CHAPTER 3

Elucidating The Mechanism of DNA-Dependent ATP Hydrolysis Mediated By DNA-Dependent ATPase A
Chapter 3: Elucidating the mechanism of DNA-dependent ATP hydrolysis mediated by DNA-dependent ATPase A

Introduction

DNA-dependent ATPases hydrolyze ATP in the presence of DNA molecule. These proteins include the helicases, topoisomerases, as well as proteins belonging to the SWI/SNF family. The energy released from ATP hydrolysis is utilized for various DNA metabolic processes; helicases utilize it for strand separation, while the SWI/SNF proteins use the energy for chromatin remodeling. Helicases and SWI/SNF protein share the common helicase motifs that have been shown to be important for the function of helicases. The mechanisms for ATP and DNA/nucleosomal binding and ATP hydrolysis have not been well studied in SWI/SNF proteins but the presence of these conserved helicase motifs in these proteins suggest that they might share similar kind of mechanisms with helicases. Helicases, as we know, are enzymes that utilize the energy from ATP hydrolysis to unwind DNA. The mechanisms of ATP and DNA binding by helicase have been well characterized through various biochemical and structural studies (Moore and Lohman, 1994; Hilbert et al. 2009; Delgoutte and Hippe!, 2002). Helicases can bind both single-stranded nucleic acid (ssNA) and double-stranded nucleic acid (dsNA) but have higher affinity towards ssNA (Lohman and Bjornson, 1996; Delgoutte and Hippel, 2002). However, there are few exceptions like RecBCD and RuvB, both of which bind preferentially to dsNA at least during the initial point of unwinding (Delgoutte and Hippel, 2002). DNA binding studies with some of the well-studied helicase like E. coli Rep and RecBCD helicase, and NS3 helicase have shown that DNA can independently bind to these proteins in the absence of ATP (Lohman and Bjornson, 1996; Ganesan and Smith, 1993, Wong et al. 1992). The mechanism for nucleic acid binding varies depending upon the oligomeric state of the helicase. Helicases have been known to function mostly as oligomers (preferably as dimer or as hexamer) but increasing
number of reports show that these proteins can function as monomers too (Delgoutte and Hippel, 2002, Mechanic et al. 1999, McGlynn et al. 2000; Xu et al. 2003). It has been assumed that homo-oligomeric forms will have at least two binding sites in case of a dimer and at least six binding sites in case of a hexamer but that has not turned out to be the case most of the time; hexameric helicases like DnaB (E. coli) and T4 (gp4) use only one or two subunit at a time to bind to ssDNA; the six subunits of Rho protein on the other hand make contact with the one RNA (Delagoutte and von Hippel, 2002). The Rep helicase from E. coli forms dimer on binding to DNA (either ssDNA or dsDNA) (Wong et al. 1992). The binding of DNA to one subunit leads to a decrease in the affinity for DNA to the other subunit (Lohman and Bjornson, 1996).

Helicases have one potential ATP binding site in the form of Walker A box (Motif I) and B (Motif II) (Caruthers and McKay, 2002). Therefore, it is expected that one ATP binds to the protein. The number of ATP binding sites is expected to vary with the oligomeric state of the protein. For example, the hexameric helicases will have one potential ATP binding site per subunit, and therefore, be theoretically capable of binding 6 molecules of ATP. E. coli DnaB helicase which functions as a hexamer has six nucleotide binding sites where three of them are high affinity and other three are low affinity binding sites (Delgoutte and Hippel, 2002; Lohman and Bjornson, 1996). A similar trend has been observed with Rho protein (Delgoutte and Hippel, 2002; Lohman and Bjornson, 1996)

All the binding studies, thus far, have shown that the binding of ATP and DNA is random but ATP hydrolysis is sequential. Further, binding of the nucleotide causes a conformational change in the protein that affects the affinity of the protein for ssDNA or dsDNA (Wong and Lohman, 1992). Studies with Rep helicase from E. coli have shown that the binding of co-factors like ATP and ADP alters the affinity of the protein for ssDNA and dsDNA and this
property is very crucial for DNA unwinding (Moore and Lohman, 1994, Hsieh et al. 1999; Wong and Lohman, 1992). Similarly, DNA repair protein RecA shows increased affinity for DNA in the presence of ATPγS and decreased affinity in the presence of ADP (Menetski and Kowalczykowski, 1985). The ADP acts as a release factor for the DNA so that the RecA protein can come on and off the ssDNA (Menetski and Kowalczykowski, 1985). However, the protein can bind to DNA in the absence of nucleotide and in fact Rep helicase monomers dimerize in presence of either ssDNA or dsDNA. Nucleotide-driven conformational change and DNA-dependent conformational alteration have also been reported for bacteriophage T7 gene 4 protein (Yong and Romano, 1995) and human mitochondrial helicase has been shown to adopt an open conformation when bound to DNA (Ziebarth et al. 2010).

Helicases undergo cycles of ligand-induced conformational alterations to mediate their unwinding activity; for e.g. in Rep helicases both DNA and ATP can bind independently to the protein but the affinity for DNA increases in the presence of ATP (Wong et al. 1992; Wong and Lohman, 1992). Furthermore, Rep helicase has a very low activity when ATP alone is present but this activity increases when the protein is bound to DNA, and the ATP itself induces a conformation in the protein that allows the protein to bind tightly to DNA (Lohman and Bjornson, 1996). However, the affinity for DNA decreases when ADP is bound to the protein, so during ATP hydrolysis the protein undergoes transition from the Protein-ATP bound state to the Protein-ATP-DNA and after ATP is hydrolyzed Protein ADP-DNA state is formed that has decreased affinity for DNA. The ADP is then released, allowing another molecule of ATP to interact with the protein (Lohman and Bjornson, 1996). Similar kinds of cycles have also been proposed for Isw2 protein, which is a member of the SWI/SNF family. Isw2 have been shown to bind to DNA both in the absence and presence of non-hydrolyzable ATP analog but ADP
promotes dissociation of Isw2 from the DNA (Fitzgerald *et al.* 2004). Isw2 also undergoes nucleotide-dependent conformational changes that are not affected by ATP hydrolysis (Fitzgerald *et al.* 2004). Besides nucleotide driven conformational changes, DNA-dependent conformational alteration have also been reported; FRET studies with SsoRad54 suggests that the binding of DNA changes the conformation of the protein (Lewis *et al.* 2008). These observations indicate that similar kind of mechanisms might be employed by SWI/SNF proteins to hydrolyze ATP and remodel the chromatin.

