CHAPTER 5

The role of Q motif in ATP binding and hydrolysis
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Introduction

Tanner et al. serendipitously discovered the ‘Q’ motif during their functional and structural studies on the DEAD box RNA helicases (Tanner et al. 2003). To establish the role of the conserved residue in these proteins, Tanner et al. 2003, inserted a glycine near the proline residue which is present 21 amino acids upstream of the Motif I in six yeast proteins (Dbp1, Dbp2, Ded1, Fal1, Prp5, and eIF4A) that were known to be essential for the S. cerevisiae strain that they are using. These mutagenized proteins were unable to support the growth of the yeast strain at temperatures between 18-36°C indicating that the mutation has a severe effect on the function of the protein. This observation made them realize that there was another important motif, present upstream of Motif I, besides the well-known seven helicase motifs. They further went on to search for the proteins that were similar in sequence to the eIF4A using BLAST. After eliminating duplicates, fragments and obvious members of other helicase families, 277 unique sequences were identified. The alignment of the these unique sequence showed the presence of conserved amino acid residues (≥ 50%) that stretch 42 amino acids upstream of the Motif I. These conserved amino acid residues included an invariant glutamine residue (99%) that stretches 17 amino acids upstream of Motif I. Besides specific residue conservation, high conservation of functional groups was also observed in these sequences; threonine and serine were found to be interchangeable, phenylalanine was the prominent aromatic residue but was replaced by tryptophan in some proteins and by tyrosine in some other proteins. To establish the importance of this motif, Tanner et al. went on to search whether this motif existed in other RNA and DNA helicases. In their search, four families were found to possess this motif. These families included the Chi family (represented by DNA helicase Rad3), SWI/SNF family
(represented by Sth1), Upf1 family (represented by Dna2) and the Ski family (represented by RNA helicase Mtr4/Dob1). In all these families there was 95% occurrence of a glutamine residue at a distance of 15-22 amino acids upstream of the Motif I.

Subsequently, in vivo studies were carried out to check the importance of this motif. Site-specific mutations were made in the conserved residues of an essential protein eIF4A in yeast. Mutation of the glutamine residue to either glutamate or alanine was found to be lethal indicating that this residue is important for the function of eIF4A (Tanner et al. 2003). Mutation of the conserved glutamine residue carried out in other members of the DEAD helicase family like Ded1, Fal1 and Prp5 also led to a lethal phenotype (Tanner et al. 2003; Cordin et al., 2004).

To further understand the function of the glutamine residue, the Q→A mutants were tested for their ATPase activity and ATP binding in eIF4A and Ded1. Mutation of glutamine to alanine led to a decrease in ATPase activity in Ded1 and eIF4A, which was found to be the result of the decreased affinity for ATP (Tanner et al. 2003; Cordin et al. 2004). Mutation of Q→E in eIF4A also led to a 48% decrease in activity as compared to the wild type but a concomitant ~3-fold increase in crosslinking to ATP was observed as compared to the wild type. Similarly in Ded1 the Q→E and Q→A mutant showed 300-fold decrease in the $K_m/k_{cat}$ value but the $K_m$ increased by only a factor 2-fold as compared to the wild type. Crystal structures of eIF4A (Benz et al. 1999) and UvrB (Theis et al. 1999) have indicated that the glutamine residue coordinates the N6 and N7 position of adenine. Recent crystal structure of DEAD box helicase eIF4AIlll and Vasa also showed that the Q motif was involved in the interaction with the adenine moiety of the nucleotide (Bono et al. 2006; Sengoku et al. 2006). In eIF4AIlll the adenine base makes specific hydrogen bond with the conserved glutamine residue. Stacking interaction of the adenine with a phenylalanine residue in the Q motif has also been mapped (Bono et al. 2006).
Similarly, the phenylalanine residue presence in the Q motif was found to participate in van der Waal interaction with an adenine moiety while the Motifs I and II were found to interact with the tri-phosphates through magnesium and the water molecule in Vasa (Sengoku et al. 2006). In addition, the Motif V and VI of Vasa also interacted with the ribose and triphosphate (Sengoku et al. 2006). Recently, mutational analysis of the Mycobacterium UvrD helicase has revealed one of the distinct properties of the conserved glutamine residue. Mutation of the conserved glutamine to alanine not only led to the decrease (20% of wild type) in activity but also to a decrease in its specificity for ATP. In this study the Q-A mutant was able to efficiently hydrolyze dATP GTP, dGTP, CTP, dCTP, UTP and TTP unlike the wild type protein which have very low efficiency to hydrolyze other NTPs besides ATP (Sinha et al. 2009).

