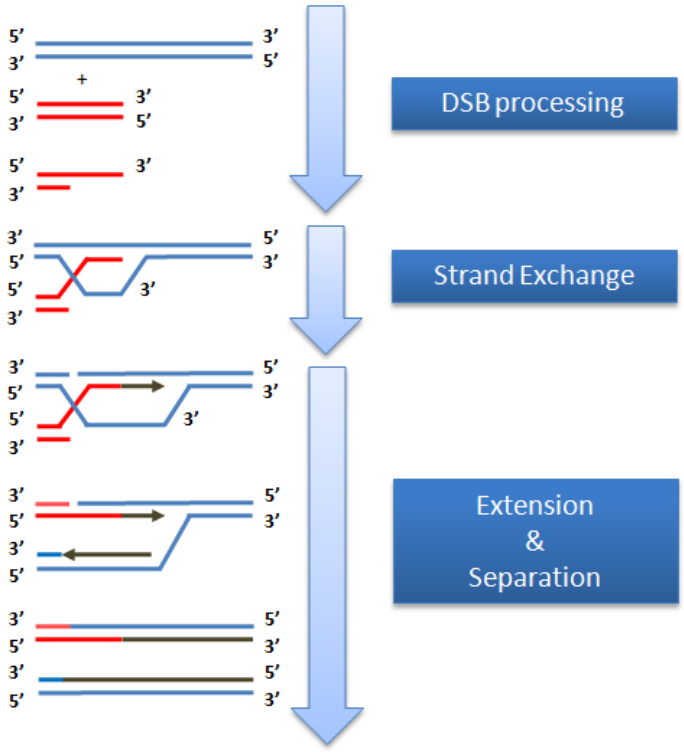


Figure 1.3: *Recombinational repair by homologous recombination. The duplex DNA fragment is first processed to obtain a long 3′ single-strand overhang. Rec A loading onto the single strand region of the DNA fragment forms a nucleoprotein filament which promotes strand exchange with a homologous donor molecule. Subsequently, two steps involving the extension of the DNA and the separation of the joined molecule complex finish the recombinational repair process.*

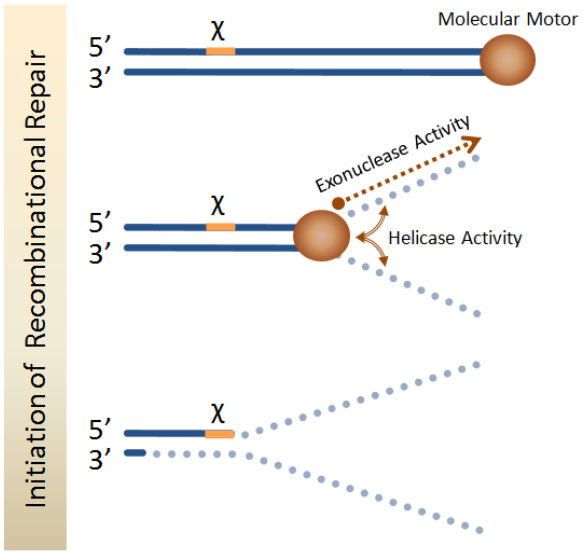


Figure 1.4: *General mechanism of action of molecular motors involved in the initiation of recombinational repair. A molecular motor, with both helicase, and exonuclease activity, translocates along a DNA molecule. Interaction with its cognate Chi sequence blocks the nuclease activity on the 3′ strand. After disassociation of the enzyme, a dsDNA fragment with a large 3′-ssDNA overhand remains.*

1.4 Goal and Outline

Chapter 2 describes the current knowledge of the AddAB molecular motor, comparing it to the well-studied RecBCD enzyme. The structure and activity mechanisms of these proteins will be discussed. In chapter 3 the the experimental techniques used in our experiments are explained. These are agarose gel electrophoresis and magnetic tweezers. Chapter 4 includes the results of our experiments. We carried out activity assays to check the functioning of the AddAB enzymes. These experiments form a series of preliminary experiments towards our final goal which is the determination of the translocation velocity of the AddAB helicase-nuclease along a single DNA molecule, using a magnetic tweezers setup. Proof of principle of the magnetic tweezers setup is reported in the second part of this chapter. Finally, we discuss the obtained results and the future steps to be taken, ending with the overall conclusions.

Chapter 2

The AddAB Molecular Motor

In Gram-negative bacteria, recombinational repair is initiated by RecBCD-like enzymes. It is widely suggested that the AddAB family of helicase-nucleases is the functional analogue of RecBCD in Gram-positive bacteria. Although both enzymes catalyze the same reaction, they show important structural differences. These structural dissimilarities suggest that both types of enzymes possess different mechanisms to catalyze the same reaction. Contrary to RecBCD, AddAB is a relatively young subject of investigation. In this chapter a general overview is given of the current knowledge of this protein. This is done by continuously comparing its features with the equivalent working mechanisms of RecBCD. The first part of this chapter is focused on the structure of these proteins, while the second part is dedicated to the activity of these molecular motors.

2.1 Structure

Composition AddAB The AddAB enzyme consists of the AddA and the AddB gene, yielding a two subunit protein with a total weight of 276 kDa. Figure 2.1(a), reproduced from Ref. [4], shows a schematic representation of the motif sequencing in AddAB. The addA subunit contains next to a nuclease motif, a 7-motif sequence, which can be attributed to the Superfamily I (SF I) of helicases. AddB contains a second nuclease motif and a walker A motif.

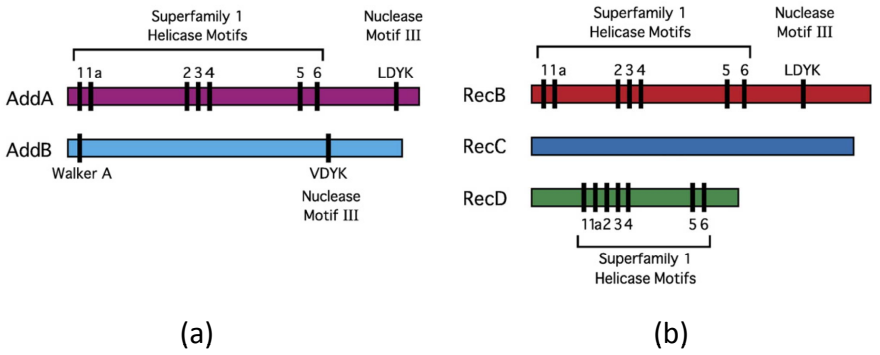


Figure 2.1: *Schematic representation of the motif organization of the AddAB and RecBCD proteins, reproduced from Ref. [4]* **(a)** *The AddA gene contains both a nuclease motif and a* 7*-motif sequence, which is characteristic for Helicase Superfamily I (SF I). The AddB subunit contains next to another nuclease motif, also a walker A motif.* **(b)** *RecBCD consists of three different subunits: RecB, RecC and RecD. RecB shows a similar motif organization as the AddA gene, containing both a SF I helicase motif and a single nuclease motif. A second SF I helicase motif is located in the RecD subunit.*

Composition RecBCD Next to the domain organization of AddAB, figure 2.1(b) shows the motif sequencing of RecBCD. This heterotrimeric molecular motor with a mass of 330 kDa consists of three different subunits: RecB, RecC and RecD. RecB contains a SF I helicase motif group next to a single nuclease motif, thereby showing major resemblance to the motif organization of the AddA gene. A second SF I helicase group is located at the RecD subunit.