Figure 3.1: Stem loop DNA used for DNA binding studies and ATPase assay.

DNA-dependent ATPase A is the bovine homolog of human SMARCAL1. The 105-kDa protein undergoes proteolysis to form an 82-kDa protein. This 82-kDa protein has been cloned and named as Active DNA-dependent ATPase A Domain (ADAAD) (Muthuswami *et al.* 2000).
ADAAD retains all the conserved helicase motifs and thus, can hydrolyze ATP in the presence of a DNA effector (Hockensmith et al. 1986; Mesner et al. 1993). Experiments have shown that the optimal DNA effector possesses double-stranded to single-stranded transition regions (Figure 3.1). It is important to note that these kind of structures does appear in chromosomes during transcription, replication, repair, and recombination where SWI/SNF proteins have been shown to be essential for the proper functioning of these processes (Flanagan and Peterson, 1999; Shen et al. 2000; Osley et al. 2007; Cherry and Baltimore, 1999; Jones and Kadonaga, 2000; Wu and Winston, 1997). Since the ATP and DNA binding properties of the SWI/SNF proteins is not very well characterized, I have taken this opportunity to use ADAAD as a model system, to understand the ATP and DNA binding properties of these group of proteins and moreover to provide a model for the ATP hydrolysis mediated by these proteins. In this chapter I will be focusing on the interactions of ATP and DNA and how DNA drives the protein to hydrolyze ATP.

**Protein purification**

The plasmid pCP101, carrying the ADAAD gene was a kind gift from Dr J. Hockensmith, University of Virginia. The plasmid was transformed in to BL21 (DE3) *E. coli* cells and the protein was overexpressed by IPTG induction. The details of overexpression and purification of ADAAD are explained in detail in the Materials and Methods section. ADAAD was purified as an 82-kDa His-tagged protein to a single band using Ni²⁺-NTA affinity column chromatography (Figure 3.2). Around 2-3 mg of protein was purified from 1 litre culture. The specific activity was calculated to be 8.0 μmole/min/mg.
Figure 3.2: Purified fraction of ADAAD that was used for in this study. Lane 1 is a marker; Lane 2 is the purified fraction of ADAAD. The molecular weight of ADAAD is 82-kDa. The arrow points to the purified ADAAD.

Results

Fluorescence study of ADAAD

Fluorescence spectroscopy

Fluorescence is the emission of light when the electrons from lowest excited state lose energy to come down to the ground state. The electron can be excited to high energy level by absorption of energy in the form of light (Figure 3.3). When the electrons from the ground gains
energy they move up into higher energy level and then lose energy in the form of fluorescence to come back to the ground state.

![Jablonski diagram]

**Figure 3.3:** One form of Jablonski diagram showing the simple concept about fluorescence.

However, all the energy that was absorbed by the electron is not dissipated in the form of fluorescence because energy is also lost due to other non-radiative processes and hence fluorescence is emitted at longer wavelengths. This phenomenon is called Stokes shift. It is important to note that all compounds do not emit fluorescence. Fluorophores can be divided into two groups; intrinsic fluorophore that occurs naturally like the aromatic amino acids, chlorophyll etc. and the extrinsic fluorophore that are added to make a sample fluoresce, for example fluorescein, dansyl, rhodamine etc.

Fluorescence intensity can be decreased by a number of ways and this decrease in intensity is called quenching. In the case of proteins, quenching can happen due to diffusional collisions, exposure of aromatic residues to solvents, and resonance energy transfer (Lakowicz, 1999). Fluorescence spectroscopy is a technique that analyzes the fluorescence of any fluorophore using a spectrofluorimeter. This technique is 1000 times more sensitive as compared
to the absorbance spectroscopy and the sample required for analysis is also minimal (Lakowicz, 1999).

Fluorescence spectroscopy has been used to study various protein-ligand interactions including protein-DNA and protein-ATP interactions (Theissen et al. 1996; Moore and Lohman, 1994).

ADAAD has 13 tryptophan residues which when excited at 295 nm show a typical tryptophan emission spectrum with $\lambda_{\text{max}}$ at 340 nm. Fluorescence quenching was observed upon addition of ATP and DNA (Figure 3.4 A and B). The quenching of fluorescence could be either due to the interaction of ATP and DNA with the protein or due to conformational change in the protein upon ligand binding leading to the exposure of the tryptophan residues to the solvent.
Figure 3.4: Representative spectra showing fluorescence quenching in presence of A) ATP and B) ssDNA.

Binding of ATP to ADAAD

I began my studies by determining whether ADAAD could bind to ATP in the absence of DNA. ATP binding to ADAAD was studied by titrating the protein (0.37 μM) with increasing concentrations of ATP and monitoring the fluorescence quenching at 340 nm. The binding reaction was carried out in a buffer containing Tris SO₄, pH 7.5, 1 mM MgCl₂ and 5mM β-mercaptoethanol at 25°C. The fluorescence data that was obtained was fitted to a one-site
saturation model because ADAAD has one possible ATP binding site in the form of Walker A motif (Figure 3.5). The $K_d$ was calculated to be $1.6 \pm 0.5 \, \mu M$ (Table 3.1)

![Graph showing ATP binding to ADAAD](image)

**Figure 3.5:** Binding of ATP to ADAAD in absence of DNA. The data was fitted to one-site saturation model and the $K_d$ was calculated to be $1.6 \pm 0.5 \, \mu M$.

**Table 3.1:** $K_d$ for ATP binding in the absence and presence of different DNA effectors was calculated using one-site saturation model.

