



ALLEGHENY COLLEGE

A Thermodynamic and Kinetic Approach to Determining Cooperative Binding in DEAD-Box Proteins

**Michael J. Albring
April 15, 2011
Biochemistry Senior Comprehensive Project
Spring 2011**

Acknowledgements

In no particular order (other than my parents obviously coming first), I would like to thank the following people for their impact on my life through the Senior Comprehensive Project, and Allegheny life in general.

I would like to start by thanking my parents, sister, and extended family for their constant love, support, and understanding throughout the past 22 years. Through all the good times, and the not so great, they have stood by me in all of my endeavors, and I really wouldn't be who I am or where I am today if it wasn't for their never-ending influence.

Dr. Garcia: I have had an absolute blast working with you for the past 3 years. You have made such an impact on my career, and I couldn't have asked for a better mentor to help me along the struggle that is college life. Thanks for all your help, but more importantly putting up with all of my antics. From spilling radiation everywhere, to Spanish Word of the Week, to your intimidating emails, to the endless amount of sarcasm... it has all made working in your lab so much fun. I value the experience like no other... and I will definitely be in touch in the future. Don't think you're getting rid of me just because I'm graduating..... I will be back.

Dr. Humphreys and Dr. VanHorn: Thank you for all of your insight over my comping experience. I value your input and thank you for sitting on my comp board. You have made the process very comfortable, and that's hard to do for a comping senior!

Erin and Jenn: What would this year have been like without you two... it would have been absolutely no fun whatsoever. I value your friendships more than I think you two know, and you have made this year so much more bearable than I would have been able to do on my own. Endless nights in the CC have been entertaining, and I look forward to the day when all of the videos we have scaring each other hit YouTube. Keep an eye out!

Megan: I love you most! We're NOT breaking up. Thank you for everything over the past several years, I'm so happy that my comp is done so now we can start having BravoTV dates again. I love all of our random adventures, addiction to reality gossip, and interesting Brooks dinner conversations. You're awesome and I hope we stay friends foreverrrrrrr...

Life Partner: You're my favorite. SO many fun times at formals, Res-Life shenanigans, and you just generally knowing how to cheer me up. I wish you nothing but the best at Vanderbilt in the fall, and I expect daily texts and weekly Skype dates because I don't want to lose touch. Stay classy and I love you.

Robert: Comping has definitely been a lot of fun. You've taught me that football players are actually some of the easiest people to scare... or maybe that's just you? Nonetheless best of luck in med school, the future, and everything else. Stay in touch!

Kristy, Mary Kate, Bruce, Robbie, Rachel, Hannah, and Abby: Thanks for listening. I share a lot with you guys and I appreciate everything over the past several years. You definitely have made life more interesting.

Stephen: You have definitely had a HUGE impact on my life. I value your friendship the most, and consider you one of the most influential people in my life. Thanks for being there through it all, and this summer is going to be awesome before we have to buckle down and be real people. Best of luck in life, and I expect daily updates into your life over the next few years.

To anyone not mentioned: I fully apologize if I left anyone out I'm running on no sleep and a coffee. Everyone I have encountered has made an impact on my life in one way or another, and I appreciate everything.

Thanks for reading this far. This is my fun, useless shout-out paragraph. So thank you to... BravoTV, Coffee, Facebook, YouTube, Spongebob, BlackBerry, My Car, Jersey Shore, Colored Paper, Lisa Lampanelli, Pretzels and Honey Mustard, The Campus Center, My Computer (for never crashing), Captain Morgan, Kathy Griffin, Britney Spears, Breaking Benjamin, Paramore, P!nk, Marilyn Manson, Skillet, Thousand Foot Krutch, The Loop, 100 Items Buffet, The Printshop, and Snowtires. These are the things in life that count.

Abstract

RNA is a vital component in maintaining biological life, and requires a high level of processing before it can achieve its mature, final structure¹. It has a high degree of flexibility, and can interact with itself, other molecules of RNA, DNA, and even proteins⁶¹. Therefore, along its folding path into its mature structure, it can form unwanted interactions that are sometimes energetically more favorable than its final active conformation². Specialized proteins are therefore employed to assist in RNA folding called RNA chaperones. An example of an RNA chaperone is DEAD-box protein A (DbpA) in *Escherichia coli*. DEAD-box proteins are ATP dependent RNA helicases, thus requiring the energy from ATP hydrolysis to separate a double stranded region of RNA⁴. DbpA exhibits what is known as cooperative binding, which occurs when the binding of its first substrate facilitates the binding of the second⁶. It is currently unknown how DbpA's observed cooperative binding occurs. Kinetic and thermodynamic parameters of DbpA suggest that an arginine within the ATP binding pocket (R334) is responsible for forming an ionic interaction which protects the ATP and facilitates RNA binding. Direct binding analyses were carried out in an attempt to verify and solidify these findings, but the majority of the experimentation period was spent troubleshooting two new protocols.

Introduction

Ribonucleic Acid (RNA) is a key component in many different areas of biological functioning. It is a biopolymer of nucleotide building blocks. These nucleotides are composed of three major components: a phosphate group, five-carbon sugar ring, and a nitrogenous base. There are four different nucleotides which make up any given RNA molecule: adenine, uracil, guanine, and cytosine. Nucleotides are distinguished from one another due to the distinct structures of the nitrogenous base (Fig. 1).

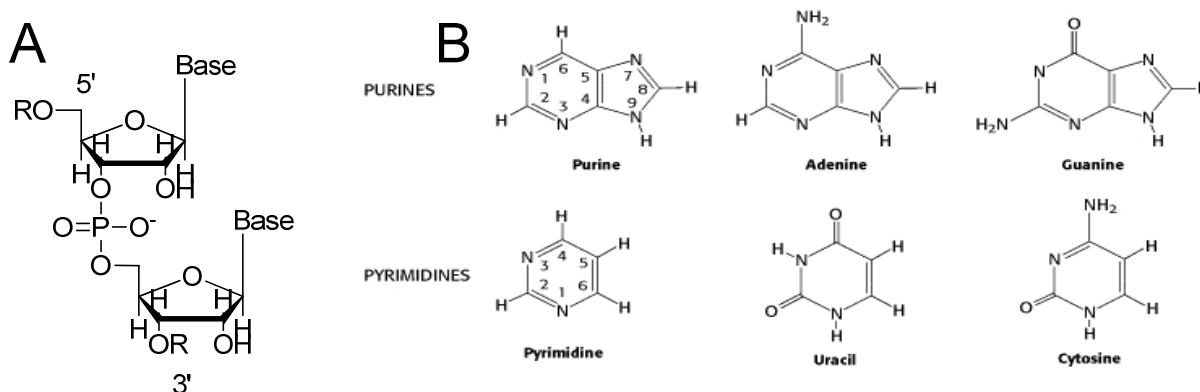


Figure 1. A) Basic building blocks of RNA linked by phosphodiester bonds. The sugar ring is numbered in a clockwise manner starting with the carbon attached to the nitrogenous base. The 5' carbon is part of the -CH₂OR group attached to the ring. Directionality of the RNA molecule is determined by the backbone, starting with the initial 5' group and ending with the 3' - OH group. B) Nitrogenous bases distinguish the nucleotides from one another.

Nucleotides are joined together via phosphodiester bonds between adjacent nucleotides. These bonds are formed between the 5' phosphate group of one nucleotide and the 3' hydroxyl group of an adjacent nucleotide. This connectivity allows the polymeric chain to possess directionality, which aids in decoding the sequence of the nucleic acid (Fig. 1). Specifically, the beginning of the polymeric RNA molecule is denoted as the 5' end, since the phosphate group attached to the 5' carbon on the sugar is exposed. The terminal end is denoted the 3' end since the 3' hydroxyl group of the nucleotide is exposed.

The linear arrangement of the nucleotides is referred to as the RNA's primary structure. RNA is typically found as single stranded structural molecules. The flexible biopolymer has 7°

of freedom which facilitates the molecule to interact with itself. The nitrogenous bases can further form hydrogen bonds with one another creating secondary structures within the RNA molecule. Double stranded helices are the most common secondary structure. Other secondary structures include hairpins, loops and bulges (Fig. 2).

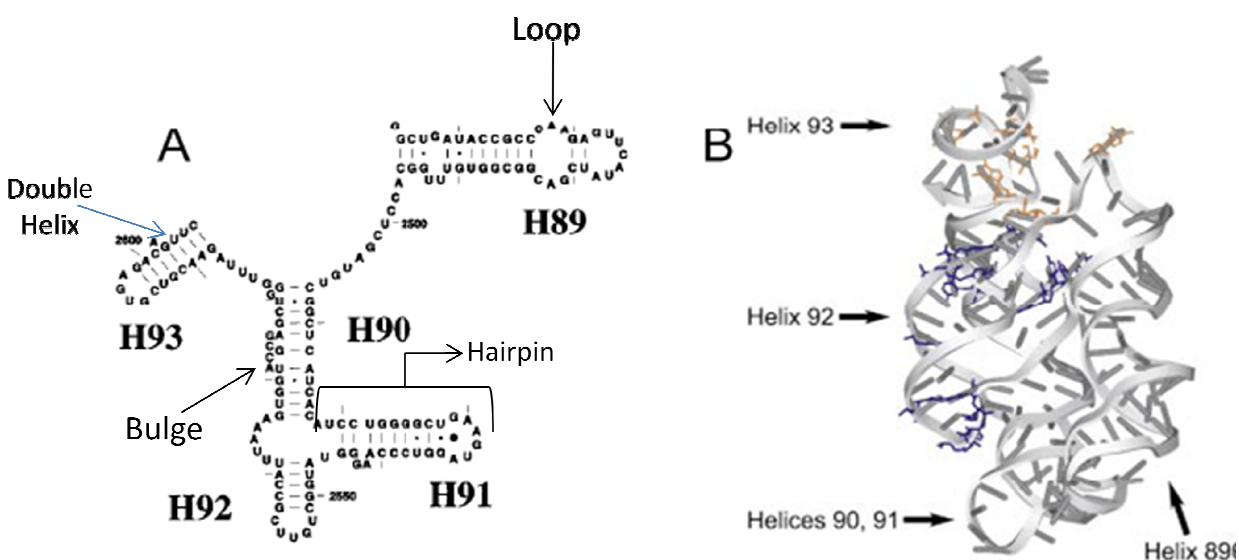


Figure 2. A) *E. coli* 23S rRNA 153mer. Shows common secondary RNA structures and those vital for DbpA binding⁶. Illustrates Hairpin 92 (H92) which is essential for protein recognition. H90 denotes the helical portion of the given hairpin. The same labeling applies to all other hairpins present. B) Tertiary RNA folding of 153mer to ensure proper function. Illustrates that structure determines function⁷. Blue nucleotides are those which bind DbpA without ATP, while those in orange only bind DbpA in the presence of ATP⁷.

Hairpins are composed of helical regions which leaves a portion of RNA unpaired, wrapping back on itself. Hairpin loops are between two and ten nucleotides in length, and are used as recognition elements for proteins that act on RNA. Tertiary interactions result from long range hydrogen bonding with other single stranded regions in the RNA⁸. A double stranded helix containing an internal short single strand of RNA is referred to as a bulge loop (Fig. 2A). Each internal break in the helix will distort the overall helix structure, allowing greater accessibility to the functional groups of the base⁸. The extent to which a bulge loop alters the RNA structure depends on the number of nucleotides in the bulge, the sequence of the nucleotides, and

nucleotides surrounding the bulge. Bulges are one to five nucleotides within a helix that lack complimentary base pairs. They are flexible and introduce a bend in the helix. Single stranded regions in hairpins, loops, and bulges allow for further interactions within the RNA molecule (Fig 2B) or even proteins⁸.