AddAB vs RecBCD When comparing the motif organization of both proteins, two important structural differences become apparent. On the one hand, AddAB contains two nuclease motifs, whereas RecBCD only contains one of these nuclease groups. On the other hand, RecBCD possesses a second helicase motif, while in addAB only one of these helicase groups is located. These structural dissimilarities suggest that both enzymes process dsDNA molecules using distinct working mechanisms.

2.2 Working Mechanisms

2.2.1 RecBCD Activity

We next discuss the working mechanism of RecBCD. This protein binds non-covalently to a dsDNA end. Subsequent ATP hydrolysis causes the enzyme to translocate along the DNA molecule, thereby unwinding the two strands of the DNA molecule. A schematic representation of the proposed mechanism used by RecBCD to produces dsDNA fragments with long ssDNA 3′-overhands, is depicted in figure 2.2.

Bipolar Translocation RecBCD′s pair of helicase motifs is responsible for a bipolar translocation mechanism, in which forward translocation of the two -

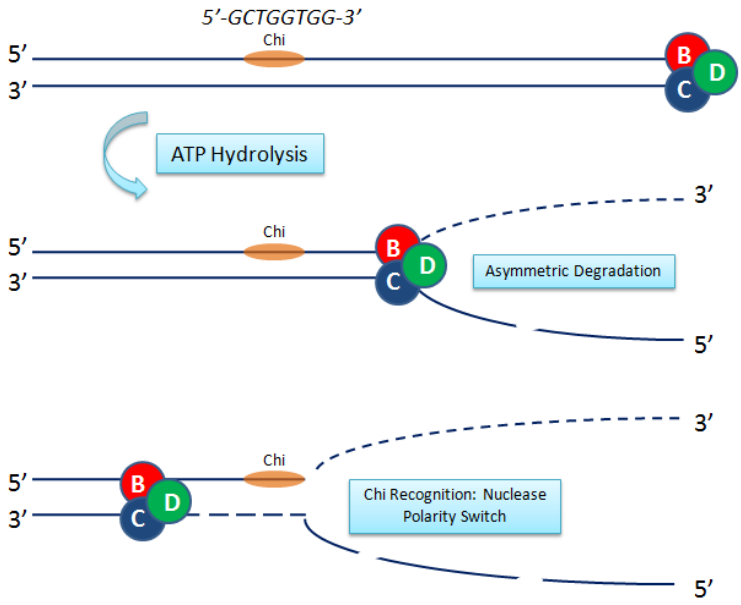


Figure 2.2: *Proposed activity mechanism for the RecBCD enzyme. Energy derived from ATP hydrolysis is used to translocate along the DNA molecule, thereby seperating the two ssDNA strands of the molecule. Prior to Chi recognition the nuclease activity of this hetero trimeric enzyme is characterized by vigorous degradation of the* $3' \rightarrow 5'$ *strand, while the* $5' \rightarrow 3'$ *strand suffers from less intensive nuclease activity. Upon recognition of the Chi sequence (5′-GCTGGTGG-3′), a nuclease polarity switch takes place, preventing degradation of the 3′-end, while nuclease activity on the contrary strand is intensified.*

oppositely polarized - helicase motors unwind dsDNA molecules [5, 6]. The high and unprecedented rate of translocation with which this process takes place, is most probably explained by the presence of two SF 1 helicase motifs.

Nuclease Activity During translocation it is assumed that the single nuclease domain is able to degrade both ssDNA strands. The crystal structure of RecBCD [7] supports this statement, since it reveals that the nuclease motif finds itself positioned near the back of the protein, making access to both ssDNA strands possible. Before Chi recognition, the nuclease activity shows a clear preference for single strand cuts on the $3' \rightarrow 5'$ strand [8].

Chi Recognition Upon recognition of RecBCD's Chi sequence, defined by 5′-GCTGGTGG-3′ [9], degradation of the $3' \rightarrow 5'$ strand is strongly attenuated, thereby changing the polarity of the nuclease activity [8]. The latter leads to an enhancement of the nuclease action on the $5' \rightarrow 3'$ strand. After dissociation of the protein from the DNA the final product is a duplex DNA fragment with a long 3′ ssDNA overhand.

2.2.2 AddAB Activity

Similarly to RecBCD, AddAB is a powerful ATPase, which processes dsDNA molecules using Chi-regulated recognition in combination with helicase and nuclease activities [10].

Helicase Activity The actual unwinding mechanism used by this enzyme is not entirely understood yet. A clear SF I helicase group located in the AddA subunit, suggests that a single-motor helicase mechanism is responsible for unwinding dsDNA molecules. However, the presence of a Walker A motif might indicate otherwise. This motif is commonly encountered in helicase superfamily groups and is vital for ATP hydrolysis: implication of AddB in helicase activities is therefore possible. The crystal structure of AddAB will provide new insights into the exact translocation mechanism of AddAB.

Dual-nuclease Mechanism The presence of a nuclease domain in each subunit of AddAB, together with the observation of symmetric nuclease activity before Chi recognition [10], led to a widely supported proposal concerning the AddAB nuclease mechanism [11]. It was stated that that each subunit of this

molecular motor is responsible for the degradation of a single ssDNA strand. The validity of this proposed dual-nuclease mechanism has not been demonstrated until recently, attributing the cleavage of the $3' \rightarrow 5'$ ssDNA strand to the AddA subunit, while the exonucleolytic degradation of the opposite strand is carried out by the AddB subunit [4].

Interaction with Chi Contrary to RecBCD's longer Chi sequence, the recognition hotspot of AddAB is a - smaller - 5 base pair sequence, defined by 5′-AGCGG-3′ [12]. Recognition of Chi by AddAB occurs during translocation, allowing AddAB to form a very stable - non-covalent - bond with this recognition hotspot [13]. After Chi recognition the nuclease activity on the $3' \rightarrow 5'$ strand is strongly attenuated, while degradation of the strand in the $5' \rightarrow 3'$ direction is not affected. This mechanism is totally different when compared to the ob-