The presence of the Q motif in SWI/SNF proteins in Sth1 (ATPase subunit of the RSC complex) was reported by Tanner et al. 2003 when they had used this protein as a bait to pull out the other members of the complex. According to Tanner et al. the glutamine residue is present about 95% or more times 19-30 amino acids upstream of Motif I in the sequences that were analyzed. In case of the SWI/SNF family, of the 47 sequences that were identified using Sth1 as the query sequence for BLAST, the frequency of occurrence of the conserved glutamine residue is 96 % and the distance of this residue is 17aa upstream of the Motif I (Tanner et al. 2003). The roles of this motif in SWI/SNF protein has not been determined but previous study with the SF2 DEAD box helicases and Powassan NS3 helicase (another SF2 helicase) suggest that this motif is involved in ATP binding and hydrolysis (Tanner et al. 2003; Cordin et al. 2004; Gallivan et al. 2003).

Previous studies with ADAAD showed that the Motif I is important for ATP hydrolysis but not for ATP binding. However, studies with a deletion construct of ADAAD (MAD33),
which possesses only the Q, I and Ia motifs showed that these motifs are sufficient for ATP binding. These observations prompted me to look for the site for ATP binding in ADAAD. The Motifs Q and Ia are the two potential sites that may be important for ATP binding as both these motifs possess aromatic amino acids that can potentially stabilize adenine binding with the protein via stacking interactions. Among these two, Q motif is the one that have been recently suspected be important for ATP binding. Moreover the involvement of Motif Ia in ATP binding have not been clearly determined. Therefore, I began these studies with site-directed mutagenesis of the Q residue to understand its involvement in ATP binding and ATP hydrolysis.

Results

Clustal W analysis of SWI/SNF proteins showed that the Q residue was highly conserved in all SWI/SNF proteins including ADAAD (Figure 5.1). In addition, a conserved aromatic residue was present just before the invariant glutamine residue in all the proteins except for the eukaryotic Rad54 where a histidine residue was present (Figure 5.1). Also, ADAAD possesses a phenylalanine residue at this position instead of the tyrosine seen in other SWI/SNF proteins. In contrast, the DEAD box helicases possess a non-polar aliphatic amino acid (leucine, isoleucine and valine) at this position. The SWI/SNF proteins have a conserved aromatic residue downstream of the conserved glutamine residue in the form of a tryptophan or a phenylalanine. Furthermore, DEAD box proteins possess a conserved phenyalanine residue (88%) upstream of the Q motif, which is not seen in the case of SWI/SNF proteins. There is also a highly conserved glycine residue downstream the glutamine residue. These observations indicate that the Q motif is also conserved in SWI/SNF proteins and it might play an important role in the function of ADAAD and other SWI/SNF proteins.
Studies using site-directed mutants

In order to have a better understanding of the Q motif, site-directed mutants were made in ADAAD in which the conserved glutamine residue was replaced with alanine (Q217A) or asparagine (Q217N). As mentioned earlier, the side chain of glutamine residue has been shown to make contacts with the N6 and N7 position of the adenine in eIF4A and UvrD (Benz et al. 1999; Theis et al. 1999). Therefore, in this study, the length of the side chain was altered by replacing the glutamine with an asparagine residue, which has a shorter side chain.

Site-directed mutagenesis

Site-directed mutant were made for the conserved glutamine residue by PCR based method using the mutagenic primer and high fidelity Pfu polymerase. pMN82 plasmid was used as template for making these mutants. The clones obtained were verified by sequencing. The scheme for generating these mutants is shown in (Figure 2.4) and the procedure is described in detail in the Materials and Methods section.