<table>
<thead>
<tr>
<th></th>
<th>$K_d$(M)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>$(1.6 \pm 0.5) \times 10^{-6}$</td>
<td>0.98</td>
</tr>
<tr>
<td>+ sIDNA</td>
<td>$(0.14 \pm 0.03) \times 10^{-6}$</td>
<td>0.96</td>
</tr>
<tr>
<td>+ dsDNA</td>
<td>$(0.09 \pm 0.01) \times 10^{-6}$</td>
<td>0.99</td>
</tr>
<tr>
<td>+ ssDNA</td>
<td>$(0.13 \pm 0.02) \times 10^{-6}$</td>
<td>0.96</td>
</tr>
</tbody>
</table>

**DNA binding to ADAAD**

ADAAD is able to optimally hydrolyze ATP in the presence of stem-loop DNA (sIDNA) (Muthuswami et al. 2000). Therefore, the interaction of ADAAD (0.37 μM) with sIDNA was studied by monitoring the fluorescence quenching on adding increasing concentrations of sIDNA. The data obtained was analyzed using a one-site model and the $K_d$ was calculated to be $19.9 \pm 4.9 \, \text{nM}$ (Figure 3.6; Table 3.2)
Figure 3.6: Binding of DNA to ADAAD in absence of ATP.

Table 3.2: $K_d$ for the interaction of DNA with ADAAD in the absence of ATP was calculated using one-site saturation model.

<table>
<thead>
<tr>
<th>Absence of ATP</th>
<th>$K_d$ (M)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIDNA</td>
<td>$(19.9 \pm 4.9) \times 10^{-9}$</td>
<td>0.98</td>
</tr>
<tr>
<td>dsDNA</td>
<td>$(64.9 \pm 11.7) \times 10^{-9}$</td>
<td>0.98</td>
</tr>
<tr>
<td>ssDNA</td>
<td>$(21.3 \pm 1.2) \times 10^{-9}$</td>
<td>0.95</td>
</tr>
</tbody>
</table>

ADAAD cannot hydrolyze ATP in presence of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) (Figure 3.7 A). There are two possible hypotheses. One of the possibilities is that ssDNA and dsDNA might not interact with ADAAD at all. The other possibility is that these molecules can interact with ADAAD but cannot induce ATP hydrolysis. Therefore, to determine whether ADAAD can bind to ssDNA and dsDNA, a 12 nt Mun I oligonucleotide identical to the 12 nt double-stranded DNA stem of the sIDNA was used as a dsDNA and an oligonucleotide 496 was used as ssDNA (Muthuswami et al. 2000).
Figure 3.7: ATPase assay and binding of ssDNA and dsDNA. (A) ATPase assay with different types of DNA. (B) dsDNA (filled square). (C) ssDNA (filled inverted triangle). (D) Comparison of binding of DNA to ADAAD in the absence of ATP. ssDNA (filled circle); dsDNA (filled square); ssDNA (filled inverted triangle).

The sequences of these oligonucleotides are presented in Table 2.2. ssDNA possesses a double-stranded region of 13 base pairs and a 12 base single-stranded region. The dsDNA and ssDNA used in this study do not have any secondary structure and do not effect any ATP hydrolysis (Figure 3.7 A). Fluorescence binding studies were carried out by titrating ADAAD (0.37μM) with increasing concentrations of ssDNA and dsDNA. The binding data showed that both these oligonucleotides bind to ADAAD with a $K_d$ similar to that of ssDNA (Table 3.2; Figure 3.7 B and C) indicating ssDNA, ssDNA and dsDNA can interact with ADAAD in the absence of ATP and all can bind to the protein in a similar manner. However, it is important to note that the total
fluorescence quenching observed upon ssDNA and dsDNA binding was only 72% that of ssDNA binding to ADAAD (Figure 3.7 D). This suggests that either the mode or binding site of these oligonucleotides is different from ssDNA binding.

The data thus far indicate that both ATP and DNA can interact with ADAAD in the absence of the other ligand. Thus, the binding of ATP and DNA to ADAAD can be described as random. In helicases, as discussed above, the binding of nucleotide to the protein increases the affinity for the DNA (Bjornson et al. 1996a; Wong and Lohman, 1992). For example, the binding of nucleotide to Rep helicases alters the affinity of the protein for ssDNA and dsDNA (Wong and Lohman, 1992; Wong et al. 1992). Similar behavior has also been observed in case of Ded1 helicase in which affinity of RNA has shown to increase in presence of ATP (Banroques et al. 2010). In DNA polymerases the binding of DNA to the protein has been shown to increase the affinity of the protein to the nucleotide (Patel et al. 1991). Even though ATP binds to ADAAD in absence of DNA, hydrolysis of ATP can happen only in the presence of DNA. Therefore, it is possible that the interactions of ATP and DNA with the protein are altered in presence of each other. Hence to prove this hypotheses, ATP binding was carried out in the presence of saturating concentration of DNA and similarly DNA binding was carried out in presence of saturating concentration of ATP.

**Binding of ATP to ADAAD in presence of DNA**

To study the interaction of ATP in the presence of saturating concentration of ssDNA, I titrated ADAAD (0.37μM) with increasing concentrations of ATP in the presence of saturated concentration of ssDNA (3μM). The data obtained was fitted to a one-site saturation model. The
$K_d$ was calculated to be 0.14 ± 0.03 μM which is approximately 10-fold less than the $K_d$ calculated for the interaction of ATP binding in the absence of DNA (Figure 3.8 A; Table 3.1).

The interaction of ATP with ADAAD was also studied in the presence of saturated concentration of ssDNA (6μM) and dsDNA (4μM) to determine if these oligonucleotides could also induce conformational changes in ADAAD. The affinity for interaction of ATP with ADAAD in the presence of ssDNA as well as dsDNA was also found to be increased by 10-fold, in a manner similar to that of ssDNA, suggesting that the interaction of oligonucleotides with the protein induces conformational change that enables ATP to bind with higher affinity (Figure. 3.8 B and C; Table 3.1). However, the total fluorescence quenching of ATP in the presence of ssDNA or dsDNA was 65% and 55%, respectively compared to that in the presence of ssDNA (Figure. 3.8 D).
Figure 3.8: Binding of ATP to ADAAD in presence of saturating concentration of DNA. (A) ADAAD was saturated with 3 μM sI DNA. (B) ADAAD was saturated with 4 μM dsDNA. (C) ADAAD was saturated with 6 μM ssDNA. In all the graphs: open circle indicates titration of ATP in the absence of DNA and filled circle indicates titration in the presence of DNA. (D) Comparison of ATP binding to ADAAD in presence and absence of DNA. of DNA (open circle), presence of sI DNA (filled circle), presence of dsDNA (open inverted triangle) and presence of ssDNA (filled inverted triangle).

**Binding of DNA to ADAAD in presence of ATP**

To study the DNA binding in the presence of ATP, ADAAD (0.37 μM) was titrated with increasing concentration of sI DNA in the presence of saturated concentration of ATP (20 μM).