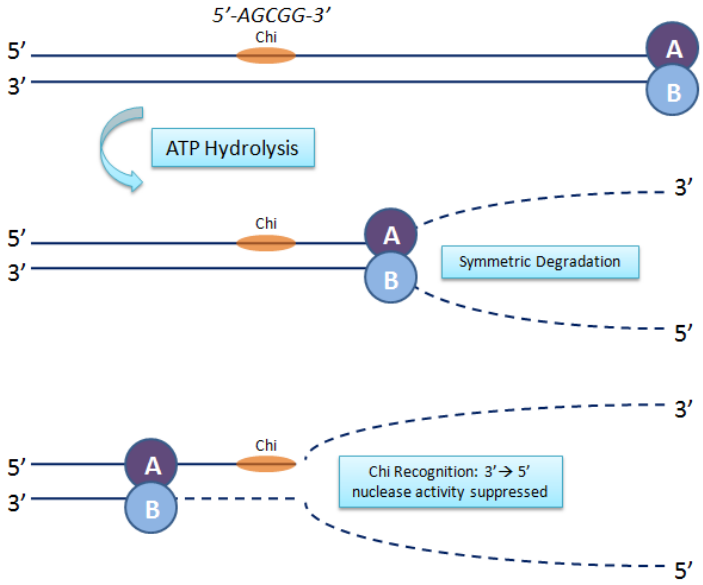


Figure 2.3: *Schematic representation of the proposed activity mechanism of the AddAB molecular motor. This two subunit protein uses energy derived from ATP hydrolysis to translocate along dsDNA molecules, while unwinding the double helical geometry of the molecule into separated ssDNA strands. Degradation of the two ssDNA strands is symmetric: each subunit of this protein contains a nuclease motif, which is responsible for the degradation of a single strand. Upon Chi recognition (5′-AGCGG-3′), the nuclease activity of the $3' \rightarrow 5'$ strand is strongly attenuated, while degradation of the opposite strand is unaffected.*

served nuclease polarity switch in RecBCD. A schematic drawing of the proposed working mechanism of the AddAB molecular motor is showed in figure 2.3.

Chapter 3

Experimental Techniques

3.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a technique used for size-dependent separation of different DNA molecules. An agarose gel consists of a matrix through which DNA molecules are able to move. The technique is based on the ability of a charged particle to move under influence of an electric field. Since DNA molecules are negatively charged, application of an electric field causes the molecules to move through the gel. The velocity at which they move, depends on the molecule′s size, shape and charge density [14]. Since their shape (linear) and charge density are usually constant, the velocity normally only depends on the size of the molecules. Staining the agarose gel with DNA fluorescent intercalating agents, makes it possible to visualize the DNA. A schematic representation showing the process of agarose gel electrophoresis is depicted in figure 3.1[1].

3.2 Magnetic Tweezers

Magnetic tweezers (MT) provide experimentalists with a single molecule technique, based on the trapping of a micrometer-sized magnetic bead in the gradient of an externally applied magnetic field. The bead is attached to a coverglass surface with a single molecule, making real-time tracking of the DNA dynamics possible.

[1]Image obtained from http://www.biochem.arizona.edu

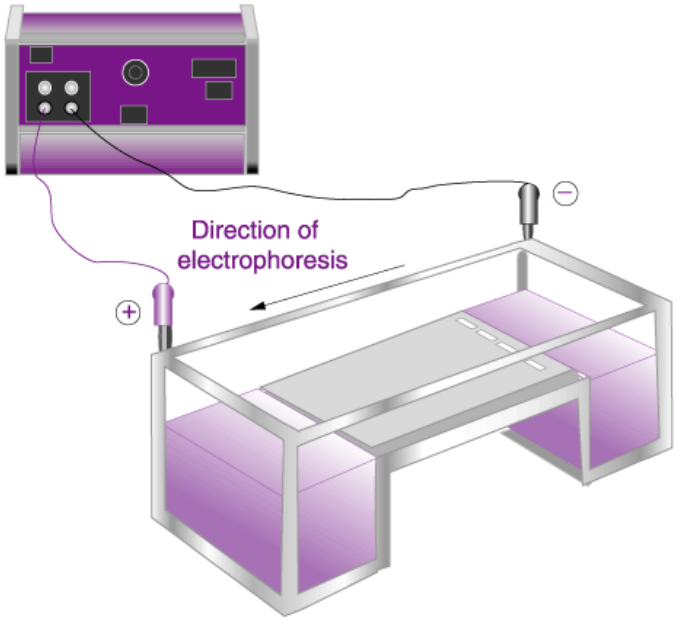


Figure 3.1: *Schematic drawing of an agarose gel electrophoresis setup. Samples are injected into the gel, which forms a matrix through which DNA molecules are able to move. Movement of the negatively charged DNA molecules is achieved by applying a static electric field across the agarose gel. Since smaller DNA molecules move faster through the gel, this method allows for separation of molecules with the same size. Visualization of the separated molecules is achieved by gel-staining and UV transillumination.*

3.2.1 The Apparatus

A MT setup similar to the one described in Ref. [15] was used in this study. MT experiments are carried out in a fabricated liquid cell, made out of two glass cover slits, thermally sealed by a 200 μm thin parafilm layer. The upper glass plate has two holes, serving as an inlet and outlet for liquid substances. The volume of the liquid cell is approximately 100 μl. In the cell, a linear dsDNA molecule is tethered by clamping one of its ends to the bottom of the cell, while the other end is attached to the magnetic bead, as shown in figure 3.3(a).

An optical image of the cell is obtained by illuminating the cell with a parallel light source, which is placed above the cell; the lower cover slit is contacted to an oil immersion objective, which is held by a piezoelectric stage. The latter allows the vertical movement of the objective′s focal plane. A CCD camera,

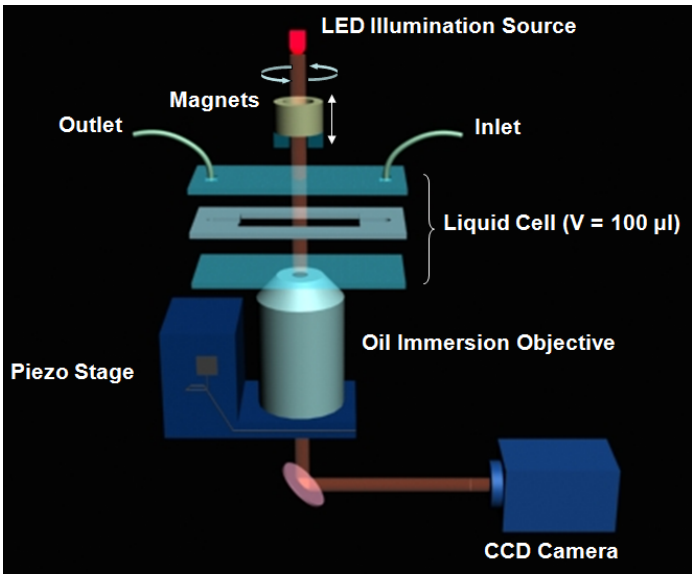


Figure 3.2: *Schematics of a magnetic tweezers (MT) setup. A liquid flowcell (volume* 100 *µl), consisting of two glass cover slits, sealed to each other by a parafilm mold, is placed into the optical path of a LED illumination source. The cell contains an inlet and an outlet, which can be used to flush the cell with liquid substances. In the cell a molecule is tethered between the bottom of the cell and a micrometer-sized bead. A magnetic field generated by two magnets, which are placed just above the liquid cell, is used to apply stretching forces on the molecules. Vertical and rotational movement of the magnets, makes it possible to change the magnitude of the applied force and supercoil DNA molecules, respectively. The bottom of the cell is contacted to an oil immersion objective, which is held by a piezo movement stage. Placement of a CCD camera in the optical path, allows a computer to acquire bead images. These are used to determine the mean vertical position of the bead, as well as the Brownian motion of the bead.*

placed at the end of the optical path, finally obtains the image of the cell. A computer is then used to acquire the images, visualizing the bead, whereafter the obtained data is analyzed. A schematic representation of a MT setup is shown in figure 3.2.