RNA must achieve complex structure for its various functional roles in a cell. These roles include but are not limited to: forming the nucleotide template in translation, adding the correct amino acid to a growing protein chain, or forming an RNA enzyme called the ribozyme. Therefore, a strong understanding of RNA structure will give insight as to the function of any given RNA. However, in order to achieve the correct structure a long single strand of RNA must be folded correctly into its final tertiary structure. Like most other cellular process, RNA folding is imperfect. This is a result of its high propensity to form alternate base pairing within a single strand of RNA. It is further suggested that RNA folding is slow, allowing more time for incorrect folding to occur³. In addition, multiple folding pathways exist which also lead to misfolded tertiary structures. These alternate interactions can lead to non-active structures, rendering the RNA incapable of performing its specific function¹.

Two different classifications of RNA folding traps are suggested. In a kinetic folding trap, the RNA folds itself incorrectly and will never refold into the active conformation, even after a substantially long period of time⁹. The switch in and out of a kinetic folding trap in turn serves as the rate limiting step in the RNA folding pathway³. The second folding trap is a thermodynamic folding trap; where the misfolded conformation is thermodynamically more stable than would be the active conformation. Occasionally thermodynamic traps can be the cause of a permanent kinetic folding trap⁹.

The folding problems are exacerbated in larger RNAs such as the ribosome. The ribosome is a large RNA-protein complex, consisting of a large and small subunit^{10,11}. Ribosomal RNAs will employ the help of proteins to ensure the correct formation and stability of secondary and tertiary structures. These accessory proteins facilitate correct folding, prevent improper folding, or fix a folding error that has already occurred^{12,13}. These accessory proteins are termed RNA chaperones, and will often times assist the RNA to fold correctly and remain a part of the final structure⁹. An example of an RNA chaperone is the RNA helicases. Helicases disrupt RNA-RNA or RNA-protein interactions during RNA folding and processing. They employ the energy of nucleotide hydrolysis to unwind helical regions and modulate structure or protein binding¹⁴.

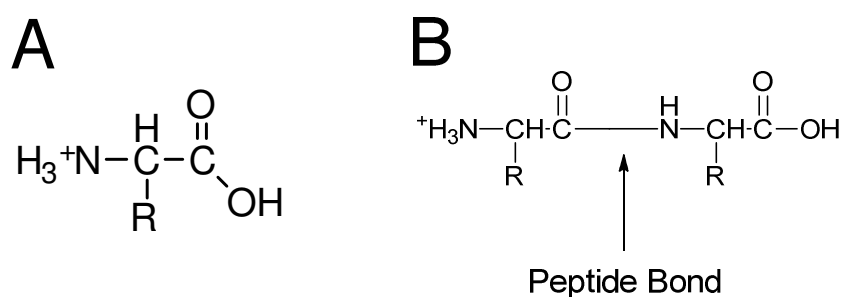


Figure 3. A) General amino acid structure. B) Building block linkage, peptide bond. R group varies between amino acids

Similar to RNA, proteins are biopolymers consisting of building blocks called amino acids (Fig. 3A). There are twenty known amino acids, each containing specific chemical properties such as aromaticity or acidity. Amino acids are linked together via an amide bond between the terminal amino end of one amino acid, and the carboxylic end of the adjacent amino acid. This bond is called a peptide bond (Fig. 3B). The linear arrangement of amino acids is known as its primary structure. Amino acids are classified as polar, non-polar, acidic, and basic. The functional groups of the protein chain hydrogen bond with each other in order to compact the biopolymer and form secondary structures such as alpha helices or beta sheets. The driving

force behind protein folding is the burial of hydrophobic functional groups from the aqueous cellular environment. Proteins can interact with their target RNA molecules by either structural recognition, sequential recognition, or a combination of both. The cellular proteins utilize various modes of RNA binding to aid in RNA folding¹⁵. For example, helicases can recognize a specific RNA sequence by interacting with single stranded regions. Other types of helicases recognize helical regions in RNA by forming ionic and hydrogen bond interactions with backbone functional groups^{16,17}.

Helicases are divided into five superfamilies based on their respective amino acid sequence conservation. The largest subgroup of helicases is the DEAD-box protein which belongs to superfamily 2 (SF2)¹⁸. DEAD-box proteins are further defined by nine conserved motifs. Motifs are conserved regions of amino acids within a protein chain. The DEAD-box is derived from the conserved amino acid sequence aspartate (D), glutamate (E), alanine (A), aspartate (D). This sequence is located in Motif II of the 9 conserved motifs within a given RNA helicase (Fig. 4). These nine conserved motifs compose part of the catalytic core of the protein, which binds both ATP and RNA in order to function¹².



Figure 4. Conserved Motifs in the catalytic core of DEAD-box proteins and their conserved amino acid sequences. If more than one amino acid residue was possible, the following abbreviations were used: a: F,W; c: D,E,H,K,R; x: any amino acid; o: S,T; h: A,F,G,I,L,M,P,V,W,Y; u: A, G; +: H, K, R⁹.

Each motif has a specific function within the DEAD-box protein to aid in substrate binding. The recently discovered Q motif is essential for both RNA and ATP binding. It recognizes the adenine base in ATP specifically (Fig. 5)^{16,19,20}. The Q motif contains a glutamine residue which forms important hydrogen bond interactions with adenine, specifically at nitrogen

6 and nitrogen 7²¹. The phenylalanine residue approximately 17 amino acids upstream from the Q motif stacks on the adenine for stronger binding²⁰.

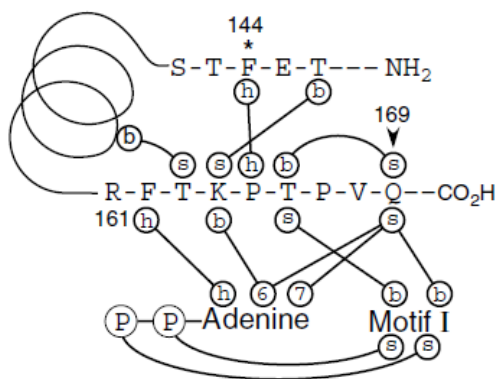


Figure 5. Interactions within the ATP binding pocket. The Q motif forms sidechain (s) interactions with N6 and N7 of the adenine base to coordinate ATP binding, and forms backbone (b) interactions with Motif I. The phenylalanine (F) upstream of the Q motif hydrophobically stacks (h) with the adenine base¹⁹.

Motif I is recognized as the Walker A motif which is responsible for binding to the α -phosphate in ATP via the last threonine residue. Motifs Ia and Ib work to bind both RNA and ATP, although the specifics are not fully known. It has been noted, however, that the last few residues interact with glutamine residues in motif II¹⁶. Motif II is the DEAD box, which is known as the Walker B motif. Motif II binds magnesium via its glutamate residue and is responsible for cleaving off the γ -phosphate during ATP hydrolysis (Fig. 6).

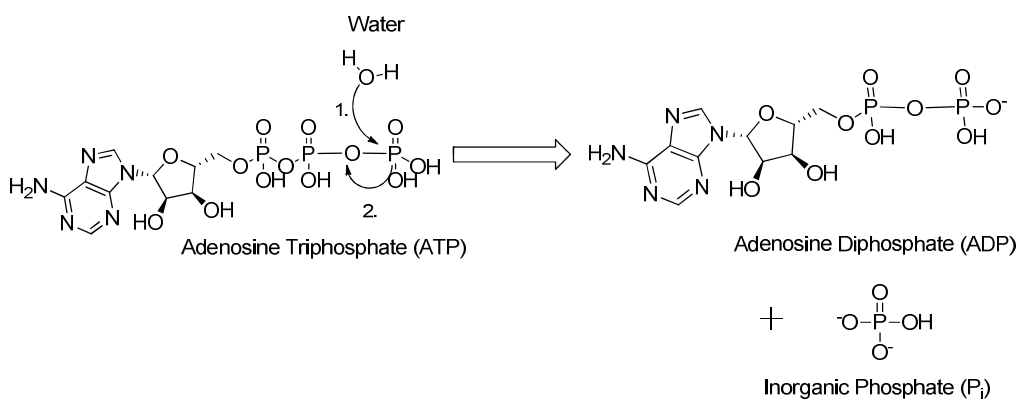


Figure 6. Mechanism of ATP Hydrolysis via cleavage of the gamma phosphate. This process releases energy used by DbpA to fuel helicase activity.

The glutamate in motif II deprotonates the bound water molecule for a nucleophilic attack of ATP¹⁸. The magnesium ion is used to coordinate the water necessary for this attack, as well as to stabilize further interactions between the ATP molecule and its DEAD-box binding site¹⁹. Motif III functions as the flexible linker region between domains 1 and 2 which allows the protein to change conformation¹⁶. Upon binding of ATP, the domains are brought closer together to achieve what is known as its closed conformation²⁸. Motif IV helps to bind RNA through interactions with its ribose-phosphate backbone through interactions with either arginine 269 or arginine 270⁴³. Its hydrophobic residues are closely located near the hydrophobic residues of motif V, suggesting interactions between the two motifs¹⁶. Motif V is suggested to also transmit the signal to the ATPase center of the protein that RNA is bound¹⁶, and the location of a histidine residue in this motif closely resembles that of Rec-A's. Rec-A is a protein whose structure is fully known and has a histidine which interacts with the γ -phosphate²². This suggests the DEAD-box protein's ability to detect the presence or absence of the γ -phosphate⁴⁴. Motif VI is important for RNA binding and helicase activity by binding the γ -phosphate of ATP, and stabilizing the transition state of ATP hydrolysis¹⁶. This is achieved through interactions with arginines at position 349 and 352 (Fig. 7)⁴³.

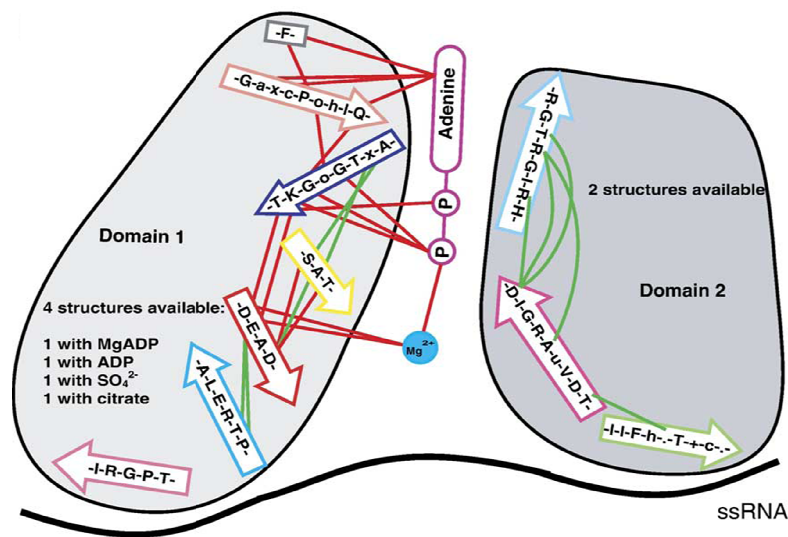


Figure 7. Domain contacts and spatial arrangement within the DEAD-box model. Motifs are color coordinated to match those in Figure 4. Interactions proven to be present are represented in red, while proposed interactions are represented in green¹⁶.