**Figure 5.1:** Sequence alignment of the SWI/SNF protein using Clustal W
Protein purification

The Q217A and Q217N mutants were purified to near homogeneity as described in the Materials and Methods (Figure 5.2). Briefly, both Q217A and Q217N were expressed as GST-tag protein and purified using Glutathione agarose resin. The proteins were eluted from the column by adding PreScission protease that cleaved GST-tag off. Subsequently, the GroEL contaminant was removed using DEAE-sepharose column.

Figure 5.2: The site-directed mutant proteins were purified as described in Materials and Methods. The GST-tag was cleaved using PreScission protease and the proteins were further purified using DEAE-sepharose column. Lane 1: Q217N; Lane 2: molecular weight marker; Lane 3: Q217A.
Q217A

ATP binding in the absence of siDNA

Replacing of glutamine residue with alanine in ADAAD also led to a loss in activity indicating that this residue is important for the activity of the protein (Figure 5.3 E). This result is similar to the one obtained in case of eIF4A and Ded1 proteins where the mutation of the conserved glutamine residue of the Q motif led to a decrease in ATPase activity as well as in affinity for ATP binding (Tanner et al. 2003; Cordin et al. 2004). In the case of Ded1 helicase, mutating the glutamine to alanine led to a 100-fold decrease in the affinity indicating that this residue was important for ATP binding. It is possible that this loss in activity was due to the decrease in affinity for ATP since Q to A mutation in Ded1 showed 100-fold higher $K_m$ value as compared to its wild type protein. Therefore, interaction of ATP with Q217A was studied using fluorescence spectroscopy. The quenching of intrinsic fluorescence was monitored on adding increasing concentration of ATP to the protein in the absence of siDNA. Analysis of the data showed that the Q217A could bind to ATP with a $K_d$ of $0.7 \pm 0.02 \mu M$, which is similar to the wild type protein. However, it is important to note that the total fluorescence quenching was only 50% as compared to that of wild type ADAAD (Figure 5.3 A)

ATP binding in the presence of siDNA

As in the wild type protein the affinity for ATP increases in presence of siDNA, therefore, the interaction of ATP with the mutant protein was studied in the presence of saturated concentration of siDNA ($2 \mu M$). Fluorescence studies showed that the same pattern that was observed with ADAAD was also seen with Q217A. There is a 7-fold increase in the ATP binding in presence of siDNA (Figure 5.3 B and F).
**Figure 5.3:** Q217A interaction with ATP and DNA. A) ATP binding in the absence of siDNA. B) ATP binding in the presence of siDNA (2μM). C) siDNA binding in the absence of ATP. D) siDNA binding in the presence of saturating concentration of ATP (20 μM). In all the cases, ADAAD is indicated by filled circles and Q217A with open circles. E) ATPase activity of Q217A as compared to ADAAD. F) Kₐ values for ATP and DNA interactions. G) Comparison of CD spectra for ADAAD with Q217A.

### siDNA binding in the absence of ATP

The role of the Q motif in DNA or RNA binding has not been clearly determined, though in Ded1 helicase this motif was speculated to regulate RNA binding (Cordin *et al.* 2004). The Q-A mutant of the Mycobacterium UvrD protein still binds to DNA but does not have any helicase activity (Sinha *et al.* 2009). siDNA binding studies for Q217A using fluorescence spectroscopy showed that the protein was able to bind to siDNA with a Kₐ of 7.0 ± 1.0 nM. This is similar to
the $K_d$ reported for the interaction of sLDNA with wild type ADAAD, thus indicating that the glutamine residue is not involved in sLDNA binding (Figure 5.3 C and F).

**sLDNA binding in the presence of ATP**

Previous studies with helicases and ADAAD showed that ATP binding increased the affinity for RNA or DNA binding (Wong et al. 1992; Wong and Lohman, 1992). Therefore sLDNA binding was studied in the presence of saturated concentration of ATP (20μM). As expected the $K_d$ for sLDNA binding decreased in the presence of ATP indicating that like ADAAD, the affinity for DNA increased in the presence of ATP for Q217A also (Figure 5.3 D and F).

However the total fluorescence quenching for Q217A is different as compared to ADAAD in all the four condition of ligand interaction. In case of ATP and DNA interaction with the protein in the absence of the other ligand, the quenching was 50%, and 60% respectively as compared to ADAAD. Similarly, the ATP binding in the presence of saturated concentration of DNA and vice versa, showed only 30% and 40% quenching as compared to ADAAD. These differences in quenching could be the result either due to differences in the amount of ligand bound to the protein or due to the differences in conformation between the mutant and wild protein.