The binding data showed that there was a ~6 fold decrease in the value of $K_d$, suggesting that the affinity of the sI DNA for the protein increased in the presence of ATP (Figure 3.9 A; Table 3.3). A similar trend was observed for ssDNA and dsDNA (Figure 3.9 B and C; Table 3.3). However, the total fluorescence quenching for ssDNA and dsDNA was only 70-80% as compared to that of sI DNA (Figure 3.9 D).
Figure 3.9: Binding of DNA to ADAAD in presence of saturating concentration of ATP. (A) Binding of ssDNA (open circle) to ADAAD. The protein was saturated with 20 μM ATP. (B) Binding of dsDNA (open square) to ADAAD in the presence of ATP. The protein was saturated with 40 μM ATP. (C) Binding of ssDNA (open inverted triangle) to ADAAD in the presence of ATP. The protein was saturated with 40 μM ATP. (D) Comparison of binding of DNA binding to ADAAD in presence of ATP. ssDNA (open circle), dsDNA (open square) and ssDNA (open inverted triangle).

Table 3.3: DNA binding in presence of saturated concentration of ATP

<table>
<thead>
<tr>
<th>Presence of ATP</th>
<th>K_d(M)</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssDNA</td>
<td>(3.0 ± 2.2) X 10^{-9}</td>
<td>0.93</td>
</tr>
<tr>
<td>dsDNA</td>
<td>(2.0 ± 1.0) X 10^{-9}</td>
<td>0.94</td>
</tr>
<tr>
<td>ssDNA</td>
<td>(3.4 ± 0.2) X 10^{-9}</td>
<td>0.97</td>
</tr>
</tbody>
</table>

The ability of ATP and DNA to facilitate a tighter binding of each other to ADAAD led us to speculate that conformational changes may drive ATP hydrolysis. Therefore, to probe the
conformational changes that may have occurred in ADAAD due to interaction with its ligands, the accessibility of tryptophan residues was monitored using quencher molecules.

**Conformational change leads to quenching of fluorescence**

Fluorescence studies of ADAAD with ATP and DNA showed quenching of the intrinsic fluorescence upon binding of the ligands. Fluorescence quenching is observed both when the tryptophan residue is directly involved in binding to the ligands as well as when there is a conformational change leading to the exposure of the tryptophan residue to the solvents. Therefore, to resolve whether the quenching observed upon interaction with the ligands was due to the binding or due to conformational changes, tryptophan residues present in ADAAD were modified using NBS in the absence and presence of ligands. NBS oxidizes tryptophan residues leading to the alteration in quenching and by conducting the reaction in the absence and presence of the ligands I hoped to determine whether the accessibility of NBS to the tryptophan residues was altered in the presence of the ligands. In this study the fluorescence quenching after NBS modification was found to be the same in the absence and presence of ligands indicating that the fluorescence quenching was due conformational change induced in the protein by the ligands (Figure 3.10).
Figure 3.10: ADAAD was titrated with increasing concentration of N-bromosuccinimide (NBS) in the absence and presence of ATP and DNA. The observed fluorescence intensity (F₀), corrected for dilution as well as inner filter effects, was plotted as a function of NBS concentration. (A). ADAAD alone. (B). After saturating with 40 μM ATP. (C) After saturating with 4 μM sIdNA. (D). Comparison of ADAAD alone (□), in the presence of ATP (□), and in the presence of sIdNA (△).

Conformational change analysis using Acrylamide quenching

Based on the binding data as well as NBS modification data, I hypothesized that ADAAD underwent conformational changes in the presence of ATP as well as DNA. To verify the hypothesis I sought to monitor the conformation changes using acrylamide. Acrylamide is a neutral quencher that has been used to probe the accessibility of tryptophan residues sensitive to changes in conformation (Lakowicz, 1999). Acrylamide quenching has been used to probe conformational changes in many proteins like glycoprotein Orosomucoid (Friedman et al. 1985), as well as to study the dATP induced conformational change in cytochrome c (Purring-
Koch and McLendon, 2000), and structural rearrangement of metalloprotein MMP-2 upon mercurial activations (Stack et al. 1996).

To probe for the change in conformation, ADAAD was titrated with acrylamide in the absence of both ATP and DNA to determine the accessibility of the 13 tryptophan residues present in this protein. Titrating ADAAD with increasing concentrations of acrylamide revealed the presence of two populations of tryptophan residues as evident from the biphasic nature of the Stern-Volmer (SV) plot (Figure 3.11). One population of tryptophan residues was relatively more accessible to acrylamide ($K_{SV1} = 9.76 \pm 0.10 \text{ M}^{-1}$) as compared to the other ($K_{SV2} = 6.7 \pm 1.0 \text{ M}^{-1}$). From the modified SV plot, the fraction accessible to the quencher ($f_a$) was estimated to be ~95% with a SV constant for the accessible fraction ($K_a$) being $9.95 \pm 0.05 \text{ M}^{-1}$.

Interestingly, when the protein (0.37 µM) was saturated with ATP (20 µM) prior to titrating with increasing concentration of acrylamide, a decrease in the value of $K_{SV1}$, $K_{SV2}$, $K_a$ and $f_a$ was observed (Figure 3.11; Table 3.4). Similarly when the protein was saturated with slDNA and then titrated with acrylamide, the two population of tryptophan were accessed differentially as evident from the quenching parameters shown in Figure 3.11 and Table 3.4. The value for $K_{SV1}$, $K_{SV2}$ and $K_a$ were reduced in the presence of slDNA as compared to ADAAD alone. However, it is important to note that the accessibility in presence of slDNA is different from that where ADAAD is saturated with ATP. Furthermore the accessibility of the tryptophan residues in presence of both slDNA and ATP is similar to that of the one saturated with slDNA alone (Figure 3.11; Table 3.4). The value for $K_{SV1}$, $K_{SV2}$ and $K_a$ were also reduced in the presence of slDNA but the most interesting observation was that when the protein is saturated with both ATP and slDNA the $K_{SV1}$, $K_{SV2}$ and $K_a$ were similar to that when the protein was saturated with slDNA alone (Figure 3.11; Table 3.4). This observation suggests that the slDNA drives the
protein to that final conformation that final conformation allows ATP hydrolysis. These findings led me to hypothesize that the conformational changes induced in the protein in the presence of ATP was different from the conformational changes induced in the presence of sIDNA (Figure 3.11; Table 3.4).