Stretching Forces A stretching force on the molecule is applied by the gradient of a static magnetic field, caused by two magnets embedded in a holder, which is positioned just above the liquid cell. The holder is attached to a motor which is able to displace the magnets in a vertical direction, such that the force

acting on the bead can be varied: bringing the magnets near the liquid cell, increases the stretching force on the molecule, while moving the magnets away from the cell, decreases the stretching force acting on the bead.

Torsional Strain A second motor, which is connected to the magnet holder, is able to rotate the magnets with respect to each other, producing perfect rotation of the magnetic bead, such that a torque can be applied on the molecules. Note that the torque imposed by the rotation of the magnetic field on the bead is much larger than the torque applied by a DNA molecule on the magnetic bead, making the latter negligible. Application of a torque on a dsDNA molecule makes supercoiling of the DNA possible, as shown in figure 3.3(b), provided that the two strands of the DNA helix do not contain any single strand nicks. If the latter requirement is not met, the molecule can freely rotate around the covalent bond opposing the nick-site, thereby immediately releasing the torque imposed by the rotating bead, preventing supercoiling.

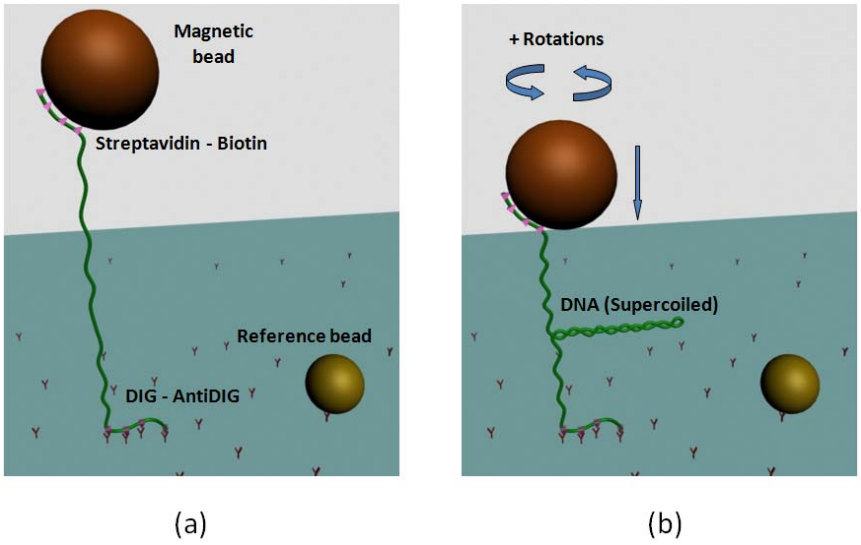


Figure 3.3: **(a)** *A DNA molecule is attached by a DIG-antiDIG bond to the surface of the liquid cell. The other end is attached to a streptavidin-coated magnetic bead. To achieve bondage to the bead, this end of the molecule is labeled with BIO molecules, which are known to form strong complexes with streptavidin. The reference bead, attached to bottom of cell, is used for relative height measurements.* **(b)** *By rotating the magnets with respect to each other, supercoiling of a nick-free DNA molecule becomes possible.*

Sample Assembly Biochemical techniques, including polymerase chain reaction (PCR) and ligation reactions, enable us to fabricate DNA molecules, with biotin (BIO) and digoxigenin (DIG) labeled handles on each of their ends, respectively (see figure 3.3). The magnetic beads are coated with streptavidin, a protein which binds to biotin. On the other hand, DIG binds to antibodies (anti-DIG), adsorbed on the bottom glass coverslit, making anchoring of the molecule to the surface possible. Body-antibody complexes are able to withstand forces up to 100 pN [16], which are substantially larger than the typically applied stretching forces (0.01 - 10 pN). After fabrication of the DNA-bead complex, the solution is injected into the liquid cell, after which the DIG-labeled ends of the DNA molecules are anchored to the anti-DIG-coated bottom of the liquid cell. A sketch of a tethered molecule in a magnetic tweezers setup is depicted in figure 3.3(a).

3.2.2 Measurement of Physical Quantities

Height measurements All height measurements are carried out by continuously monitoring and comparing the intensity of the diffraction rings of two distinct beads. The first bead is the one to which the molecule is attached; the second bead is a reference bead which is attached to the bottom of the liquid cell (see figure 3.3). To do this a diffraction ring intensity profile is made of each bead by moving the objective′s focal plane in a vertical direction, thereby measuring the intensity of the diffraction rings as a function of position of the objective′s focal plane. These calibration files are made at a fixed stretching force. The resulting calibration files are used during measurements in combination with an interpolation algorithm to determine height difference between the two beads. This can be done with a spatial resolution of 10 nm [17]. This relative-height measurement minimizes any effect induced by the drift of the setup′s components, since the DNA bead and the reference bead are evenly affected by any form of drift.

Force Measurement The force applied by the magnetic field on the bead is determined by analyzing the Brownian motion of the bead. Random collisions of water molecules on the bead displace the bead out of its equilibrium position, with an amplitude that strongly depends on the applied force: the larger the applied stretching force, the smaller the amplitude of the Brownian motion is and vice versa. The situation is schematically depicted in figure 3.4: for small

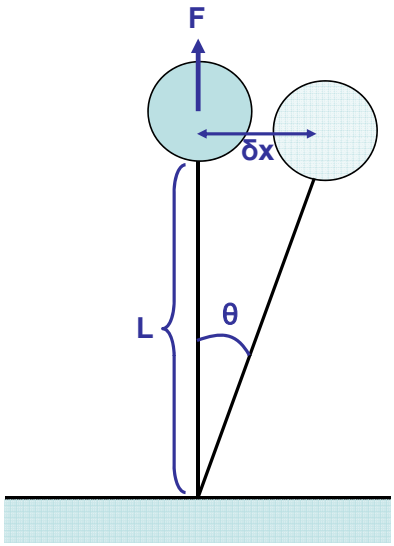


Figure 3.4: *After application of a stretching force F, yielding an extension length L of the molecule, the bead is displaced with respect to its equilibrium position by a distance δx, under influence of Brownian motion. The magnitude of the force is given by* $F = \frac{k_B T L}{<\delta x^2>}$*: by measuring both L and δx the value of the stretching force can be calculated.*

values of θ a restoring force F_r, which can be approximated by $F \cdot \theta$, acts on the bead, in which F is the magnitude of the upward stretching force applied by the magnetic field. If the displacement amplitude $\delta x << L$, the restoring force can be rewritten as $F_r = F\frac{\delta x}{L}$. Treating this system as an elastic spring, which motion is described by Hooke′s law, an effective spring constant κ_R is introduced, which equals $\frac{F}{L}$. The elastic energy of this spring is then given by $\frac{1}{2}\kappa_R < \delta x^2 >$, in which $< \delta x^2 >$ is the mean square displacement of the Brownian motion. This energy equals the energy provided by the Brownian motion, being equal to $\frac{1}{2}k_B T$. After rearrangement of terms this yields an expression for the stretching force F given by:

$$F = \frac{k_B T L}{< \delta x^2 >}. \tag{3.1}$$

By measuring the mean square displacement of the bead, a value for the stretching force F can be obtained. A disadvantage of this technique is that long measurement times are needed to accurately obtain a value for $< \delta x^2 >$. The

mean squared displacement is measured experimentally by Fourier transforms of the bead′s Brownian motion, as done by Strick [18].