The binding pocket of DEAD-box proteins for their ATP substrate has been highly studied structurally, and the specific amino acids responsible for ATP binding and hydrolysis have been identified. As illustrated by Figure 7, the ATP binding pocket is located between the two catalytic domains of a DEAD-box protein; therefore residues in both domains have important interactions with the ATP molecule. Within motif I, lysine 53 (K53) contacts the β and γ phosphates through hydrogen bonding. Threonine 54 (T54) helps to coordinate a magnesium ion, which forms important contacts with the β and γ phosphates as well²². Motifs II and IV also play a key role in ATP coordination and stabilization. Aspartate 153 (D153) and glutamate 154 (E154) in motif II facilitate coordination of the magnesium ion, while arginine 334 (R334) of motif IV binds all three phosphates. E154 coordinates a magnesium ion to the binding site which helps to stabilize the binding pocket by contacting the β and γ phosphates of ATP. D153 binds ATP through the coordination of this Mg²⁺ ion¹².

The amino acid residues more directly involved with ATP binding are represented in Figure 8. Figure 8 contains proposed amino acid residues responsible for ATP binding specifically in DEAD-box protein A (DbpA) in *E. coli*, the protein under investigation. R331, R334, and T54 make direct contact with the ATP molecule, while serine 184 (S184), threonine 186 (T186), aspartate 156 (D156), and histidine 327 (H327) are involved with interactions between Domains 1 and 2. These interdomain interactions are important to maintain the structure of the binding site, and can also assist in the protein structural changes upon ATP binding (Fig. 8)²³. R331 of motif IV further stabilizes ATP binding by interacting with the γ -phosphate²³.

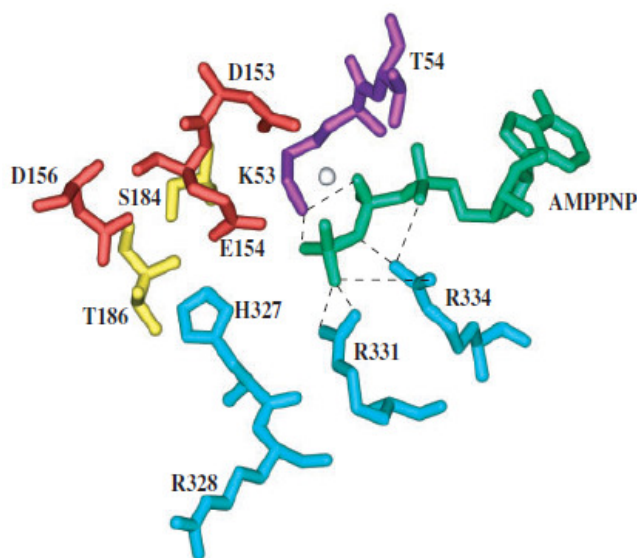


Figure 8. Important residues in the proposed ATP binding pocket of DbpA and the interactions they form. These residues are modeled off of the currently known ATP binding pocket for the *Vasa* protein. The circle represents the associated magnesium ion necessary for water and ATP binding²³.

Structurally, DEAD-box proteins are suggested to contain two Rec-A like domains which are separated by a flexible linker (Fig. 9)^{14,24,25}. Rec-A is a DNA-dependent protein that contains a Walker A and Walker B motif separated by a phosphate loop²². The Walker A and B motifs in Rec-A are suggested to also be present in DEAD-box proteins, and mediate ATP binding and

hydrolysis. The domains of the DEAD-box are classified as Rec-A-Like due to the structural similarities. The flexible linker allows the protein to undergo the conformational opening and closing of the two Rec-A-Like domains during the ATP hydrolysis cycle²².

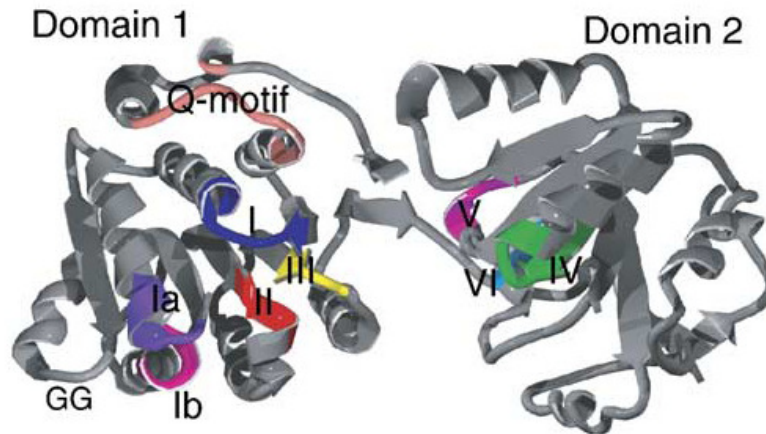


Figure 9. Crystal structure for eIF4A, the archetype for DEAD-box proteins. Notice Domain 1 and Domain 2 are separated by the flexible linker of Motif III in yellow. Motifs are color-coded to correspond to the linear arrangement of Motifs in Figure 4¹⁶.

Furthermore, catalytic domains of the DEAD-box are also flanked by an N- or C-terminal extension which further facilitates and stabilizes the nucleotide binding²². For example, the *E. coli* DEAD-box protein DbpA has a C-terminal extension that recognizes Hairpin 92 of the 153mer. The 153mer is a fragment of RNA 152 nucleotides in length. It is a component of the 23S subunit in the *E. coli* ribosome. This binding helps target the DEAD-box protein to its RNA. The two core domains form an ATP binding pocket as well as an RNA binding surface. As illustrated in Figure 7, ATP binds at a cleft between the two core domains, and the RNA molecule binds to the bottom surface of the core. The motifs interact with each other to sense RNA and ATP binding¹⁶. Thus, upon RNA binding, ATPase activity is stimulated^{24,26,27}. Upon the binding of one substrate, it is noted that certain DEAD-box proteins increase their ability to bind the second substrate. This characteristic is classified as cooperative binding. It has been

previously shown that with DEAD-box protein A (DbpA) in *Escherichia coli*, exhibits such behavior⁶.

The exact mechanism of DEAD-box mediated RNA disruption is currently under debate. However, a general consensus does exist and is termed the destabilization model. The two catalytic domains of the DEAD-box protein can either adopt an open or closed conformation, based on whether or not ATP and RNA are bound (Fig. 10). The switch between the open and closed conformations is the driving force behind DEAD-box protein activity. The core shifts from the open to closed conformation once both substrates are bound, thus initiating ATP hydrolysis. Therefore, conformational change is dependent on the nature of the ATP bound, specifically the γ phosphate²⁸. Upon the completion of ATP hydrolysis, the γ phosphate is released and causes protein structural changes. Its release causes the bound magnesium ion in the pocket to change location, and in combination with disrupted interdomain contacts, the enzyme changes its conformation²⁸. The DEAD-box produces ADP as a result, which has a lower affinity to remain bound, thus promoting its dissociation. Following the dissociation of ADP, the protein reverts to its open conformation for an additional round of hydrolysis^{29,30}. This activity can help refold RNA that has incorrectly folded itself as a result of a kinetic or thermodynamic folding trap.

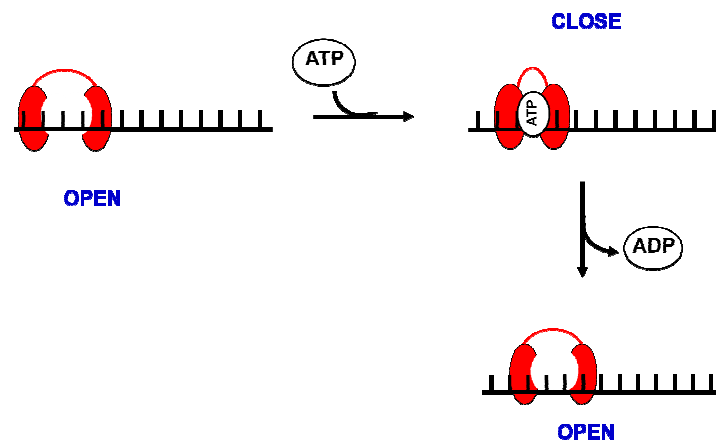


Figure 10. Cartoon representation of the open-close conformation cycle of DEAD-box proteins minus the N- or C-terminal extension.

As mentioned earlier, folding traps are very common in larger RNA molecules³. Therefore it can be concluded that erroneous folding traps are especially potent in the folding process of ribosomal RNA (rRNA). Since the ribosome is so large, it requires a highly complex folding process and involves several proteins. DEAD-box proteins are highly involved in the process of assembling the ribosome¹. Ribosome assembly is a highly complicated process, and its structural integrity is vitally important to protein synthesis and cell survival. Their structures are believed to be highly conserved across all domains, which further emphasizes the need for correct folding. Ribosomes are composed of three folded RNAs bound to several different proteins³¹. The rRNAs are synthesized as a single strand from a DNA template, which may fold prematurely. It has been noted that very rarely will a newly synthesized rRNA fold quickly into its final structure¹. Thus, it is believed that RNA obtains a significantly different structure as the mature ribosome is assembled³². The initial incorrect folding must be disrupted and corrected to yield a mature and functional ribosome. This process of refolding requires the energy from the hydrolysis of ATP. This energy is used to drive the helicase activity of proteins, such as DEAD-box proteins, to remold the RNA-RNA and RNA-protein interactions³².

For example, the *E. coli* DEAD-box protein DbpA is directly involved with ribosome assembly of the 23S rRNA in the large ribosomal subunit (Fig. 11B). In contrast with other DEAD-box proteins, DbpA is highly specific for its RNA substrate and will not bind any other RNA variant. Not only does DbpA recognize the RNA structurally, but it also incorporates strict sequence-specific binding^{33,34}. It specifically recognizes a short 153 nucleotide fragment which stimulates full ATPase activity (Fig. 11A)³⁵. Specifically, the C-terminal extension of DbpA binds to hairpin 92 within the 153 nt fragment of domain V and docks the catalytic core of the protein at various areas in the RNA. DbpA recognizes hairpin 92 by interacting with its single stranded loop³².