Table 3.4: Fraction of tryptophans exposed to the acrylamide in presence and absence of ATP and DNA

<table>
<thead>
<tr>
<th></th>
<th>$K_{SV1}(M^{-1})$</th>
<th>$K_{SV2}(M^{-1})$</th>
<th>$K_a$</th>
<th>$f_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAAD alone</td>
<td>9.76 ± 0.14</td>
<td>6.70 ± 1.0</td>
<td>9.95 ± 0.05</td>
<td>0.95 ± 0.002</td>
</tr>
<tr>
<td>ADAAD + ATP</td>
<td>7.28 ± 0.40</td>
<td>4.25 ± 0.61</td>
<td>8.0 ± 1.1</td>
<td>0.96 ± 0.005</td>
</tr>
<tr>
<td>ADAAD + sIDNA</td>
<td>5.73 ± 0.13</td>
<td>3.09 ± 0.17</td>
<td>8.75 ± 0.25</td>
<td>0.85 ± 0.005</td>
</tr>
<tr>
<td>ADAAD + ssDNA</td>
<td>4.28 ± 0.33</td>
<td>2.85 ± 0.27</td>
<td>5.15 ± 0.25</td>
<td>0.87 ± 0.04</td>
</tr>
<tr>
<td>ADAAD + sIDNA + ATP</td>
<td>5.63 ± 0.5</td>
<td>2.75 ± 0.08</td>
<td>7.25 ± 0.85</td>
<td>0.9 ± 0.06</td>
</tr>
<tr>
<td>ADAAD + ssDNA + ATP</td>
<td>3.88 ± 0.18</td>
<td>2.42 ± 0.28</td>
<td>5.16 ± 1.13</td>
<td>0.87 ± 0.03</td>
</tr>
</tbody>
</table>

Figure 3.11: Stern–Volmer plots. ADAAD was titrated with acrylamide in absence and presence of ATP and DNA. (Filled square) protein alone; (open square) in presence of saturating concentration of ATP; (filled circle) in presence of saturating concentration of sIDNA; (open circle) in presence of saturating concentrations of sIDNA and ATP; (filled triangle) in presence of ssDNA; and (open triangle) in presence of saturating concentration of ssDNA and ATP.
The conformational changes in the protein were also studied in the presence of ssDNA alone. The values for $K_{SV1}$, $K_{SV2}$, $K_a$ and $f_a$ in the presence of protein saturated with ssDNA alone or together with ATP were found to be different from those obtained when the protein was saturated with slDNA or slDNA together with ATP (Figure 3.11; Table 3.4). From these results, I concluded that the conformational changes induced by the type of DNA effector in the presence of ATP are also critical to ATP hydrolysis. Thus, ADAAD is able to hydrolyze ATP only in the presence of slDNA but not in the presence of ssDNA.

In helicases, ATP hydrolysis has been shown to be responsible for conformational changes in the protein. Therefore, to understand whether ATP hydrolysis was responsible for the conformational changes in ADAAD too, ATPase activity of ADAAD was measured at 25°C and in the absence of regeneration buffer. The regeneration buffer contains pyruvate kinase and phosphoenolpyruvate that converts the ADP, generated by ATP hydrolysis mediated by ADAAD, to ATP. While recording fluorescence, the regeneration system was omitted from the buffer therefore ATPase activity was recorded in the absence of regeneration system. As shown in Figure 3.12, there is no detectable ATP hydrolysis at 25°C and in absence of a regeneration buffer suggesting that the conformational changes measured in our experiments is due to the interaction of the ligands with the protein.
**Figure 3.12:** (A). ATPase activity of ADAAD in presence of sIDNA at 37°C (○), and at 25°C (○). The reaction at 37°C was done exactly as described in Materials and Methods. The reaction at 25°C was executed in the presence of 50 mM Tris.SO₄ pH 7.5 buffer containing 1 mM MgSO₄, and 5 mM β-mercaptoethanol. The reaction was incubated at 25°C for the indicated time points. The reaction was stopped and the amount of phosphate released was quantitated as described in Materials and Methods. It is important to note that this reaction buffer lacks the pyruvate kinase and phosphoenolpyruvate regenerating system, as we did not use them while recording fluorescence spectra. The reaction was performed at 25°C, as this was the condition under which the fluorescence spectra were recorded. 1 μg of protein was used for these assays.

**Model for ATP hydrolysis in presence of a DNA effector**

Based on the binding data, it is evident that ATP and sIDNA can independently bind to ADAAD forming [E.ATP] and [E.sIDNA] respectively. Subsequently, sIDNA interacts with [E.ATP] forming [E.ATP.sIDNA] and similarly ATP interacts with [E.sIDNA] forming [E.sIDNA.ATP].

The binding data suggests that ADAAD interacts with ATP and sIDNA in a random manner resulting in the formation of [ADAAD.ATP.sIDNA] or [ADAAD.sIDNA.ATP] ternary complex. The formation of these ternary complexes would eventually lead to ATP hydrolysis. Similarly, the binding of ssDNA and dsDNA is also random but the ternary complexes [ADAAD. ATP.ssDNA] or [ADAAD.ssDNA.ATP] cannot hydrolyze ATP (Figure 3.13).
Figure 3.13: Model for interaction of ATP, sldNA, ssDNA and ATP hydrolysis.

The fluorescence quenching studies suggest that the interaction of DNA (sldNA or ssDNA) with ADAAD drives the final conformation of the ternary complex. This, therefore, implies that even though ATP and DNA can interact randomly with the protein, there is an inherent sequentiality to the reaction underneath the apparent randomness.

Based on the fluorescence data, there are four possible theoretical models for ATP hydrolysis (Figure 3.14).
Figure 3.14: Theoretical models for interaction of ATP and DNA with ADAAD leading to ATP hydrolysis.

Model I

In the first model, ATP and DNA can bind to ADAAD independent of each other. Binding of ATP leads to a conformational change that allows DNA to bind to ADAAD with higher affinity leading to the formation of [ADAAD.ATP.DNA] complex. Similarly, binding of DNA to ADAAD in the absence of ATP leads to a conformational change that allows ATP to bind with 10-fold higher affinity leading to the formation of [ADAAD.DNA.ATP] complex. 