Chapter 4

Results

In this chapter we report on the results of our experiments. The first part of the chapter is focused on activity assays to test the activity of the AddAB proteins. The second part discusses the tuning of the magnetic tweezers setup: a single molecule technique that is fundamental for our future studies on protein-DNA interactions.

4.1 AddAB Activity Assays

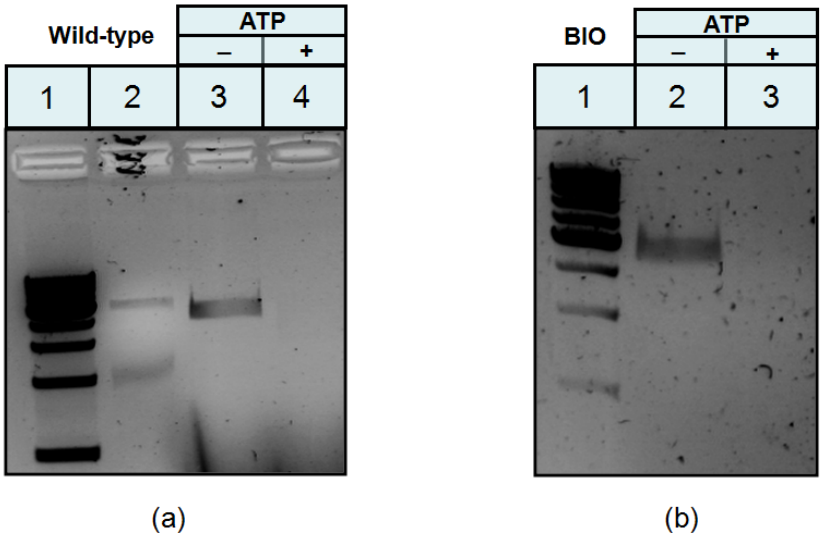
Wild-type AddAB (wt-AddAB) and an equally functional biotynilated version of this protein (BIO-AddAB) were provided by the Dillingham group (Department of Biochemistry, University of Bristol). To test the activity of these molecular motors we carried out a series of *in vitro* activity assays and used agarose gel electrophoresis to analyze the results.

The proteins were incubated with a linear dsDNA substrate (DNA-χ^0) that does not contain any Chi recognition spots in its nucleotide sequence. Processing of DNA-χ^0 by AddAB should therefore lead to fully degraded dsDNA. The dsDNA fragment was 5290 base pairs (bp) long and contained 4 bp 5′-overhands on each of its ends. In figure 4.1, recorded images of two agarose gels are depicted, showing the results of the activity assays of wt-AddAB and BIO-AddAB, respectively.

4.1.1 Wild-type AddAB

Standard activity assays of wt-AddAB consist of the incubation of wt-AddAB together with DNA-χ^0, using a 5:1 particle ratio, respectively. These experiments are analyzed through agarose gel electrophoresis. When sufficient ATP is present in the reaction volume, we expect wt-AddAB to process the DNA substrate. This is translated into the absence of the DNA band representing the initial DNA-χ^0 substrate in the agarose gel.

The result of this wt-AddAB activity assay is depicted in figure 4.1(a). In



Activity Assays wt-AddAB and BIO-AddAB

Figure 4.1: **(a)** *The result of a standard activity assay in which the activity of wild-type AddAB (wt-AddAB) is examined. When no ATP is added to the reaction volume, the initial DNA substrate is not processed by the protein, yielding a clearly observable DNA band in lane* 3*. Incubation of wt-AddAB and DNA-*χ^0 *in the presence of ATP leads to the disappearance of this band. In lane* 2 *the results of a heat denaturing experiment can be observed. Next to a band that stands for the double stranded DNA-*χ^0 *substrate, a second band is observed that corresponds the corresponding ssDNA strands of this substrate. Finally, lane* 1 *shows a* 1 *kbp dsDNA reference ladder.* **(b)** *The same experiment as explained in subfigure (a) is carried out using the biotynilated version of AddAB (BIO-AddAB). The observation of a high-intensity DNA band in lane* 2 *indicates that BIO-AddAB does not show any sign of activity when no ATP is added to the reaction volume. Addition of ATP to the reaction leads to the disappearance of the dsDNA band in lane* 3*.*

lane 4 the experiment is carried out in the presence of ATP. As expected, this lane shows the absence of DNA-χ^0. The result of the control experiment in the absence of ATP (lane 3) shows the unprocessed DNA product. The detailed reaction conditions of these experiments are reported in appendix A.1.

If the nuclease activity of wt-AddAB is blocked, the presence of only helicase activity should yield ssDNA fragments, with the same length as the initial DNA-χ^0 substrate. Therefore, to proof that the protein shows ***both*** helicase and nuclease activity, we needed demonstrate that our agarose gel is capable of visualizing ssDNA, when equally sized ssDNA fragments are present at relatively high concentrations. We therefore included a heat denaturing experiment (appendix A.2) in lane 2 using the same amount of DNA-χ^0, as used in the lanes 3 and 4. It can be clearly observed that ssDNA fragments can be visualized. We can therefore attribute the observed activity of wt-AddAB in lane 4 to both helicase and nuclease activity.

Finally, lane 1 shows a 1 kbp dsDNA ladder used for DNA size comparison.

4.1.2 BIO-AddAB

Next, we carried out an equal experiment using BIO-AddAB to check the activity of the biotynilated protein. As can be observed in lane 3 of the agarose gel image depicted in figure 4.1(b), incubation of BIO-AddAB and DNA-χ^0 in the presence of ATP yields the disappearance of the band that represents the initial DNA substrate.

The result of the control experiment in which no ATP was added to the reaction volume can be observed in lane 2. As expected, the band representing the unprocessed DNA-χ^0 substrate is clearly visible. We therefore assign the observed activity of BIO-AddAB to correct functioning of both the helicase and nuclease behavior of the protein. For the reactions conditions we refer to appendix A.1.