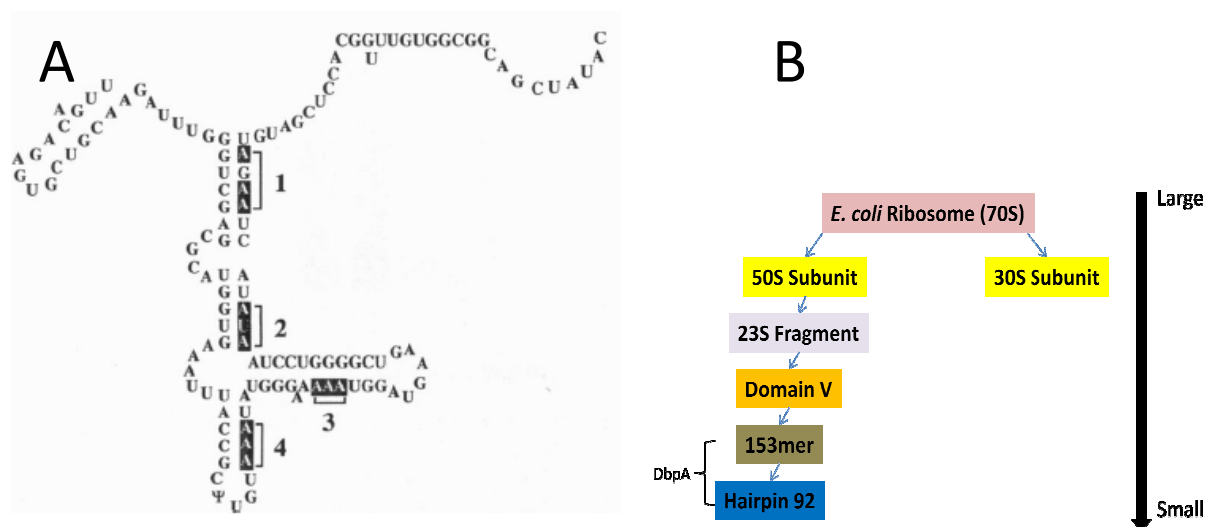


Figure 11. A) Structure and sequence of the 153 nucleotide fragment required for DbpA function. In a previous study, stems indicated 1-4 are sequentially recognized by DbpA and are important in triggering ATP hydrolysis⁶⁴. B) Flowchart to aid in the understanding of where DbpA specifically binds and acts in the ribosome.

The specific role of DbpA is to assist with the formation of the peptidyltransferase center of the large ribosomal subunit and to fuse the subunits together. The peptidyltransferase center of the ribosome is located between the large and small subunits, and is responsible for forming the peptide bond between amino acids in the growing protein chain. A previous study illustrated this fact by analyzing DbpA function with a series of mutant proteins. The noteworthy portion

of this study involved the mutant protein containing an amino acid mutant at the position 331 arginine (R331). R331 is an amino acid residue in motif IV within the ATP binding pocket of DbpA that binds the γ -phosphate. It is said to act as the “arginine finger” and stabilizes the transition state of ATP hydrolysis²³. R331 was mutated so the amino acid residue at position 331 was no longer an arginine, but an alanine (R331A). Alanine is non-reactive and therefore this mutation eliminated the possibility for any interaction with the amino acid at position 331. As a result of this mutation, the R331A protein increased production of the 50S and 30S ribosomal subunits, but decreased the fusion of the subunits into the mature and active 70S ribosome³⁶. This suggests that the subunits were folded incorrectly and were prevented from combining with one another, illustrating DbpA’s role in the formation of a mature ribosome.

In addition to the functional characterization of DbpA, this protein has also been extensively biochemically characterized. Its binding affinities for both ATP and RNA have been determined and show a cooperative binding between the two substrates, meaning the binding of one substrate facilitates the binding of the other. The dissociation constant (K_d) of RNA in the presence and absence of ATP suggests its cooperativity. A protein’s K_d is the concentration of substrate at which half the protein is bound. The smaller the K_d value, the higher the protein’s affinity for its substrate (Appendix B). In the absence of ATP, the 153mer has a K_d of 0.44 nM. In the presence of AMPPNP (non-hydrolyzable analog), the K_d decreases to 0.05 nM (Fig. 12)⁶. These studies also have shown that helix 89 and hairpin 92 are both required for the cooperative binding of RNA. For example, the RNA R80 (80 nucleotide fragment that is missing helix 89) has an RNA binding K_d of 0.97 and 2.0 in the presence and absence of AMPPNP, respectively (Fig. 12)⁶. Since the K_d does not decrease upon the binding of ATP, the protein does not exhibit cooperative behavior. The loss is a result of the removal of

H89 from the 153mer structure to form the 80mer. This helix, therefore, is suggested to be a necessary recognition element, in addition to hairpin 92, for the binding of DbpA to its RNA substrate⁶.

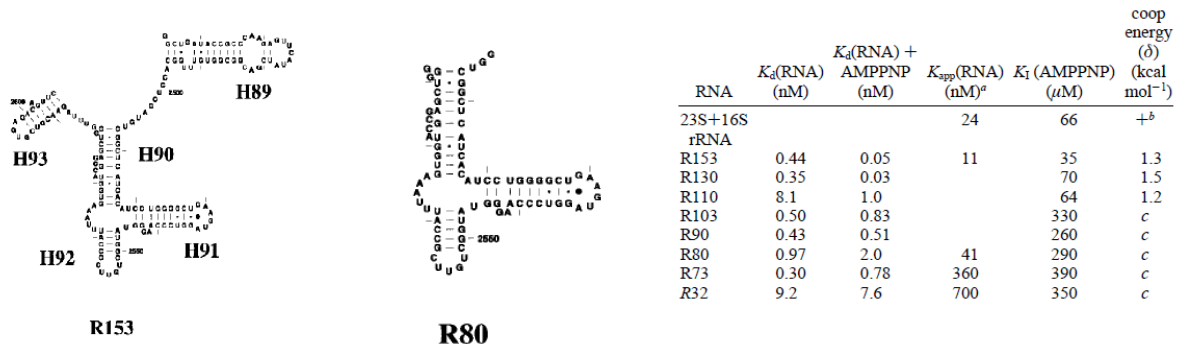


Figure 12. Cooperative binding of DbpA to 153mer and 103mer, respectively. Data shows helix 89 is required for cooperative binding of ATP and RNA⁶. RNA notation represents the number of nucleotides present in the overall 153mer structure or substructure⁶.

DbpA has been further studied using the Michaelis-Menton Enzyme Kinetic model (Appendix A)³⁸. As illustrated in Figure 13, as substrate concentration increases *in vitro*, the rate of reaction increases until it reaches saturation (100% enzyme is bound to substrate). This is considered the maximum reaction rate ($V_{\max}$). Dividing the $V_{\max}$ by the concentration of the protein can give the maximum rate of catalysis (k_{cat}), or ATP processed per minute per enzyme (Appendix A). The Michaelis Constant (K_m) is similar to the dissociation constant (K_d) and represents the concentration of substrate at which half of the enzyme is bound. It is calculated as a result of enzyme kinetic activity, in contrast to the K_d value which is determined by directly analyzing protein-substrate binding. K_m approximates the dissociation equilibrium and gives insight into the binding affinity of the enzyme. Thus, a lower K_m value indicates a higher affinity of a protein for its substrate³⁷.

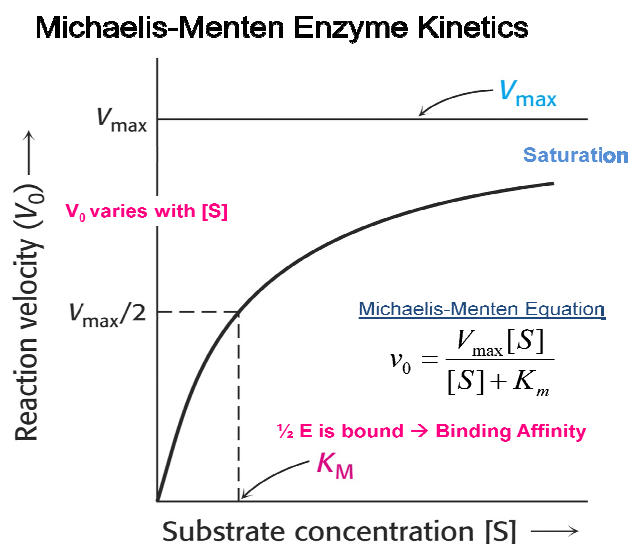


Figure 13. Representative Michaelis-Menten Enzyme Activity Graph. E denotes enzyme, $V_{\max}$ denotes maximum rate of reaction, K_M denotes the Michaelis constant.

One major assumption under the Michaelis-Menton model is that one enzyme binds one substrate (Appendix A). In the case of DbpA, two substrates are required for ATP hydrolysis stimulation and helicase activity. Therefore, in order to perform Michaelis-Menten kinetics, one substrate must be present in saturating amounts to ensure that all enzymes are bound to the first substrate, allowing the usage of the Michaelis-Menten equation (Appendix A).

One major question that remains unanswered for DbpA is: Which types of contacts are responsible for the cooperative behavior of DbpA with only certain RNAs? The current hypothesis is that cooperativity is mediated by additional ionic contacts within the ATP binding pocket that which form as a result of RNA binding to the protein (Fig. 14). In theory, these additional interactions form a protective barrier around the ATP molecule to protect it from the environment². In order to determine if these additional interactions are present and which amino acids are responsible for them, a series of experiments were run on wild type DbpA and four associated mutants.

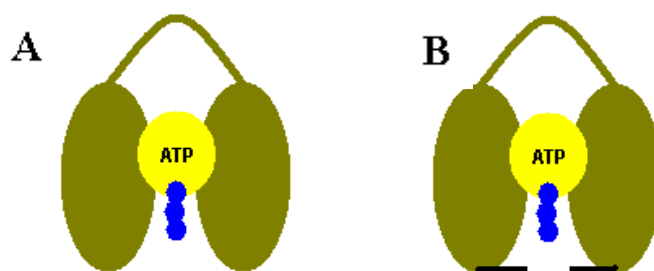


Figure 14. A) Non-cooperative interaction of DbpA (green) with ATP. B) Additional ionic interactions (black) leading to the cooperative binding model. In the non-cooperative model, ATP binding does not induce the additional ionic interactions to form the protective salt bridge.

I have previously analyzed the Michaelis-Menten parameters as a function of salt for wild type DbpA and the S184A, T186A, H327A, and R334A mutants. Mutants were selected because they had an effect on the RNA binding cooperativity of DbpA, but did not render the protein inactive²³. In the mutant protein, the amino acid under investigation is mutated to an un-reactive alanine. This eliminates the possibility of the amino acid interacting with ATP or other residues in the binding pocket. These mutants were used to determine the cooperative nature of the ATP binding pocket in DbpA. Different RNAs can result in either cooperative or non-cooperative binding. Therefore, the mutants are used to give insight as to which amino acid or motif might be responsible for sensing the type of RNA bound.

Ribosomal RNA was present in high concentrations in all experiments within the given salt range to ensure substrate saturation. The Michaelis-Menton values were determined by running a high throughput ATPase assay (Appendix C) on wild type DbpA and the four mutants to measure production of ADP over time.