Therefore, to determine whether the conformation was altered, the global conformation of Q217A mutant was estimated by CD spectroscopy. The CD spectra showed that the global conformation of mutant was almost similar to that of wild type ADAAD thus confirming the mutation to alanine does not affect the structure of the protein (Figure 5.3 G). These observations, therefore, indicate that the conserved glutamine residue in ADAAD is not involved in ligand interaction like the glutamine residue in eIF4A and UvrD but it is important for
catalysis as reported in other DEAD box helicases and Powassan NS3 helicase  (Tanner et al. 2003; Gallivan et al. 2003; Cordin et al. 2004).

**Q217N**

The crystal structure studies of eIF4A and UrvD suggested that the side chain of the glutamine residue was making contacts with the N6 and N7 position of the adenine ring of ATP (Benz et al. 1999; Theis et al. 1999). So in this study glutamine was mutated to asparagine, which has shorter side chain to see whether the length of the side chain is important for ATP hydrolysis (Figure 5.4).

The ATPase activity assay showed that shortening of the side chain does not affect the ability of the protein to hydrolyze ATP (Figure 5.5 E). The $K_{DNA}$ and $V_{max}$ were calculated to be 3.12 ± 0.02 nM and 0.0019 nmole/min respectively (Table 4.1). The $K_{DNA}$ for Q217N was 3-fold greater as compared to the wild type protein while the $V_{max}$ was slightly higher than the wild type (0.0013 nmole/min) (Figure 5.5; Table 5.1). The $k_{cat}$ was also found to be slightly higher than the wild type protein (Table 5.1). The $k_{cat}/K_m$ was found to be 2-fold less as compared to ADAAD.

![Figure 5.4: Structure of Glutamine and Asparagine](image)

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Table 5.1: Kinetics parameters for ADAAD versus Q217N

<table>
<thead>
<tr>
<th></th>
<th>$k_DNA$ (nM)</th>
<th>$V_{max}$ (nmole/min)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ min$^{-1}$M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAAD</td>
<td>0.93 ± 0.04</td>
<td>0.0013</td>
<td>19.06</td>
<td>20.5</td>
</tr>
<tr>
<td>Q217N</td>
<td>3.1 ± 0.02</td>
<td>0.0019</td>
<td>27.85</td>
<td>8.9</td>
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Figure 5.5: ATPase assay of ADAAD and Q217N with increase concentration of ssDNA

ATP binding in absence of DNA

To understand the interaction of ATP with the mutant, increasing concentration of ATP was titrated with the mutant protein. The binding data showed that the ATP could bind to Q217N with a $K_d$ of 1.28 ± 0.3 μM, which is very similar to the wild type ADAAD, suggesting that interaction of ATP does not require the glutamine residue (Figure 5.6 A and F).
ATP binding in the presence of sLDNA

ATP binding in the presence of saturated concentration of DNA was also done to determine if the mutant could bind to ATP with increased affinity in the presence of sLDNA. The fluorescence binding data showed a 5-fold decrease in the $K_d$ value for ATP in the presence of DNA indicating an increase in the affinity in a manner similar to the wild type ADAAD (Figure 5.6 B and F). Furthermore the total fluorescence quenching is 80% as compared to ADAAD suggesting that there may be a difference in the amount of ligand bound or the conformation due to ligand binding is different in Q217N mutant.

![Graphs and data](image)

**Figure 5.6:** Q217N interaction with ATP and DNA. A) ATP binding in the absence of sLDNA. B) ATP binding in the presence of sLDNA (2μM). C) sLDNA binding in the absence of ATP. D) sLDNA binding in the presence of saturating concentration of ATP (20μM). In all the cases, ADAAD is indicated by filled circles and Q217A with open circles. E) ATPase activity of Q217N as compared to ADAAD. F) $K_d$ values for ATP and DNA interactions G) Comparison of CD spectra for ADAAD with Q217N