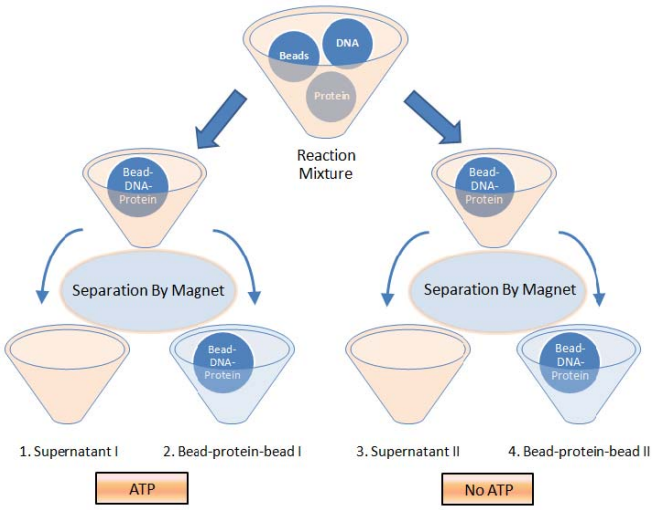
Again, lane 1 represents a util 1 kbp dsDNA ladder for DNA size analysis.

4.2 AddAB-Bead Activity Assays

Next, we tested the ability of biotynilated AddAB to process DNA when bound to streptavidin coated magnetic beads. This is a prerequisite to proceed to future MT experiments. Our goal was twofold. Firstly, to check if BIO-AddAB binds to streptavidin coated beads. Secondly, if when bound to the bead, the biotynilated enzyme retains its ability to process DNA. To find an answer to these questions, we carried out a series of *in vitro* experiments, which are discussed in the next sections.

4.2.1 BIO-AddAB

To carry out this experiment, we incubated in the absence of ATP the previously used DNA-χ^0 substrate, together with BIO-AddAB and streptavidin coated magnetic beads in our standard reaction buffer. The ratio dsDNA-ends/BIO-AddAB/Beads was approximately $20 : 50 : 1$. We considered that subsequent



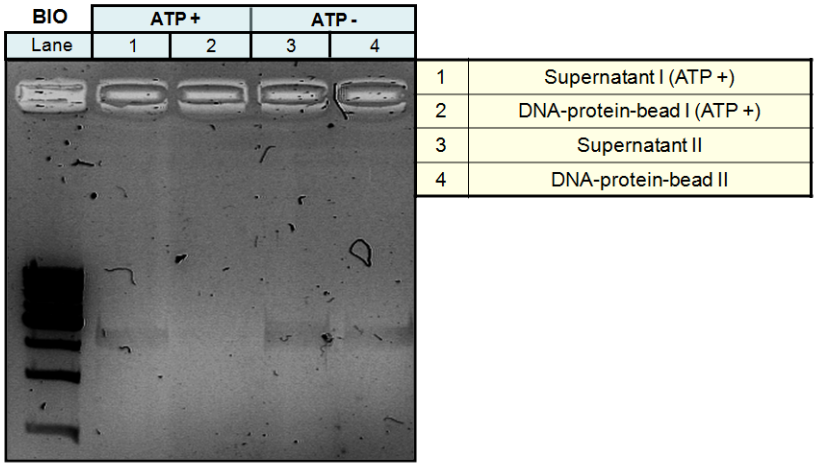
AddAB-Bead Activity Assay Experiment

Figure 4.2: *A reaction mixture was prepared containing BIO-AddAB, DNA and streptavidin coated magnetic beads. After splitting the mixture in two equal volumes, a magnet was used to separate the formed Bead-DNA-protein complexes from the remaining supernatant. Next, activity assay experiments were carried out adding ATP containing buffers to aliquots* 1 *and* 2 *and no-ATP containing buffers to aliquots* 3 *and* 4.

incubation of this mixture, should lead to the formation of complexes in which the BIO-AddAB binds to the streptavidin coated beads and to one of the ends of the DNA molecules. To test the activity of these complexes we followed the following procedure.

The mixture was first split into two aliquots of equal volume. The DNA-protein-bead complexes were separated from the supernatant using a magnet, obtaining a total amount of four tubes. Aliquot 1 contained the supernatant and aliquot 2 the DNA-protein-bead complexes. Aliquots 3 and 4 contained the same as aliquots 1 and 2, respectively. A clear overview of this procedure is depicted in figure 4.2.

Next, we added ATP containing buffers to aliquots 1 and 2 and non-ATP containing buffers to aliquots 3 and 4. After addition of ATP, a reaction time



BIO-AddAB-Bead Activity Assay

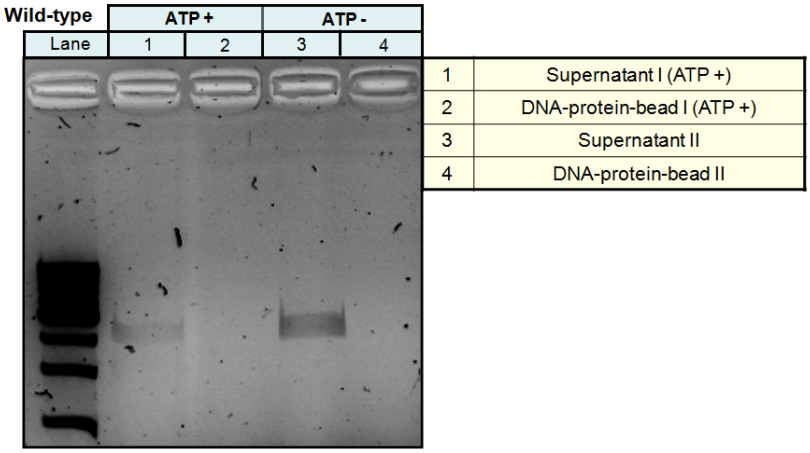
Figure 4.3: *The agarose gel image shows the results of an experiment in which the activity of BIO-AddAB is tested, while bound to micrometer-sized streptavidin coated magnetic beads. Complexes were fabricated in which BIO-AddAB proteins were bound to both the magnetic beads and to the end of dsDNA molecules. After separation of the complexes from the supernatant both remaining reaction volumes were tested on protein activity. Activity of BIO-AddAB is demonstrated by comparing lanes* 2 *and* 4*. The same reactions conditions with the presence of ATP as the only variable shows that BIO-AddAB retains its activity, while being a part of these complexes; this can be deduced from the disappearance of the DNA band in lane* 2*. Similar intensities of the observed bands in lanes* 1 *and* 3*, suggest that all BIO-AddAB is bound to the magnetic beads.*

of 10 minutes was implemented at a temperature of 37°C. For detailed reaction conditions we refer to appendix A.3. The resulting samples were run through an agarose gel. In figure 4.3 the results of this experiment are depicted.

All lanes, with the exception of lane 2 showed the presence of DNA. The absence of DNA in lane 2 suggests that the activity of the BIO-AddAB is conserved even when the protein is bound to the streptavidin coated magnetic beads. This statement is enforced by the presence of DNA in lane 4 in which the absence of ATP is the only difference with respect to the reaction carried out in aliquot 2. Furthermore, roughly equal intensities of the bands observed in lanes 1 and 3 suggest that there was no BIO-AddAB present in aliquot 1. This would imply that all proteins were bound to the magnetic beads.