The K_m in my analysis was measured with respect to salt concentration which gave insight to cooperative binding. The slope of $\log(K_m)$ vs. $\log[\text{salt}]$ sets an upper limit to the number of ionic interactions within the ATP binding pocket^{39,40}. The wild type DbpA as well as

the S184A, T186A, and H327A mutants all had a slope of about one, thus indicating at most one ionic interaction (Fig. 16A and B). Interestingly, the R334A mutant had a slope of approximately zero which indicates that the intermolecular interactions with ATP are not ionic suggesting that arginine 334 in DbpA mediates the proposed important ionic contacts. If an important ionic interaction was present, an increase in K_m would be noted as salt increased. This would be a result of the salt bridge's ability to protect ATP binding at low concentrations yielding a lower K_m , yet showing difficulty protecting binding at higher salts, showing a higher K_m . These trends were observed as expected with wild type DbpA, S184A, T186A, and H327A mutants as expected.

a

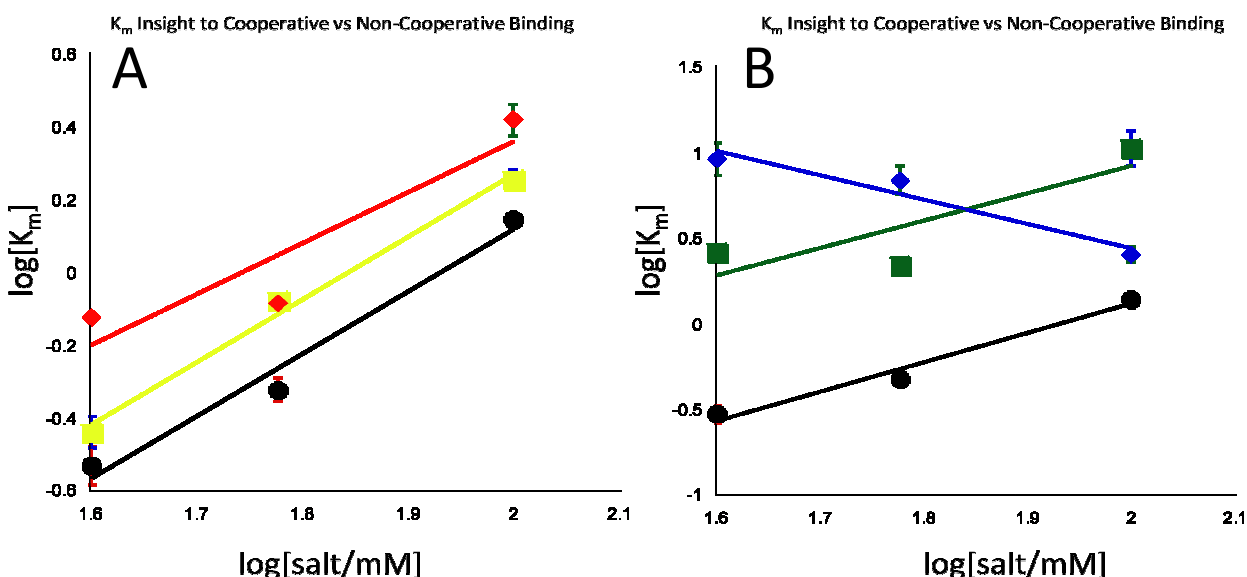


Figure 16. Salt effect on K_m . A) Wild Type (black) 1.72, S184A (yellow) 1.7, T186A (red) 1.4. B) DbpA (black), H327A (brown) 1.6, R334A (blue) -1.4. The slope of the lines represents the maximum number of ionic interactions within the protein in question³⁹. DbpA has a slope of 1.72, S184A was 1.7, T186A was 1.4, H327A was 1.6, and R334A was -1.4. All mutants had a weaker initial K_m than the wild type, and R334A was the only mutant without a slope similar to that of the wild type. This suggests R334 has an effect on DbpA's cooperative binding. Values are an average of 3 trials.

In addition to analyzing the K_m for the proteins, the maximum rate of reaction per enzyme can also give insight towards the cooperative nature of DbpA. Figures 17 A and B show

the R334A mutant has the largest decrease k_{cat} as a function of salt, suggesting it is the most catalytically sensitive to the increasing salt environment. This sensitivity of the mutant to increasing salt suggests the elimination of the protective bridge, exposing the ATP to electrostatic competition. The catalytic rate of DbpA and the S184A, T186A, and H327A mutants that show cooperative ATP binding is unaffected or modestly affected by an increase in ionic strength. This shows that the cooperative ionic interaction are protecting the hydrolysis of ATP from its environment, thus giving roughly the same rate of hydrolysis regardless of salt environment. In contrast, if a protein demonstrates non-cooperative ATP binding, sharp decrease in k_{cat} is observed since the gate ionic interaction is not present to protect hydrolysis, as observed with the mutant arginine protein (R334A).

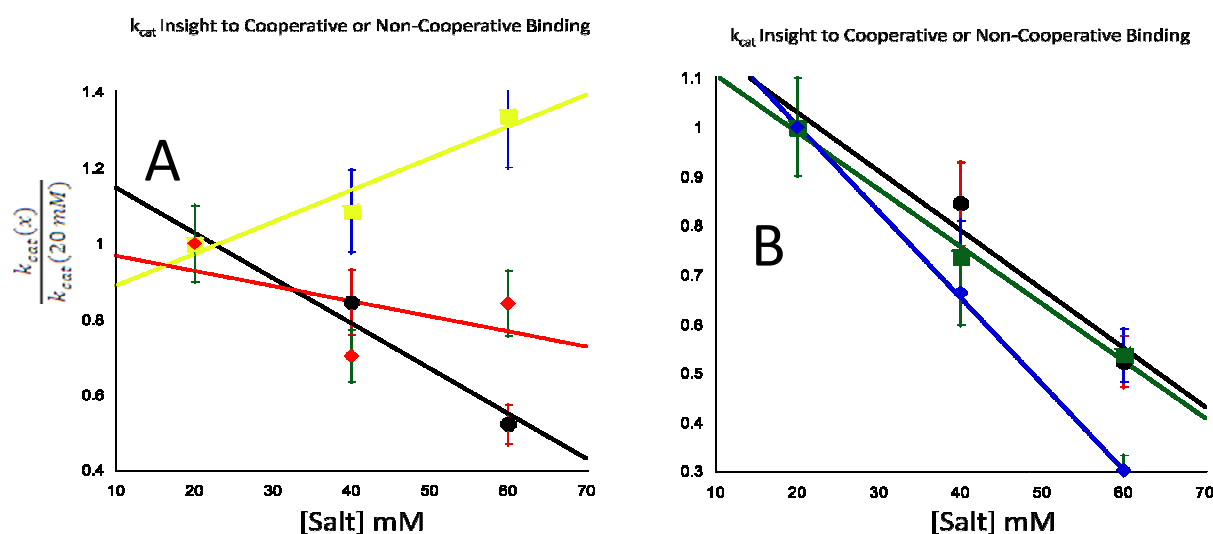


Figure 17. Effect of salt on k_{cat} for A) DbpA (black), S184A (yellow) T186A (red). B) DbpA (black) H327A (brown) and R334A (blue). Note how R334A is the most catalytically sensitive to the increasing salt environment, decreasing activity as salt levels increase. This suggests the ATP binding pocket is not protected by the proposed ionic interactions that result from DbpA's cooperative nature. Values are an average of 3 trials.

The next step towards understanding the cooperative nature of DbpA is to directly analyze ATP binding, as opposed to analyzing the kinetic parameters of the Michaelis-Menten model. High Throughput Filter Binding and Spin Column Assays were employed to measure the

dissociation constants of ATP binding for DbpA and the selected mutants at varying salt concentrations. With these methods, a radioactively labeled non-hydrolyzable analog of ATP, ATP γ S (Fig. 18), will bind to DbpA in the presence and absence of RNA, allowing a direct measure of its binding, not hydrolysis. ATP γ S is non-hydrolyzable due to the replacement of oxygen on the γ -phosphate with a sulfur atom. This sulfur atom will be radioactively labeled with ^{35}S . This substitution greatly diminishes ATP-hydrolysis, by disabling the coordination of magnesium and water that is necessary for ATP-hydrolysis. This is caused by the electronic and size differences between sulfur and oxygen⁴¹.

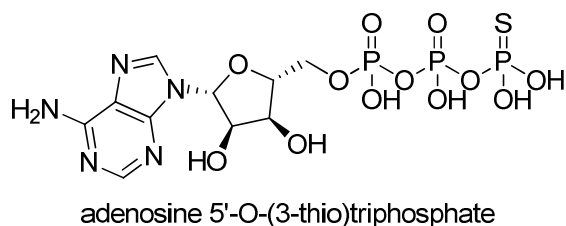


Figure 18. ATP γ S, a non-hydrolyzable analog of ATP. ATP γ S still binds correctly to DbpA but does not undergo hydrolysis without an associated magnesium ion⁴¹.

While a significant amount of biochemical information exists regarding DbpA, it remains to be determined which interactions lead to its cooperative nature. With the use of Spin Column Assays and High Throughput Double-Filter Binding Assays, the direct effect of salt concentration on ATP binding can be analyzed. Wild type DbpA as well as the four mutants, S184A, T186A, H327A and R334A, were used to analyze the interactions responsible for cooperative ATP binding. In conjunction with the known kinetic parameters for DbpA, as well as the direct binding analyses, I have attempted a comprehensive analysis of the DbpA ATP binding pocket.

Materials and Methods**ATPase Assay, RNA Titration**

NADH/PEP Dilution Buffer (10 mM Hepes pH 7.5, 50 mM KCl, 1mM DTT) was used in order to prepare 200 mM NADH and PEP stocks. rRNA from *E. coli* was serially diluted with an RNA Dilution Buffer (50 mM Hepes pH 7.5, 50 mM KCl, 5 mM MgCl₂) 10 times in a 12 well dilution strip, leaving only dilution buffer in the last two wells for a blank measurement. A 1.25X Master Mix was created of 125 mM Tween 20, 62.5 mM Hepes pH 7.5, 12.5 mM MgCl₂, 1.25 mM DTT, 1.25 mM PEP, 1.75 mM NADH, 12.5 mM PK/LD, 7.5 mM ATP, specific concentration of protein (50 nM DbpA, 150 nM S184A Mutant, 150nM T186A Mutant, 250 nM H327A Mutant, 250 nM R334A Mutant), and desired concentration of KCl. 40 µL of 1.25X Master Mix was added to a specified well in a 384-well plate, and the reaction was initiated with 10 µL of rRNA from the serial dilution. The 384-well plate was spun quickly at 2,000 rpm and placed in the spectrophotometer. The absorbance of each well was read at 338 nm, 5 flashes per well every 30 seconds for 15 minutes. The pathlength of each well was then calculated spectrophotomically. Assays were run on rRNA, 153mer RNA, and 80mer RNA. Data was analyzed in accordance with Appendix D.

ATPase Assay, ATP Titration

NADH/PEP Dilution Buffer and RNA Dilution Buffer were prepared as per the rRNA Titration Protocol (above). ATP was serially diluted water 10 times in a 12 well dilution strip, leaving only water in the last two wells for a blank measurement. A 1.25X Master Mix was created containing 125 mM Tween 20, 62.5 mM Hepes pH 7.5, 12.5 mM MgCl₂, 1.25 mM DTT, 1.25 mM PEP, 1.75 mM NADH, 12.5 mM PK/LD, 250 nM RNA (either rRNA or 153mer), specific concentration of protein (50 nM DbpA, 150 nM S184A Mutant, 150nM T186A Mutant, 250 nM H327A Mutant, 250 nM R334A Mutant), and desired concentration of KCl. Remaining

protocol was identical to an RNA titration, and data was analyzed in accordance with Appendix D.

153mer and 80mer Folding Protocol

The 153mer rRNA was refolded into its correct structure each time an experiment was performed. The desired starting concentration of 153mer was added to 50 mM Hepes and 25 mM KCl. This solution served as the first well in the 10 step dilution series in accordance with the rRNA Titration Protocol. The solution was heated for 2 minutes between 90 and 95 °C in a heating block. The block was then removed and allowed to cool to approximately 35 °C by sitting at room temperature. 60 mM MgCl₂ was added to cool 153mer to obtain a final concentration of 1 mM MgCl₂. Solution sat at room temperature for 5 minutes. Newly folded 153mer RNA was then diluted serially and used in the given experiment.