<table>
<thead>
<tr>
<th></th>
<th>ATP(-DNA) μM</th>
<th>ATP(+DNA) μM</th>
<th>DNA(-ATP) nM</th>
<th>DNA(+ATP) nM</th>
</tr>
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<tbody>
<tr>
<td>ADAAD</td>
<td>1.5 ± 0.07</td>
<td>0.14 ± 0.02</td>
<td>3.8 ± 0.8</td>
<td>0.8 ± 0.03</td>
</tr>
<tr>
<td>Q217N</td>
<td>1.3 ± 0.3</td>
<td>0.26 ± 0.02</td>
<td>7.2 ± 1.3</td>
<td>1.5 ± 0.01</td>
</tr>
</tbody>
</table>
DNA binding

The interaction of sLDNA with the mutant protein was also found to be similar to ADAAD both in the absence and presence of ATP. The $K_d$ for sLDNA binding alone was calculated to be $7.2 \pm 1.5$ nM and in the presence of saturated concentration of ATP (20μM) the affinity increased by 5-fold (Figure 5.6 C, D and F). Hence the ATP and sLDNA binding are almost similar to ADAAD. Based on these observations it is evident that the conserved glutamine is not involved in ATP binding as opposes to what has been observed in eIF4A, UvrD, and Ded1. As mentioned earlier there are differences in the total fluorescence quenching between Q217A and ADAAD during ligand interaction. As CD analysis of the mutant proteins suggests that the global conformations are similar to ADAAD, there is a possibility that the local conformation change has altered in these proteins. To probe for the alterations in local conformations, acrylamide quenching studies were done to determine whether the 13 tryptophan residues in these mutants were in the same environment as in the wildtype.

![Figure 5.7: Stern-Volmer plots for ADAAD, Q217N and Q217A. Empty circle (○): ADAAD; filled circle(●): Q217N; and upright triangle(Δ): Q217A.](image-url)
Quenching studies of Q217A had revealed remarkable differences in the local conformation of the mutant as compared to ADAAD. In contrast, the local conformation of Q217N was found to be almost similar as that of ADAAD (Figure 5.7). However, the quenching studies of Q217N in the presence saturated concentration of ATP and DNA together showed that in the presence of the ligands the mutant protein undergoes conformational change leading to the alteration in the quenching profile. (Figure 5.8). In contrast, the Q217A mutant does not show any difference in the quenching pattern in the presence of ATP and DNA indicating that even though the ligands are able to bind to the protein, they are not able to alter the local conformation of the protein. Finally, the quenching parameters clearly indicate that the two populations of tryptophan present in ADAAD and Q217N are more accessible than the two populations of tryptophans present in Q217A (Table 4.2). This clearly indicates that the ADAAD and Q217N have more open conformation as compared to Q217A.

Table 5.2: Quenching parameters for ADAAD, Q217N and Q217A

<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</td>
<td>12.7 ± 2.0</td>
<td>1.51 ± 0.2</td>
<td>12.7 ± 2.6</td>
<td>0.99</td>
</tr>
<tr>
<td>Q217N</td>
<td>10.5 ± 0.8</td>
<td>4.14 ± 0.5</td>
<td>12.8 ± 1.4</td>
<td>0.94</td>
</tr>
<tr>
<td>Q217A</td>
<td>4.4 ± 0.5</td>
<td>3.02 ± 0.6</td>
<td>5.5 ± 0.7</td>
<td>0.90</td>
</tr>
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</table>

As discussed earlier in this chapter, the Q motif possesses additional conserved residues besides the invariant glutamine residue. A close examination of the residues flanking the glutamine residue showed the presence of a conserved proline and some aromatic residues. The function of these residues has not been determined in SWI/SNF proteins. Moreover as we have seen mutation of the glutamine to alanine does not affect the ATP and DNA binding even though
it does change the local environment of the tryptophan residues. Therefore, to map the ATP binding site and DNA binding site, deletion constructs were made in which the entire Q motif was removed. This construct, MAD53, contained Motifs Ia, II, III, IV, V, and VI.

Figure 5.8: Stern-Volmer plots for ADAAD, Q217N and Q217A in the presence of saturated concentrations of ATP (20µM) and DNA (2µM) together.