Model II

Model III

Model IV
this model it is assumed that both [ADAAD.ATP.DNA] and [ADAAD.DNA.ATP] complex are capable of mediating ATP hydrolysis.

Model II

In the second model, it is assumed that ATP and DNA bind sequentially to the protein. The protein first binds to DNA forming a binary complex to which ATP then binds to form the [ADAAD.DNA.ATP] complex. This complex mediates the hydrolysis of ATP.

Model III

In this model it is assumed that both ATP and DNA can independently interact with ADAAD forming the binary complex [ADAAD.ATP] and [ADAAD.DNA] respectively but interaction of DNA with [ADAAD.ATP] complex to form the ADAAD.ATP.DNA complex does not lead to ATP hydrolysis. ADAAD has to bind DNA first forming [ADAAD.DNA] complex that interacts with ATP leading to the formation of [ADAAD.DNA.ATP] complex. This complex is capable of hydrolyzing ATP.

Model IV

This model is similar to Model III. It is assumed that [ADAAD.DNA.ATP] complex can hydrolyze ATP but [ADAAD.ATP.DNA] cannot hydrolyze ATP. However, in this model it is additionally assumed that the [ADAAD.ATP.DNA] complex is converted to [ADAAD.DNA.ATP] complex, which eventually lead to ATP hydrolysis.

Deriving a theoretical framework for ATP hydrolysis by ADAAD in presence of sLDNA:

For the models shown in Figure 3.14, the following equations were derived.

Abbreviations used: E = ADAAD; A = ATP; D = sLDNA
In all these models, we have chosen pre-equilibrium rather than the steady state hypothesis for all our models since the $K_d$ values obtained for ADAAD suggests that the values are true binding constants.

**MODEL I:** In the first model, I have proposed that both pathways results in product binding such that ATP hydrolysis can be mediated by both [E.ATP.DNA] as well as [E.DNA.ATP] complex.

The dissociation constants corresponding to the various equilibria in Model I are given as follows:

\[
K_d = \frac{[E][A]}{[EA]}; \quad K_D = \frac{[E][D]}{[ED]}; \quad K_A = \frac{[E][A]}{[EDA]}; \quad [EDA] = [EAD]
\]

The total enzyme concentration, $E_{\text{total}} = [E] + [EA] + [ED] + [EDA]$

The velocity of the reaction is $v = k_2[EDA]$, where $k_2$ is the rate constant for product formation.

\[
v = \frac{v_{\text{max}}[A][D]}{K_d + K_d'[A] + K_d'[D] + [A][D]}, \quad \text{where} \quad v_{\text{max}} \text{ is the maximal velocity of the reaction and} \quad K_d' = \frac{K_d}{K_d' + K_d'}
\]

\[
\frac{1}{v} = \frac{1}{v_{\text{max}}} \left\{ \frac{1}{[D]} \left( K_d + \frac{K_d'[A]}{[A]} + \frac{K_d'[D]}{[D]} \right) \right\}
\]

\[
K_d = \frac{[A][K_d'(c/m) - 1]}{1 - K_d'[(c/m)]}, \quad \text{where} \quad m \text{ and } c \text{ are the slope and intercept respectively of the plot of } 1/v \text{ vs } 1/D
\]

**MODEL II:** In presence of both ATP and DNA the binding of the ligands occurs sequentially with ATP following DNA binding, resulting in the formation of [E.DNA.ATP] complex;

The dissociation constants corresponding to the various equilibria in Model II are given as follows:

\[
K_D' = \frac{[E][D]}{[ED]}; \quad K_A = \frac{[E][A]}{[EDA]}
\]

The total enzyme concentration, $E_{\text{total}} = [E] + [ED] + [EDA]$. 

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\[ E_{\text{total}} = [E] \left[ 1 + \frac{[D]}{K_D'} + \frac{[D][A]}{K_D'K_A} \right] \]

\[ v = k_2 [EDA] \]

\[ v = \frac{v_{\text{max}} [A]}{K_A K_D' \left\{ \frac{1}{D} + \frac{1}{K_D'} (1 + \frac{[A]}{K_A}) \right\}} \]

\[ \frac{1}{v} = \left( \frac{K_A K_D'}{v_{\text{max}} [A]} \right) \frac{1}{[D]} + \frac{K_A}{v_{\text{max}} [A]} (1 + \frac{[A]}{K_A}) \]

The \( K_A \) for \([E.DNA]\) complex may be calculated as \( K_A = \frac{[A]}{K_D' (c/m) - 1} \)

**MODEL III:** Although either ATP or DNA can bind ADAAD, only the interaction of DNA with ADAAD followed by binding ATP results in productive binding.

The dissociation constants corresponding to the various equilibria in Model III are given as follows:

\[ K_A' = \frac{[E][A]}{[EA]} ; K_D' = \frac{[E][D]}{[ED]} ; K_D = \frac{[EA][D]}{[EAD]} ; K_A = \frac{[ED][A]}{[EDA]} ; [EDA] \neq [EAD] \]

The total enzyme concentration, \( E_{\text{total}} = [E] + [EA] + [ED] + [EDA] + [EAD] \)

\[ E_{\text{total}} = [E] \left[ 1 + \frac{[A]}{K_A} \left( 1 + \frac{[D]}{K_D'} + \frac{[D][A]}{K_D'K_A} \right) \right] \]

Assuming that only DNA-bound protein is capable of hydrolyzing ATP,

\[ v = k_2 [EDA] \]

\[ v = \frac{v_{\text{max}} [A][D]}{K_A K_D' \left\{ \frac{1}{K_A} + \frac{[D]}{K_D'} (1 + \frac{[A]}{K_A}) \right\}} \]

\[ \frac{1}{v} = \left( \frac{K_A K_D'}{v_{\text{max}} [A]} \right) \frac{1}{[D]} + \left( \frac{1}{K_D'} + \frac{[A]}{K_D'} + \frac{[A]}{K_D K_A} \right) \]

The \( K_A \) for \([E.DNA]\) complex may be calculated as

\[ K_A = \frac{[A]}{K_D' \left( \frac{c}{m} \right) - 1} - \frac{K_{D'A'}}{K_D K_A} \]