4.2.2 Wild-type AddAB

As a control experiment, the same experiment was carried out using wt-AddAB instead of BIO-AddAB. The results are shown in figure 4.4. In lanes 2 and



wt-AddAB-Bead Activity Assay

Figure 4.4: *An identical experiment as discussed in figure 4.3 was carried out using wild-type AddAB (wt-AddAB). The depicted image of an agarose gel represents the result of this experiment. In lane* 2 *and* 4 *no DNA is observed, indicating that wt-AddAB is unable to bind to streptavidin coated beads. Therefore, all wt-AddAB is expected to be located in the remaining supernatants. Incubation of this supernatant with ATP shows signs of protein activity, since the intensity of the DNA band observed in lane* 1 *is less bright than the band observed in lane* 3.

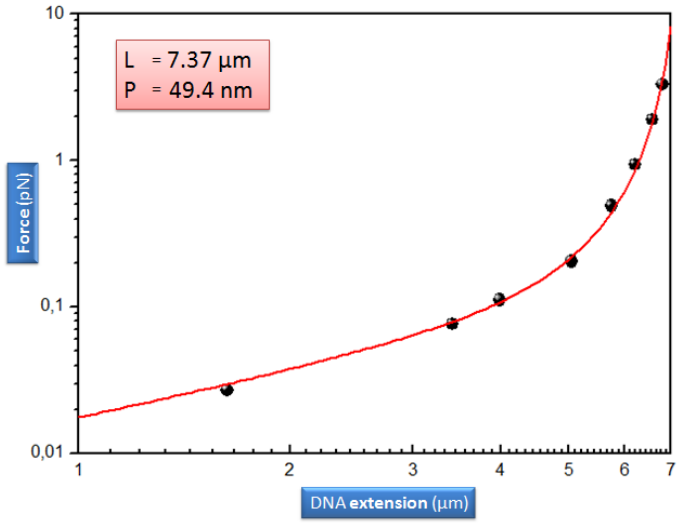


Figure 4.5: *The data of force-extension measurements carried out on a* $\approx$ 7.45 μm *long dsDNA molecule, were fitted to the WLC model, which describes the elasticity of the molecule. Fit parameters are* L= 7.37μm *and* P= 49.4 *nm*

4, corresponding to aliquots in which magnetic beads were present, no DNA is observed. This is an expected result, proving the inability of wt-AddAB to bind to streptavidin coated beads. In lane 1 and 3 presence of DNA is observed, although the observed intensity of the band in lane 3 is much larger compared to lane 1. The latter suggests nuclease and helicase activity of the wt-AddAB protein, although not all DNA was degraded. A detailed description of the reaction conditions is reported in appendix A.3

4.3 Toward a Magnetic Tweezers Activity Assay

Now that we demonstrated that the activity of BIO-AddAB is unaffected *in vitro* when this protein is bound to magnetic beads, as a next step we want to investigate the processing of dsDNA molecules by this protein in a MT setup.

4.3.1 Tuning the magnetic tweezers

The MT sample was fabricated following the protocol described in Ref. [15]. A dsDNA plasmid[1] (20678 bps) was linearized using the restriction enzymes NotI

[1]A kind gift from the Molecular Biophysics group, Delft University of Technology

and XhoI. PCR was used to fabricate Digoxigenin and Biotin labeled small dsDNA fragments which were cut with the same restriction enzymes NotI and XhoI, respectively. After ligation of the remaining labeled dsDNA handles to the longer DNA fragments, dsDNA molecules with lengths ranging between 21.8 and 22.0 kbps were obtained, corresponding to a molecule length of ≈ 7.45 μm (considering 1 bp ≈ 0.34 nm).

Calibration of the MT was carried out by determining force-extension curves of these dsDNA molecule constructs. These force-extension curve describe the elasticity of the biomolecules in the elastic regime. The curves were well fitted to the worm-like chain (WLC) model [19, 20], using two fit parameters: the length of the molecule L and its persistence length P. The latter quantity is the length over which the orientation of a molecule segment remains unchanged. We compared our obtained fit parameters to the length of our DNA molecule and the theoretical value of P which is approximately 50 nm [21].

Figure 4.5 shows the data of a force-extension curve obtained for one of the dsDNA molecules. The data points were fitted to the WLC model yielding the fit parameters L= 7.37μm and P= 49.4nm, in good agreement with the expected values.

Chapter 5

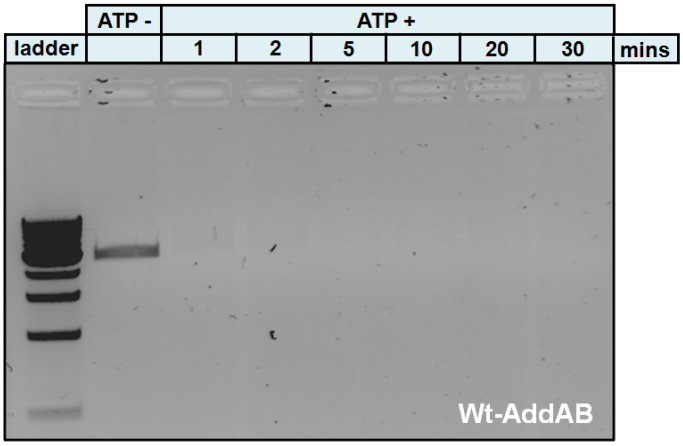
Discussion

In this chapter we discuss the different experiments that we carried out. The results are briefly highlighted in combination with other remarks of importance when considering these experiments.

5.1 Gel Activity Assays

We have demonstrated the activity of wt-AddAB and BIO-AddAB proteins to process dsDNA molecules *in vitro*. In these experiments the reaction time was set to 10 minutes. However, other experiments suggest that under the same conditions as the experiments described in sections 4.1.1 and 4.1.2 (DNA 1 nM, wt-AddAB 5 nM, ATP 1 mM), wt-AddAB needs less time to fully degrade the DNA, as becomes clear from the results depicted in figure 5.1. In this picture the results of an agarose gel activity assay of wt-AddAB are showed, in which each lane is characterized by a different reaction time. It can be clearly observed that most of the DNA is degraded before the first minute is passed.

We also noticed that the activity of wt-AddAB and BIO-AddAB strongly depends on the storage temperature of the protein. Immediate use of protein which was stored at $-80°$C, yielded high activity, judging from the agarose gel activity assays. However, re-freezing the protein at $-20°$C strongly attenuated the protein activity within a time frame of 24 hours to a point at which no activity could be observed. Meanwhile, use of these molecular motors when re-stored at $-80°$C did not significantly affect the activity of the proteins.



Time Activity Assay wt-AddAB

Figure 5.1: *An image of an agarose gel, showing the result of an experiment in which the activity of wt-AddAB is tested, with the reaction time as the only variable. One can clearly observe that most of the wt-AddAB is degraded in less than* 2 *minutes.*

5.2 Magnetic Tweezers Experiments

We have carried out force extension measurements on single dsDNA molecules using a MT setup. The results of these measurements could be successfully fit to the WLC model, describing the elastic behavior of the DNA molecules in a correct manner. Besides the exemplified example in this report, we have carried out the same measurements on several other DNA molecules, yielding good fit parameters close to the theoretically expected values of the length and persistence length of each molecule. However, more statistics on these measurements are desired in order to fully characterize the working of this MT setup.