Fluorescence studies with deletion construct MAD53

The Q and Motif I have been reported to be involved in ATP binding in helicases, but the binding studies with site-directed mutations in these two motifs in ADAAD showed no defect in ATP binding even though the ATPase activity of these mutants was affected. Therefore, to further delineate the role of Q and I motif, ATP and sDNA binding studies were carried out using deletion construct MAD53 which was made by Nimish Khanna, a project student in the
lab, using gene specific primers and the protein was further purified by Meghna Gupta, a project student in the lab. MAD53 lacks the Motifs Q and I but retains all the other conserved motifs.

**ATP binding**

The interaction of ATP with MAD53 in the absence of slDNA was studied using fluorescence spectroscopy and the $K_d$ for this interaction was determined to be $1.4 \pm 0.2 \, \mu\text{M}$, which was surprising because it was almost similar to ADAAD binding with ATP alone (Figure 5.9 A and E). Similarly, ATP binding in the presence of saturated concentration of slDNA (2 $\mu\text{M}$) was found to be increased by ~10-fold (Figure 5.9 B and E). These observations thus implicate that the Motifs I and Q are not required for ATP binding. The total fluorescence quenching was also found to be only 35% of the ADAAD, this could be due to the fact that the mutant protein contains two tryptophan residues less than the full length protein. It could also be due to differences in conformation between MAD53 and ADAAD.

**DNA binding**

The interaction of slDNA with MAD53 was also studied and the $K_d$ was calculated to be $8.3 \pm 0.9 \, \text{nM}$, which was approximately two-fold higher than the ADAAD protein (Figure 5.9 E). The total fluorescence quenching was also found to be only 45% as compared to that of ADAAD (Figure 5.9 C). We had previously seen that DNA binding to ADAAD in the presence of saturated concentration of ATP (20 $\mu\text{M}$) showed an increase in the affinity. However, the interaction of slDNA with MAD53 in the presence of saturating concentration of ATP (20$\mu\text{M}$) was found to be the same as that in the absence of ATP indicating that there is no increase in the affinity for slDNA as observed in the case of ADAAD (Figure 5.9 D and E). This suggests that the Motifs Q and I are important for DNA binding in the presence of ATP. Therefore,
comparison of ATP and DNA binding studies of MAD53 and ADAAD shows that the ATP binding does not involve the Motifs Q and I, but they are necessary for inducing the conformational change that allows the siDNA to bind tightly. The conformation that allows the DNA to bind tightly could be achieved either by the interaction of ATP or by the interaction of siDNA itself with these two motifs.

**Figure 5.9:** MAD53 interaction with ATP and siDNA. A) ATP binding in the absence of siDNA. B) ATP binding in the presence of siDNA (2 μM). C) siDNA binding in the absence of ATP. D) siDNA binding in the presence of saturating concentration of ATP (20 μM). E) Kd value for ATP and siDNA interaction. In all the cases, ADAAD is indicated by filled circles and MAD53 with open circles.

<table>
<thead>
<tr>
<th></th>
<th>ATP(μM)</th>
<th>ATP+ satsiDNA(μM)</th>
<th>DNA(nM)</th>
<th>DNA+ATP sat(nM)</th>
</tr>
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<tbody>
<tr>
<td>ADAAD</td>
<td>1.5 ± 0.07</td>
<td>0.14 ± 0.02</td>
<td>3.81 ± 0.78</td>
<td>0.82 ± 0.01</td>
</tr>
<tr>
<td>MAD53</td>
<td>1.4 ± 0.18</td>
<td>0.16 ± 0.00</td>
<td>8.31 ± 0.87</td>
<td>9.56 ± 0.68</td>
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How does ATP bind to the protein? Reports thus far have suggested that the interaction occurs via the phosphate residues as well as the adenine ring. Therefore, to understand the contribution of the adenine ring and the phosphate residues with respect to ATP binding, I studied the interaction of adenine and ADP with ADAAD. ADP and adenine titrations were done with ADAAD and the $K_d$ for ADP and adenine was calculated to be $1.4 \pm 0.08 \mu M$ and $1.35 \pm 0.07 \mu M$ respectively (Figure 5.10 A and B). Thus, both adenine and ADP can interact with ADAAD with a $K_d$ similar to that of ATP. This indicates that the adenine ring by itself can bind to ADAAD and that the calculated $K_d$ is a contribution of stacking interactions alone. To determine whether the interaction with phosphate residues is important for high affinity binding, the interaction of adenine with ADAAD in the presence of s1DNA was studied. Binding studies show that the $K_d$ was for the interaction of adenine with the protein in the presence of s1DNA was $0.16 \pm 0.01 \mu M$. Thus, the adenine ring alone is sufficient for tighter binding to ADAAD (Figure 5.10 D).