**MODEL IV:** Both ATP and DNA can bind to ADAAD in random order followed by the other ligand. The \([E.DNA.ATP]\) form is the active one and the \([E.ATP.DNA]\) is either unproductive or undergoes a conformational change to \([E.DNA.ATP]\) for ATP hydrolysis.
The dissociation constants corresponding to the various equilibria in Model IV are given as

\[ K_{1} = [E][A] / [EA]; \quad K_{D} = [E][D] / [ED]; \quad K_{D} = [E][A] / [EDA]; \quad K_{C} = [EAD] / [EDA]; \]

\[ [EAD] \neq [EAD] \]

The total enzyme concentration, \( E_{\text{total}} = [E] + [EA] + [ED] + [EDA] + [EAD] \)

Assuming that [EDA] but not [EAD] is capable of giving product and that EAD can convert to give EDA with a dissociation constant of \( K_{C} \), we obtain

\[ v = \frac{v_{\text{max}}[A]}{K_{A}K_{D} \left( \frac{1}{[D]} \left[ 1 + \frac{[A]}{K_{A}'} + \frac{[A]}{K_{D}'} + \frac{[A]}{K_{C}K_{K}} \right] \right)} \]

\[ 1 = \frac{1}{[A]} \frac{K_{A}K_{D}'}{v_{\text{max}}} + \frac{1}{[A]} \frac{K_{A} + K_{C}}{v_{\text{max}}} \]

The \( K_{A} \) for [E.DNA] complex may be calculated as

\[ K_{A} = \frac{K_{C} + 1}{\left( [A] / (K_{D}'c/m - 1 + K_{D}'c / mK_{A}' \right)} \]

And \( K_{C} \) for conversion of [E.ATP.DNA] to [E.DNA.ATP] as

\[ K_{C} = \frac{c - mK_{A}'}{K_{D}'(A + K_{A}')} \]

Table 3.5: Comparison of \( K_{ATP} \) values calculated from different models.

<table>
<thead>
<tr>
<th>( K_{ATP} ) (M)</th>
<th>Calculated from Model I</th>
<th>Calculated from Model II</th>
<th>Calculated from Model III</th>
<th>Calculated from Model IV</th>
<th>Experimentally determine from ATP hydrolysis</th>
<th>Experimentally determine from fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{\text{max}} ) (nmoles of Pi release)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From slope</td>
<td>4.7</td>
<td>0.31x10^{-3}</td>
<td>4.2</td>
<td>3.8</td>
<td>2.4</td>
<td>NA</td>
</tr>
<tr>
<td>From intercept</td>
<td>4.2</td>
<td>4.2</td>
<td>7.9</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( K_{ATP} ) (M)</td>
<td>-27.6x10^{-6}</td>
<td>0.21x10^{-3}</td>
<td>1.2x10^{-5}</td>
<td>0.14x10^{-6}</td>
<td>0.13x10^{-6}</td>
<td></td>
</tr>
</tbody>
</table>
From the theoretical models, the $K_{\text{ATP}}$ (dissociation constant for interaction of ATP with ADAAD in the presence of saturating concentration of DNA) was calculated for each model by substituting the $K_d$ values obtained from fluorescence studies. The $V_{\text{max}}$ was calculated from the plot of $1/v$ versus $1/\text{DNA}$ that was obtained by ATP hydrolysis using the $\text{sLDNA}$ (Muthuswami et al. 2000). The values for $K_{\text{ATP}}$ calculated from the proposed models were compared with $K_d$ value for ATP binding in the presence of $\text{sLDNA}$ through fluorescence studies. As shown in Table 3.5 the $K_{\text{ATP}}$ calculated from Model IV matched with the $K_d$ value calculated from fluorescence studies thereby indicating that ATP hydrolysis by ADAAD in presence of $\text{sLDNA}$ occurs according to framework provided by Model IV. From these calculations it is evident that the formation of [ADAAD.DNA.ATP] ternary complex is favorable for ATP hydrolysis suggesting the reaction progresses sequentially despite the fact that both ATP and DNA can interact with ADAAD in a random manner. Efforts were made to determine whether the order of addition of ATP and DNA in the reaction mix affects the ATPase activity of the protein. ADAAD was pre-incubated either with ATP or with DNA before the addition of the reaction buffer and the other ligand. The amount of inorganic phosphate (Pi) released was monitored as a function of time. However the release of Pi was found to be almost the same in both the reactions (Figure 3.15)
Attempts were also made to capture and differentiate $[\text{ADAAD.ATP.DNA}]$ and $[\text{ADAAD.DNA.ATP}]$ ternary complexes by CD spectroscopy. The secondary structure of ADAAD, $[\text{ADAAD.DNA}]$, and $[\text{ADAAD.ATP}]$, $[\text{ADAAD.DNA.ATP}]$, and $[\text{ADAAD.ATP.DNA}]$ was recorded as explained in the Materials and Methods (Figure 3.16). However, differences were difficult to discern.

From these experiments two possibilities emerge. It is possible that the two complexes really have similar structures and are both active. The other possibility is that the interconversion of $[\text{ADAAD.ATP.DNA}]$ to $[\text{ADAAD.DNA.ATP}]$ is very fast to be captured either by simple CD spectroscopy or by ATPase activity assay.
Discussion

Active DNA-dependent ATPase A Domain (ADAAD) is a member of the SWI/SNF family of proteins (Flaus et al. 2006). The parent protein of ADAAD is a 105-kDa protein called DNA-dependent ATPase A that was first isolated from calf thymus tissues (Hockensmith et al. 1986). The 105-kDa protein undergoes proteolytic cleavage to yield two polypeptides of molecular masses 68- and 82-kDa. All the three polypeptides exhibit ATPase activity only in the presence of DNA (Hockensmith et al. 1986; Mesner et al. 1993). The 82-kDa is called as ADAAD and peptide sequencing shows that it lacks the N-terminal region but retains the entire seven helicase motifs (Muthuswami et al. 2000). ADAAD shows maximum activity in the presence of DNA molecules possessing single-stranded to double-stranded transition regions (e.g. stem-loop DNA). However, single-stranded and double stranded DNA without any secondary structures do not function as ATPase effectors (Muthuswami et al. 2000).
In this study, I have attempted to elucidate the mechanism of interaction of ATP and DNA interaction with ADAAD resulting in ATP hydrolysis.