TLC Plate Assay

Running Buffer was made containing 750 mM LiCl and 37.8 mM Formic Acid. Per 500 mL of Running Buffer, 15.9 g of LiCl was added to 300 mL of ddH₂O and was allowed to go into solution. Then 18.9 mL of 1 M Formic Acid was added and then brought to a total volume of 500 mL. 5X Binding Buffer was made containing 250 mM Hepes, 25% glycerol, 5 mM DTT, 25 mM MgCl₂, and desired concentration of KCl. Next, a 5X RNA Buffer was made containing 1X Binding Buffer and 4 μM 153mer RNA. A 5X Radioactive ATP Mix was made containing 1X Binding Buffer, 25 mM ATPγS (cold), 0.517 nM ATPγS (hot), 25 mM MgCl₂, and 25 mM DTT. A 5X Protein Buffer was made containing 5X of the 5X Binding Buffer and 500 nM of the desired protein. For a no protein control reaction, a final Reaction Buffer was made containing 1X Binding, RNA, and ATP Buffer. ATP binding was measured in presence and absence of RNA to determine cooperativity. Binding reactions sat at room temperature for one

hour then 45 mM EDTA pH 8 was added to stop the reaction. TLC plate was pre-run in water and dried. The TLC Plate was spotted with 0.5 μ L of reaction, and the TLC plate ran in the Running Buffer for 30 minutes. The plate was allowed to air dry and the plate was exposed to a phosphor-imaging screen. The screen's phosphorescence was detected and the intensity of the each initial spot and upstream region was recorded. The % ATP hydrolyzed was calculated by dividing the intensity of the upstream region (contained radioactively labeled ATP γ S) by the intensity of the initial spot.

Spin Column Binding Assay

Wild type DbpA only was tested with RNA and without RNA. No mutants were tested using the Spin Column Assay. The Binding Buffer contained 100 mM Hepes pH 7.5, 20 mM MgCl₂, 2 mM DTT, 10% glycerol, 50 nM radioactively labeled ATP γ S, 1.6 μ M RNA (if applicable) and the desired concentration of KCl. The protein was serially diluted by half over a total of six reaction tubes. The first reaction tube in each series contained 5 μ M of protein in 1X Buffer to a total of 50 μ L. Once each protein was diluted by half six times, the reaction tubes were incubated at room temperature for 1.5 hours. Wash Buffer was made containing all elements of the Binding Buffer without ATP or RNA. Each Spin Column was washed with 100 μ L of Wash Buffer by spinning the column at 3,000 rpm for 2 minutes, or just enough to wet the filter. The tubes were then spun again inverted to remove any excess liquid that did not pass through the filter. After incubation period, 1 μ L of Reaction Mix from each tube was spotted on a TLC Plate to measure total radiation. The remaining Reaction Mix was added to a Spin Column, and spun at 3,000 rpm very briefly, just enough time to collect approximately 1 μ L of filtrate. 1 μ L of filtrate from each tube was spotted on the TLC plate, and was exposed to the phosphor-imaging screen after drying. The screen's phosphorescence was detected and the

intensities of the filtrate and total ATP were determined. The fraction of free ATP was calculated by dividing the intensity of the filtrate by the intensity of the total ATP. The fraction free was then plotted as a function of protein.

Filter Binding Assay

The binding of DbpA and mutants was identical to the Spin Column Binding Assays with 6 dilutions of protein starting at 5 μ M. The Binding Buffer contained 10.3 nM hot ATP γ S, as opposed to the 50 nM used in the Spin Column Binding Assay. Soak Buffer (50 mM Hepes pH 7.5 and desired concentration of KCl) and a Wash Buffer (50 mM Hepes pH 7.5, 10 mM MgCl₂, and desired concentration of KCl) were made during incubation time. The Wash Buffer remained on ice while the nitrocellulose and hy bond membranes soaked in the soak buffer for one hour. After the 1.5 hour reaction incubation, 1 μ L of reaction mix was spotted on a TLC plate. This was used to measure total radiation for the reaction. The blot for the experiment was set up in accordance with Appendix F. 75 μ L of ice cold wash buffer was passed through specified wells, followed by 25 μ L of the binding reaction. For 30 mM KCl and lower, 75 μ L of Wash Buffer was then passed through the specified wells. For 60 mM KCl, 50 μ L of Wash Buffer was then passed through the specified wells. For any salt over 50 mM, no Wash Buffer was passed over the specified wells. Washing was avoided to prevent washing the radioactive ATP γ S completely through the filters. The blot was vacuumed for an additional 10 minutes to ensure all reaction mix passed through the filters. The filters were separated and allowed to dry at room temperature. Nitrocellulose filter and TLC plate were exposed to a phosphor-imaging screen. The phosphorescence of the plate was detected, and the intensities were used to analyze free ATP in accordance with Appendix G.

Results and Discussion

RNA Folding of 153mer and 80mer

The exact binding location of DbpA to its rRNA substrate has been determined previously as Hairpin 92³². Therefore, any subfragment of rRNA containing H92 could be used in the assays. I worked with two smaller fragments of rRNA, both of which contained Hairpin 92. These two sub-fragments were the 153mer and the 80mer. These rRNA strands are 153 and 80 nucleotides in length, respectively. Any RNA must be in its correct mature structure before it can perform its given function. Therefore, in order for DbpA to bind as efficiently and as tightly as possible to the 153mer RNA, it was necessary to ensure that the 153mer and the 80mer were folded correctly. If Hairpin 92 was not formed in these fragments, DbpA's binding would be extremely weak. Previous experiments had suggested that the RNA was not folded correctly so it was necessary to determine which standard RNA folding protocol would work best for the 153mer and the 80mer. The first heating option was to heat the RNA for 60 seconds at 95 °C, 3 minutes at 65°C, then cool to room temperature for 5 minutes. The kinetic parameters as a result of this heating protocol were extremely lower than expected, with the k_{cat} for 153mer at 47.43 ATP min⁻¹ enzyme⁻¹ as opposed to the expected value around 300 ATP min⁻¹ enzyme⁻¹ (Fig. 19). Its K_m was significantly higher than expected, proving to be 376 µM instead of 159 µM (Independent Study data, not shown). The second heating protocol proved to be inaccurate, this time heating the RNA for 1 minute at 90 °C then immediately transferring the sample to ice. Figure 20 illustrates that neither the 80mer nor the 153 mer achieved a saturation curve, and thus the results were inconclusive. Therefore, it was necessary to discontinue the use of this protocol and test a third method of RNA folding. The final heating protocol is the one described in the materials and methods section. It proved highly accurate for the 153mer, illustrating a full saturation curve. It yielded a k_{cat} of 140.64 ATP min⁻¹ enzyme⁻¹ and a K_m of 59 µM. While the k_{cat} was lower than the expected 300 ATP min⁻¹ enzyme⁻¹, the resulting Michaelis-Menten curve

shows the RNA was in the correct conformation and reached saturation. The K_m recorded was lower than previously conducted experiments, therefore suggesting the binding of DbpA to the 153mer was tight (Fig. 21). The third heating protocol unfortunately illustrated no effect on the 80mer. Therefore, I decided that the 80mer could not achieve saturation and thus discontinued analyzing it.

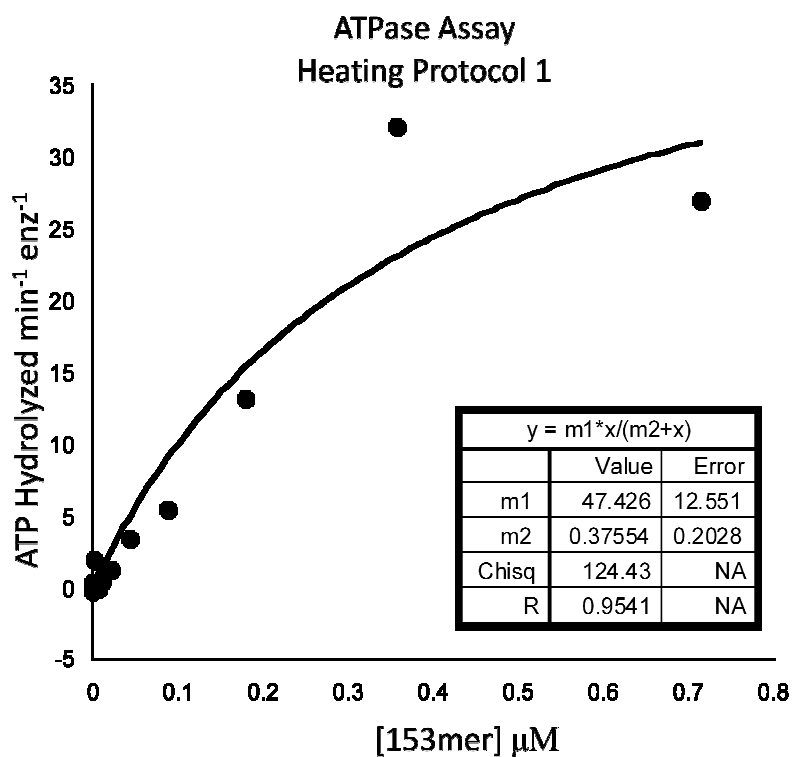


Figure 19. Results showing Heating Protocol 1 (heat 60 seconds at 95 °C, 3 minutes at 65°C, then cool to room temperature for 5 minutes) was ineffective at folding the 153mer. Protocol was not attempted on the 80mer.

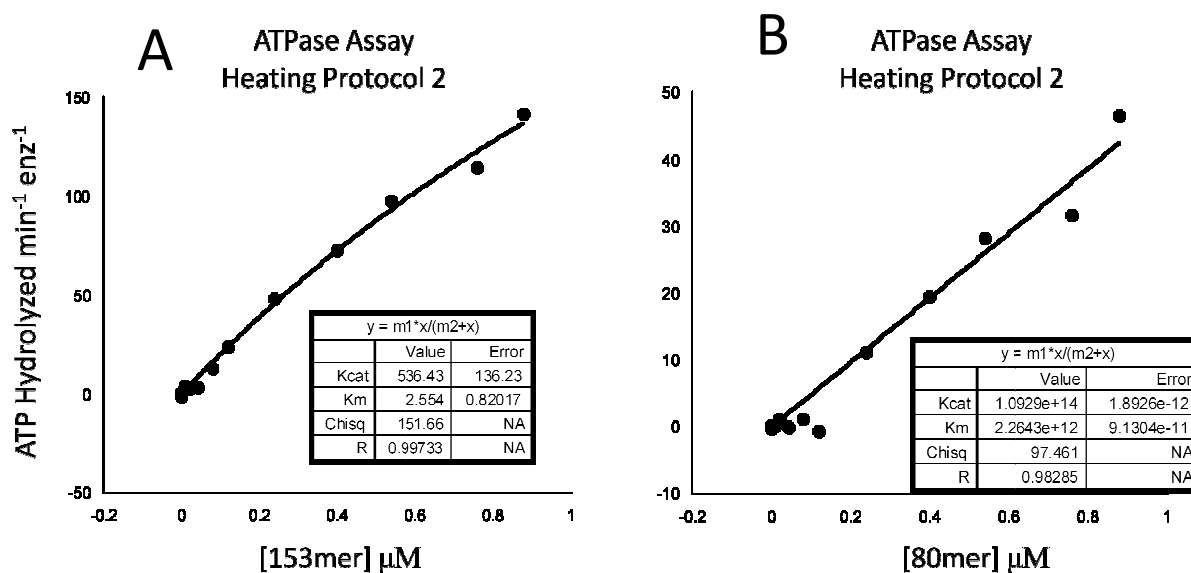


Figure 20. Results from Heating Protocol 2 (heat 1 minute at 90 °C then immediately transferring the sample to ice). This test proved unreliable for both the A) 153mer and the B) 80mer.