We were not able to find any coilable DNA molecules among our used samples (L= 7.45 μm). This could have been caused by deterioration of the used DNA substrate, in the form of single-strand nicks along the individual strands. However, in recent experiments with significantly shorter DNA molecules (1.2 μm), which are not discussed in this report, coilability of DNA molecules is clearly demonstrated, resembling results as obtained by Strick [22].

5.3 Future Work

We demonstrated the activity of BIO-AddAB while bounded to streptavidin coated magnetic beads. This result opens the door for future studies of this particular protein-DNA interaction in a magnetic tweezers setup. Successful implementation of this experiment makes real time monitoring of AddAB-translocation along double strand DNA molecules possible.

For this purpose, constructs as schematically depicted in figure 5.2 have to be examined in the magnetic tweezers. In this experiment the streptavidin coated magnetic beads are covered with biotynilated AddAB. One of these proteins is expected to bind to the double strand end of a dsDNA molecule. Fabrication of dsDNA molecules with one DIG labeled end then enables us to tether these molecules between the bottom of the liquid cell and the trapped bead-protein complex. Successful insertion of these constructs into our setup should allow for height measurements of the magnetic beads. Addition of an ATP containing buffer to the liquid cell, should therefore enable us to study the processing of DNA molecules by these bead-protein complexes.

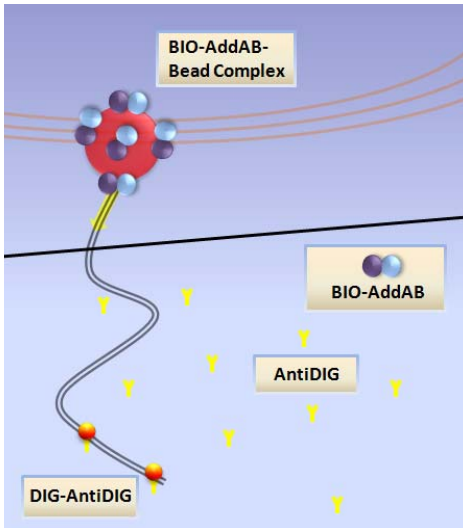


Figure 5.2: *Schematic drawing of the construct needed to study AddAB-DNA interaction in a magnetic tweezers setup. A dsDNA molecule is tethered by attaching one its (DIG-labeled) ends to the anti-DIG coated bottom of the liquid cell. The other (double strand) end of the molecule is bound to a BIO-AddAB protein, which is attached to a streptavidin coated magnetic bead. The bead is attracted by an externally applied magnetic field.*

Contrary to the MT measurements discussed in this report, the dsDNA molecule is indirectly attached to the magnetic bead through a non-covalent binding of the protein to the double strand end of the molecule. The tensile forces that the latter bond can withstand are difficult to predict. It is therefore an interesting challenge to study the the stability of the entire construct, when an upward force is working on the magnetic bead caused by the magnetic field.

Anyhow, with the results presented in this report, successful functioning of this future MT experiment looks promising.

Chapter 6

Conclusions

In this chapter we briefly summarize our conclusions:

- We demonstrated the activity of wt-AddAB and BIO-AddAB by means of *in vitro* experiments.
- We presented proof of BIO-AddAB′s ability to bind to streptavidin coated magnetic beads.
- We showed that BIO-AddAB retains its activity, when the protein is bound to streptavidin coated magnetic beads.
- Next, we carried out successful force extension measurements on dsDNA molecules in our magnetic tweezers setup.
- Fitting the results of the force extension measurements to the theoretical WLC model yielded fit parameters (molecule length, persistence length) corresponding to the theoretical expected values. We therefore conclude that our magnetic tweezers work adequately.

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Appendix A

Reaction Conditions

A.1 Wild-type AddAB and BIO-AddAB Activity Assays

Incubation The experiments described in sections 4.1.1 and 4.1.2 are carried out at a temperature of 37°C and the reaction time was set to 10 minutes, excluding 2 additional minutes of incubation time prior to the addition of the wtAddAB/BIO-AddAB. Reaction volumes of 16 μl were prepared in 1.5 ml aliquots. As a reaction buffer, we used our standard reaction buffer, containing 25 mM Tris-acetate (TrisOAc) at pH 7.5 and 2 mM magnesium acetate (MgOAc). For every DNA-χ^0 molecule in the reaction volume (1 nM), 5 wt-AddAB or BIO-AddAB proteins (5 nM) were present. When protein activity was required, 1 mM ATP was added to the aliquots. Note that the excess of free magnesium ions compared to the ATP concentration, meets the requirement for the presence of nuclease activity [4]. Disulfide bonds between proteins are minimized by addition of 1 mM Dithiothreitol (DTT). Furthermore, the reaction volume contained 2 μM E. coli single strand binding (SSB) protein.

Stop buffer To stop the reactions, the reaction volumes were treated for 10 minutes with a 2× stop buffer, containing 100 mM EDTA, 1 mg/ml Proteinase K and 2.5% (w/v) sodium dodecyl sulfate (SDS).

Agarose Gel Electrophoresis After addition of 8 μl of loading dye containing, 0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll-400, 10 mM Tris-HCl (pH 7.5) and 50 mM EDTA (pH 8.0), the samples were run through a 1% agarose gel, which was stained with SYBR Green.

A.2 Heat Denaturing Experiment

To carry out the heat denaturing experiment as described in section 4.1.1, a total mixture of 32 μl was prepared containing 1 nM of the DNA-χ^0 substrate in our standard buffer (see appendix A.1). Heat denaturing was done by warming up the reaction volume for 5 minutes at 90°C and immediately cooling the sample down at a temperature 4°C during a second time-window of five minutes. After addition of 8 μl of loading dye (see appendix A.1), the sample was analyzed by agarose gel electrophoresis.

A.3 Protein-Bead Assays

The overall conditions of the experiments discussed in sections 4.2.1 and 4.2.2 are the same as the conditions discussed in appendix A.1.

A mixture of DNA-χ^0 (1 nM), wt-AddAB or BIO-AddAB (5 nM) and streptavidin coated beads ($\approx$ 100 pM) with a diameter of 1 μm was prepared; all suspended in a 25 mM TrisOAc (pH 7.5) and 2 mM MgOAc buffer solution, also containing DTT (1 mM) and E. coli SSB (2 μM). The DNA and the proteins were incubated for 2 minutes at 37°C, before the magnetic beads were added. Next, the reaction mixture was split in two equal volumes.

The a magnet was used to separate the DNA-protein-bead complexes (BIO-AddAB: section 4.2.1) or the magnetic beads (wt-AddAB: section 4.2.2) from the remaining supernatant. We subsequently suspended the bead(-protein) complexes in our standard reaction buffer, also containing DTT and E. coli SSB in equal concentrations as mentioned in appendix A.1.

After selective addition of ATP and non-ATP containing buffers each of the aliquots comprised a total volume 16 μl. The reaction time was set to 10 minutes at a temperature of 37°C. Reactions were stopped with a 2$\times$ stop buffer (see appendix A.1). Before addition of the gel loading dye and the subsequent agarose gel (1%) electrophoresis, the magnet was used again to remove the magnetic beads from the samples.