The interaction of ADAAD with ATP and DNA was studied using fluorescence spectroscopy. Binding studies shows that both ATP and DNA can bind to ADAAD independent of the other ligand. Interestingly, ADAAD can also interact with ssDNA and dsDNA like Rep helicase, Sth1 and Rad54 (Wong and Lohman, 1992; Saha et al. 2002; Tanaka et al. 2002). Rad54, for example, has also been shown to bind to both ssDNA and dsDNA but only dsDNA is able to elicit ATP hydrolysis (Tanaka et al. 2002).

Interaction of the protein with DNA results in a 10-fold increase in the affinity of ADAAD for ATP. Similarly, there is an enhanced DNA binding when ADAAD was saturated with ATP. The enhanced binding of ATP or DNA in the presence of the other ligand has also been observed in YxIN, a RNA helicase in B. subtilis (Theissen et al. 2008) and SsoRad54cd (Lewis et al. 2008). Nucleotide- and DNA-induced conformational changes have also been observed in helicases like DnaB (Nakayama et al. 1984), Helicase II (UvrD) and Rep protein (Chao and Lohman, 1990) as well as in E. coli transcription terminator Rho protein (Bear et al. 1985). Limited proteolysis in these proteins showed that the pattern of digestion is different from the unbound protein with the ATP bound and DNA bound protein. These observations showed that ADAAD can interact independently with either ATP or DNA and that there is one site for ATP and another for DNA binding.

The observations made in this chapter indicate that like helicases the interaction of ATP and DNA with ADAAD is random. Binding of the nucleotide causes a conformational change that allows DNA to bind tightly. Similarly DNA binding causes a conformational change that
allows ATP to bind with higher affinity. However, the increase in affinity for ATP due the DNA-driven conformation has not been documented in helicases or any SWI/SNF protein. Even though ATP and DNA can interact independently with ADAAD, their affinity for ADAAD increases in presence of each other and ATP hydrolysis happens only when both the ligands are present. This prompted us to consider the existence of two pathways that convert the inactive protein in to an active one. One of the pathways is that ATP binds to ADAAD causing a conformational that allows DNA to bind tightly. The protein then undergoes a change in the conformation that allows ATP hydrolysis to happen. In the other pathway, DNA first interacts with the protein then ATP binds followed by ATP hydrolysis. The theoretical models drawn to address the existence of these two pathways suggest that they are not equivalent. Further, the model suggests that the [ADAAD.ATP.DNA] complex could be converted to [ADAAD.DNA.ATP] complex before it could hydrolyze ATP. Fluorescence quenching data also supports this argument because if we look at the conformation of ADAAD in the presence of ATP and DNA, it is the DNA that dictates the final conformation and not ATP. However, the order of addition of ligand does not appear to influence the ATPase activity as the ATPase assays showed that there is no difference in the Pi released in both cases. This could be either because the two complex are active or the conversion from [ADAAD.ATP.DNA] to [ADAAD.DNA.ATP] is too fast to be able to detect by this assay.

Another important observation is that the protein is active only in the presence of sDNA and not in the presence of ssDNA and dsDNA. From this study we can say that the DNA binding site has two subsites or contacts that fits the sDNA in such a way that the single-stranded region interacts with one subsite and the double-stranded region with the other subsite.
The fact that the protein becomes active only in the presence of sIDNA that has both single- and double-stranded region suggests that the DNA effector needs to simultaneously contact both these subsites thereby driving the protein to a conformation that can hydrolyze ATP. Neither ssDNA nor dsDNA can simultaneously bind both subsites and hence, cannot effect the conformational change required for ATP hydrolysis.

It is also interesting to note that ATP hydrolysis does not happen when only ATP is bound to the protein. The crystal structure of Rad54 showed that the binding of DNA allows the Motif II (DExx) to change from an inactive β conformation to an active α conformation that allows ATP hydrolysis to happen (Subramanya et al. 1996; Durr et al. 2005). When the protein is in the inactive β conformation the DE residues are not able to make contacts with the ATP. Thus, it is possible that the switch occurs only in the presence of DNA and therefore, ATP hydrolysis happens only when DNA is bound to the protein. Further, Motif VI in RNA helicase eIF4A and Motif IV in UvrD have also been shown to be important for ATP binding and hydrolysis (Pause et al. 1993; Hall and Matson, 1997). Additionally, the crystal structure of SsoRad54 has shown that the protein has two domains where the Motifs I, Ia, II and III are in one domain (domain 1) and the Motif IV, V and VI are present in the other domain (domain 2). In the crystal structure, the domain 2 is rotated 180° and the conserved motifs are facing outward from the catalytic domain making them far apart to form a catalytic site for ATP hydrolysis. However, biochemical analysis suggests that the domain 2 rotates to align the conserved motif to the active site during ATP hydrolysis. From these findings we can propose a model for ADAAD that for ATP hydrolysis to happen all the motifs that are required for binding ATP and for catalysis should be aligned correctly and this is achieved only by the binding of sIDNA.
Modification of tryptophan with NBS demonstrated that the quenching of tryptophan fluorescence was due to the conformational changes driven by ATP and/or DNA binding. Acrylamide quenching studies also showed that there are two populations of tryptophan residues that were differentially accessible to the quencher. The change in the Stern-Volmer constants on addition of ligand suggested that local environment of these two tryptophan residue were perturbed. This further proved that the conformational changes took place only upon ligand binding. Further, the Stern-Volmer constants, $K_{SV1}$ and $K_{SV2}$, decreased on adding ATP or DNA indicating that the tryptophan residues became less accessible, which could imply that ADAAD adopts a closed conformation on ligand binding. The crystal structure of SWI/SNF and helicase has shown that the two RecA like domain are joined together by a flexible hinge region that allows two domains to rotate with respect to each other (Durr et al. 2006; Durr et al. 2005). Moreover helicase and some members of SWI/SNF proteins like Rad54 have been known to translocate along the DNA through either an ‘inch-worm’ model or a rolling model (Cairns, 2007; Delgoutte and Hippel, 2002). Both these models require that the protein undergo a series conformational change upon ligand binding and ATP hydrolysis.

Acrylamide quenching studies revealed that the sLDNA binding is the one that drives the protein to a conformation that enable the protein to hydrolyze ATP. Even though ssDNA can interact with the protein, quenching studies showed that the conformation of the protein in presence of ssDNA is different from sLDNA-bound form indicating ssDNA cannot force the protein to that active form.