Is the interaction with the phosphate residues critical for s1DNA binding? To understand this question, s1DNA binding in the presence of adenine was studied. The $K_d$ for s1DNA binding in the presence of adenine was calculated to be $1.20 \pm 0.09 \text{nM}$ which is almost similar to the ADAAD, thus indicating that adenine ring alone is able to induce a conformation that allow s1DNA to bind tightly (Figure 5.11).
Figure 5.10: ADAAD interaction with ADP and Adenine. A) ADP in the absence of siDNA binding B) Adenine binding in the absence of siDNA C) Comparison of the ADP, Adenine binding with ATP D) Comparison of ATP and Adenine binding in the presence of siDNA (2 μM) E) CD spectra of ADAAD in the presence of ATP (Black), Adenine (Red) and ADP (Green) F) Stern-Volmer plot of ADAAD in the presence of ATP (Black) and Adenine (Red). In all cases ATP is represented by filled circle, ADP by inverted triangle and Adenine by open circle.
Finally, quenching studies were carried out to monitor the local conformation due to adenine binding because the total fluorescence quenching during adenine titrations both in the absence and presence of DNA was found to be 80% and 60% as compared to ATP (Figure 5.10 C). Quenching data revealed that the local conformation induced in the protein by the interaction with adenine was indeed different from the conformation induced due to ATP binding (Figure 5.10 D). The CD spectra of ADAAD in the presence of adenine, ADP and ATP were also different indicating that the global conformations induced in the protein are not the same (Figure 5.10 E and F). Thus, these results suggest that while the adenine ring contributes to the binding affinity by stacking interactions, the phosphate residues contribute to the conformation of the protein required for ATP hydrolysis.

![Graph](image_url)

**Figure 5.11:** ADAAD interaction with DNA in the presence of saturated concentration of Adenine (20μM)
Discussion

The Motif Q is present upstream of Motif I in all the SWI/SNF proteins. Besides the conserved glutamine residue, this motif also contains a conserved aromatic amino acid residue just before as well as seven amino acid downstream of the highly conserved glutamine residue. In addition, these proteins also possess a conserved glycine residue three amino acid downstream of the conserved glutamine residue. The Q motif in the SWI/SNF proteins is slightly different from that present in the DEAD box helicases. In the DEAD box helicases there is a conserved phenylalanine residue 17 amino acids upstream of the Q motif, which was not found in the case of SWI/SNF proteins.

Mutation of the conserved glutamine residue to alanine in ADAAD led to complete loss of activity. A similar loss in ATPase activity had also been observed in the case of DEAD helicase proteins, Ded1 and eIF4A (Tanner et al. 2003; Cordin et al. 2004). However, unlike the Ded1 protein where the affinity for ATP was affected (K_m increases by ~100-fold), Q217A could still bind to ATP with the same affinity as that of the wild type both in the absence and presence of ssDNA.

Fluorescence data also indicated that the interaction of DNA with the Q217A mutant was similar to that of the wild type protein both in the absence and presence of ATP. This data agrees well with the observation that mutation of the conserved glutamine residue in Mycobacterial UvrD does not affect the DNA binding of the protein, though in case of Ded1 helicase, the motif appears to be important for RNA binding (Sinha et al. 2009). The interaction of RNA with the Q→A mutant of Ded1 has not been determined, yet the Q→E mutant has been shown to possess reduced affinity for the RNA substrate both in the absence and presence of
AMP-PNP (Cordin et al. 2004). The reduced affinity for the RNA substrate could also have resulted from a defect in nucleotide binding ($K_m$ is 3-fold more as compared to wild type) because studies have shown that ATP enhances the RNA affinity.