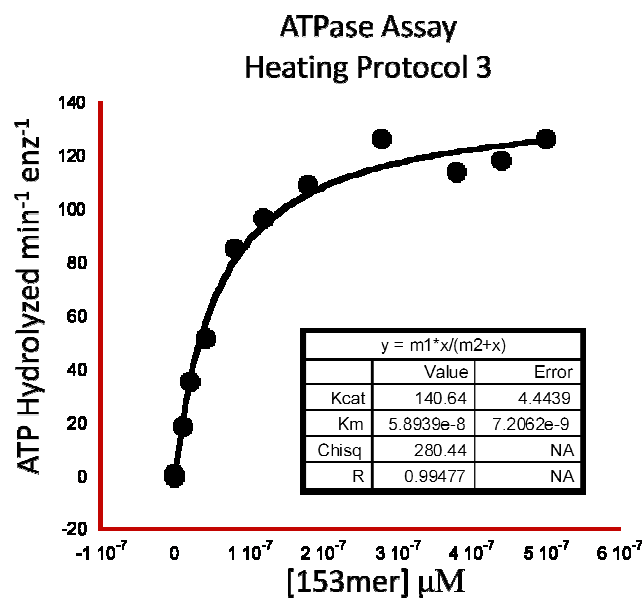


Figure 21. Heating Protocol 3 (see Materials and Methods) proved to be the best for the 153mer and was used in all future experiments.

After the correct folding of RNA was obtained, the next step was to determine what concentration of 153mer would saturate DbpA to eliminate it as a factor in equilibrium binding analysis. A series of RNA titrations were completed at varying salt levels to determine what minimum concentration of RNA could be used. It was observed at both low salt (30 mM KCl)

and high salt (100 mM KCl) that approximately 700 nM of the 153mer could stimulate maximum ATP hydrolysis (Fig. 22). To error on the side of caution, 800 nM RNA was used in all direct binding assays.

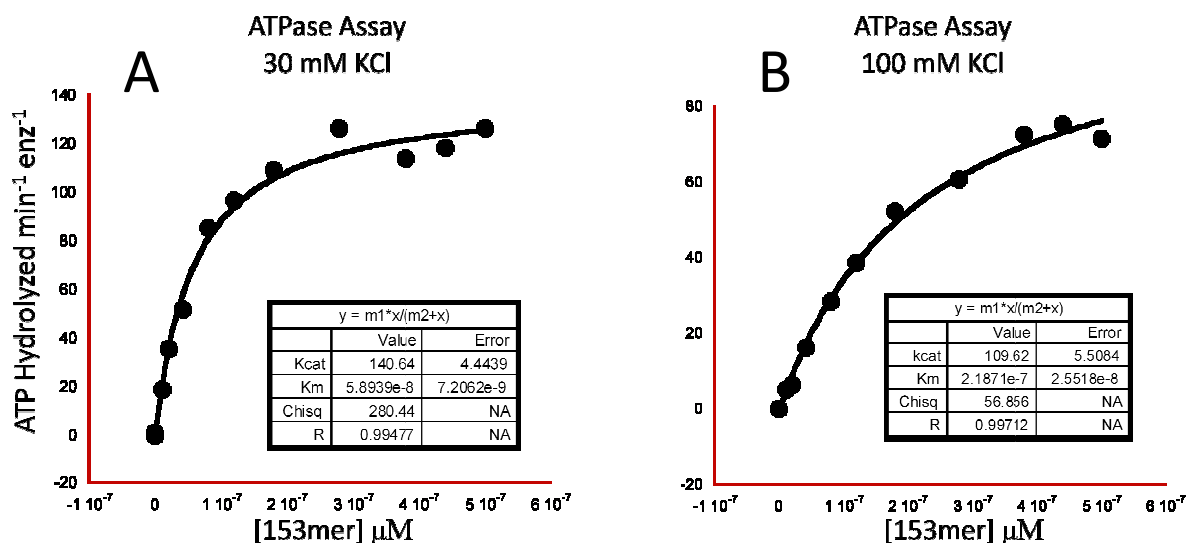


Figure 22. ATPase assay results. A) ATPase assay using wild type DbpA at 30 mM KCl. B) ATPase assay using wild type DbpA at 100 mM KCl. Data used to help determine saturating concentration of RNA to be used in binding assays.

TLC Plate Analysis

The next step to working towards a direct binding analysis was to conduct Thin Layer Chromatography (TLC) assays. This assay was conducted to justify that the ATP γ S will not hydrolyze when bound to a protein. The assay was conducted at low and high salt, to show that the binding and lack of hydrolysis was independent of the salt concentration in the environment. At both high (100 mM) and low (30 mM) salt, wild type DbpA and all associated mutants were unable to hydrolyze the ATP γ S (Fig. 19). A hydrolysis control experiment was conducted on a kinase, a protein which specifically functions to remove phosphates from ATP. Even the kinase was unable to hydrolyze the ATP γ S. This is justified by the lack of a radioactive spot upstream from the initial spot site. If hydrolysis were to occur, a solid spot would appear upstream due to the cleavage of the gamma phosphate off of the ATP as a result of the higher polarity of the free

phosphate in comparison to the hydrolyzed ATP molecule. Therefore, this analog proved to be a great choice when analyzing binding to DbpA and associated mutants.

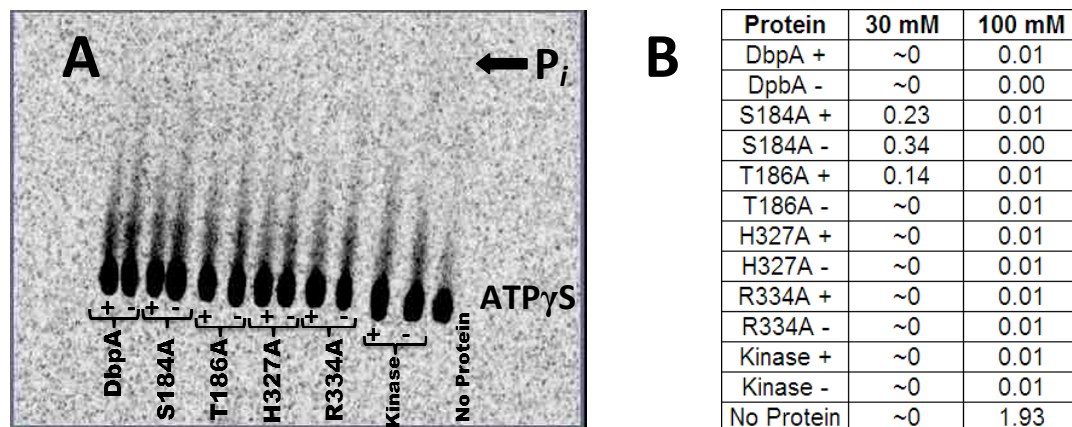


Figure 23. TLC assay results showing no protein was able to hydrolyze the ATP γ S. **A)** Example TLC radiation results. Note how there is no band upstream of the spot site, suggesting little to no ATP γ S was hydrolyzed by the protein. **B)** Numerical values for the percent of ATP γ S that was hydrolyzed by a given protein at a given salt concentration, with and without RNA. All values are significantly low and negligible, further illustrating ATP γ S is unhydrolyzable by the proteins in question.

Spin Column Binding Assay

In order to generate a positive control for the filter binding assays, spin column assays were conducted on wild type DbpA only. By the use of radioactivity, the spin columns allow a direct and accurate measure of the protein-ATP interaction. With this method, ATP, RNA, and DbpA are allowed to bind with one another at varying concentrations of DbpA. They are then added to a spin column containing a filter. This filter will prevent any protein and bound ATP γ S from passing through, allowing free ATP γ S to pass. Therefore, the radioactivity of both can be determined, and the fraction of ATP γ S can be determined. The method is sensitive and allows for accurate measurements without damaging effects on the overall protein. However, this method is very tedious and time consuming and therefore was only employed briefly in an attempt to determine a positive control. In the first Spin Column Assay, the columns containing the binding reaction buffer were spun for too long and at too high of an RPM. Therefore, the physical strain on the reaction more than likely disrupted the protein structure and yielded

inaccurate results. Therefore, the next time the assay was conducted I decreased the speed at which the columns were spun, as well as the amount of time they spun in hopes to avoid denaturing the protein.

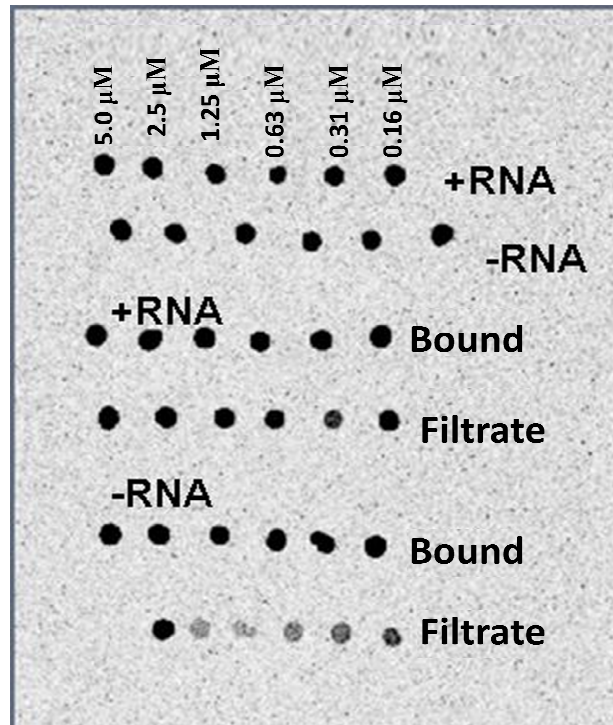


Figure 24. Example Spin Column raw data. In theory there should have been a noticeable decrease in the intensity as protein concentration decreased. The obtained results were not as definitive as predicted.

The results from this experiment gave linear correlations as expected, but the data was inconclusive when comparing the with and without RNA results (Fig. 25A and B). The major inconsistency between the two was the values of the y-axes. The reaction containing RNA should have had larger values on its y-axis because theoretically there should have been less free ATP. At worst the axes would have been the same, but the graph with RNA should not have smaller values when compared to the reaction without RNA. The slopes correlate to the K_d of the protein for its substrate. With RNA resulted in a slope of 2.98, correlating to a K_d of 0.34 μM . Without RNA yielded a slope of 10.782, correlating to a K_d of 0.093 μM . These values are inconsistent with the current literature. According to a previous study, the K_d of DbpA for ATP

is $21\mu\text{M}$ in the presence of an RNA substrate⁶. My result is approximately 60-fold under what the determined K_d value should be, suggesting the need for yet another trial. The final Spin Column protocol was altered slightly, now placing samples on a cellulose 300, F-254 TLC plate to expose to the phosphor-imaging screen, instead of filter paper, which was used in the previous two assays. No significant correlation could be determined from the obtained results. It was ultimately concluded that the spin columns used in the assay were far too big for the small volume of reaction mixture, that protein was stuck to the filter.