Mutation of glutamine to asparagine does not affect the activity of the protein as the specific activity of the Q217N and ADAAD were found to be almost similar (Figure 4.6 E). The $k_{cat}$ and $V_{max}$ were found to be slightly higher as compared to ADAAD (Table 4.1). The $K_{DNA}$ was also 3-fold more as compared to ADAAD, leading to a 2-fold decrease in the $k_{cat}/K_m$ ratio. However, the binding data shows that the ATP and DNA binding are similar to that of the wild type ADAAD indicating that the shorter side-chain of the asparagine does not affect the binding parameters though it appears to be critical for ATP hydrolysis. This is in contrast to the data obtained from the crystal structure of eIF4A and UvrD where the side chain of the conserved glutamine residue has been shown to make contacts with the N6 and N7 position of the adenine base. In the case of ADAAD either the side chains of glutamine residue are not necessary to make the contact with the nucleotide or the length of side chain in Q217N is sufficient to make the contact. Therefore, from these studies I concluded that the functional group of glutamine is required for ATP hydrolysis only.

It is also important to note that the mutation of the glutamine residue to alanine changes the local conformation of the tryptophan residues to a great extent. The quenching studies indicate that there are two populations of tryptophan residues in ADAAD, Q217N, and Q217A. The local conformation of the tryptophan residues is very crucial for the activity of the protein because in both ADAAD and Q217N quenching is almost similar (within experimental error) whereas in Q217A the quenching studies indicate that there is a drastic change in local
These findings suggest that the glutamine residue might be important to maintain the structure of the active site required for catalysis.

Mutation of glutamine to alanine in DEAD box proteins has been shown to be lethal. Crystal structure studies with eIF4A has shown that the P loop is in a closed conformation as opposed to the general open conformation that is found in other DNA and RNA helicases. The open conformation is associated with the bound ligand and in eIF4A when ATP binds, the conserved glutamine and serine residues form interactions with the threonine and glycine residue of Motif I. These interactions are not seen in the closed conformation state of eIF4A. Therefore, it is possible that the glutamine residue in ADAAD is not involved in direct contact with ATP or DNA but may have some interactions with other residues of the protein that are important to maintain the active site of the protein. This interaction is presumably important to maintain the active site of the protein. It is possible that the glutamine residue is involved in maintaining an open conformation and when the glutamine is mutated, the interaction necessary to maintain this conformation is lost leading to a conformation that does not allow the proper accessibility of the aromatic residues.

Studies with the mutants of Motif I and the conserved glutamine residue of Motif Q clearly indicate that these residues are not involved in ATP and DNA binding but are important for catalysis. However, there is a possibility that the ATP and DNA binding involves a combination of residues and therefore mutating one residue at a time might not really affect the binding of the ligands. Therefore, deletion constructs were used to study the importance of these motifs. Studies with the deletion construct, MAD53, indicated that the interaction of ATP with the mutant protein in the absence and presence of sldNA was similar to that of ADAAD. A comparison of this data with the deletion construct MAD33 which has the Motif Q, I and Ia
suggest that the Motif Ia might be the site for interaction with ATP. The Motif Ia in ADAAD has three tryptophan residues that may provide the site for stacking of the adenine ring of ATP. Interestingly, the binding of sLDNA to MAD53 in the presence of ATP was that same as that in the absence of ATP suggesting Motif Q and I might be needed for the high-affinity interaction of DNA with the protein.

To further understand the mechanism of interaction of ATP with ADAAD, binding studies were done using ADP and adenine as substrates. The titration of ADP and adenine with ADAAD showed that the $K_d$ were similar to that of ATP suggesting that the adenine ring is sufficient to mediate the interaction of ATP with ADAAD. Further, a 10-fold increase in the binding affinity of adenine to the protein was observed in the presence of sLDNA suggesting the interaction measured by fluorescence spectroscopy was due to the stacking interaction between the adenine ring and the protein.

sLDNA binding done in the presence of adenine also showed that there was an increased affinity for sLDNA suggesting that adenine is able to induce a conformation that allows sLDNA to bind tighter to ADAAD. However, quenching studies with ADAAD in the presence of saturated concentration of adenine showed that the local conformation is different from the one where ADAAD was saturated with ATP. Similarly, comparison of the CD spectra of ADAAD in the presence of ATP and adenine showed that the conformation induced by adenine is different from the conformation induced by ATP, suggesting that the phosphate moieties plays a role in maintaining the appropriate conformation of ADAAD required for ATP hydrolysis.