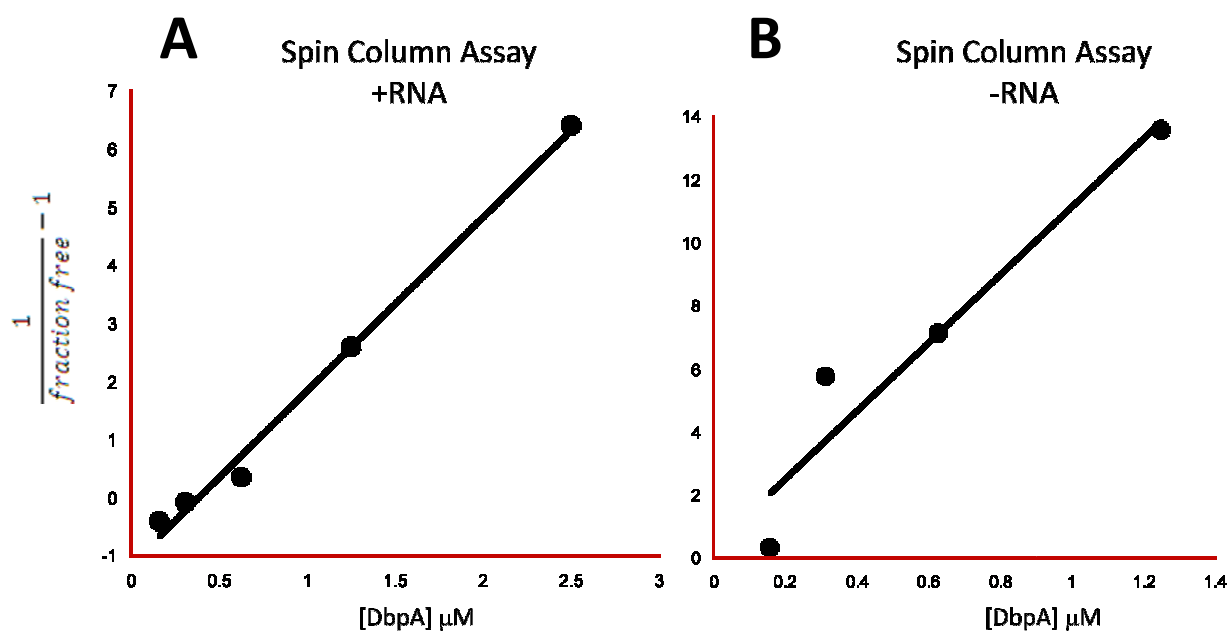


Figure 25. Spin column assay results A) with RNA and B) without RNA. The slope of the lines correlates to $1/K_d$ for a given protein. The observed slope of the +RNA graph was 2.98, translating to a K_d of $0.34\mu\text{M}$. The observed slope of the -RNA graph was 10.782, translating to a K_d of $0.093\mu\text{M}$. These results are inconclusive, since we would expect lower values on the y-axis in the presence of RNA.

Filter Binding Assay

Since the Spin Columns kept yielding inconclusive results, the next step was to look for an alternative method to directly analyze the binding parameters of DbpA. The method needed

to be quick, efficient, and able to be easily adapted to a high-throughput method. The suggested Filter Binding Assays met all the above criteria and appeared to be a logical next-step towards obtaining relative information. The new method was optimized to obtain reproducible and reliable data in accordance with Appendix F. The first issue the protocol gave was with the hy-bond filter. The hy-bond filter is a membrane with a positive charge that in theory should trap ATP and RNA. However, this filter did not seem to be trapping any of the unbound ATP once the reaction mix had passed through the initial nitrocellulose filter (Fig. 26A). This hindered the determination of the total intensity which was to be used in data analysis in accordance with Appendix F. In order to calculate total intensity to be used in analysis, 1 μL of each reaction was spotted on to a cellulose 300, F-254 TLC plate before passing it through the filters (Fig. 26B). The intensity of TLC was multiplied by 25 since 25 μL of reaction was filtered through the membranes (Appendix F).

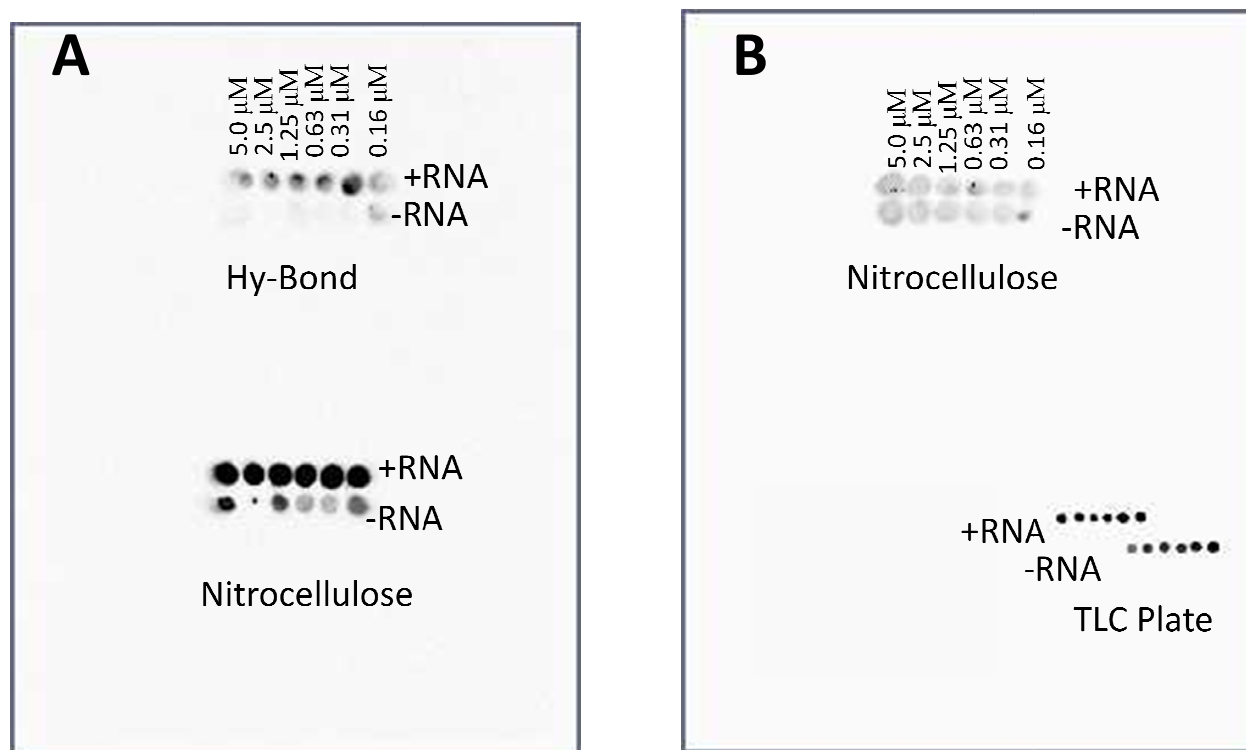


Figure 26. A) Filter binding assay at 30 mM KCl. Hy-bond did not appear to be trapping free ATP γ S. B) Filter binding assay at 30 mM KCl using TLC plate to spot reaction to use for total intensity. Experiment performed on wild type DbpA only and protein concentration decreases from left to right.

These protocol adjustments proved beneficial, although it was still observed that the binding of the protein to the RNA was still weak. This hinted back toward the initial problem of the 153mer not being folded correctly. The subsequent filter binding assay was completed after the aliquot of 153mer had been refolding according to the heating protocol from determined by the ATPase assays. While the radiation image looked promising, once the data was analyzed and plotted, there were only a few mutants that worked, as well as the wild type DbpA. A linear correlation was noted for the highest three protein concentrations for the wild type DbpA and the H327A mutant at 30 mM (Fig. 27). The DbpA had a recorded K_d of 152.3 μ M, while the H327A mutant had a K_d of 748.5 μ M, neither of which are close to the published value of 21 μ M⁶. On a positive note, the wild type K_d was significantly close to the published K_m for DbpA, 120 μ M, illustrating the fact that K_d and K_m are in fact similar measures of binding affinity. The increase

in K_d with the induction of the H327 mutant shows ATP is bound significantly weaker with the mutant. All other mutants tested with this method showed extremely weak binding, with an inconsistent linear regression.

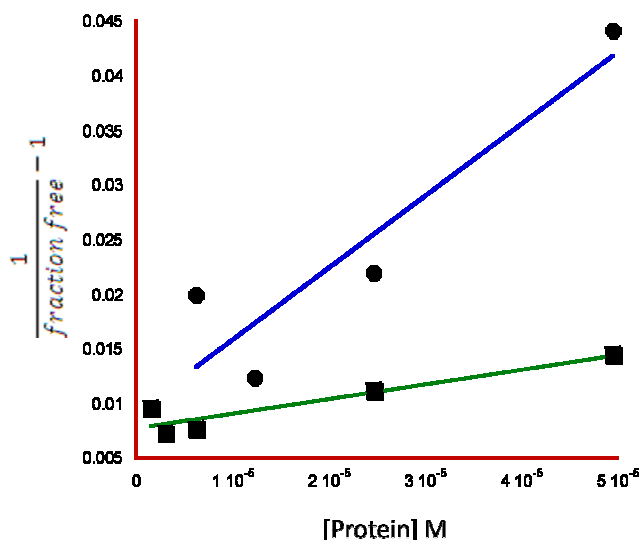


Figure 27. Filter binding analysis of wild type DbpA (blue) and H327A (green) mutant at 30 mM KCl after refolding by heating of the 153mer RNA. The slope of the line for wild type DbpA was 565.54, which translated to a K_d of 152.3 μM . The H327A mutant had a slope of 133.6, which translated to a K_d of 748.5 μM .

As salt concentration was increased, even weaker binding was observed for all proteins analyzed. It was therefore estimated that the K_d values for increasing salt were approximated as greater than 1 mM. Again, these values are substantially greater than the previously published K_d and K_m values for wild type DbpA and the associated mutant proteins (Table 1). This data is consistent with the published K_m value for H327A of 2000 μM ²³.

Protein	30 mM KCl	50 mM KCl	80 mM KCl	100 mM KCl
DbpA	152.3 μ M [*]	>1 mM	>1 mM	>1 mM
S184A	>1 mM	>1 mM	>1 mM	>1 mM
T186A	>1 mM	>1 mM	>1 mM	>1 mM
H327A	748.5 μ M [*]	>1 mM	>1 mM	>1 mM
R334A	>1 mM	>1 mM	>1 mM	>1 mM

Table 1. Estimated K_d values for DbpA and the associated mutants. (*) values have been experimentally determined, while the remaining values have been estimated based on current observations and experimental troubleshooting.

The filter binding experiments show a strong linear correlation at high protein concentrations. Therefore, it is suggested that the starting concentration of protein used in the dilution sets was too low. It appears that the simple act of increasing the starting concentration of protein would solve this problem, however increasing the concentration much further would cause the filter to clog easily, and yield even more inaccurate data. The observed binding parameters of DpbA and the associated mutants were less than consistent from trial to trial, and were even further inconsistent with the current published data for both the K_d and K_m values. It can easily be concluded that the protocol for the Filter Binding Assay is still imperfect and needs significant improvements. I am currently attempting to revamp the filter binding protocol in order to obtain a method free from the errors encountered over the course of the past few months.

Conclusion

The attempt to determine cooperative binding in DbpA has posed several problems with the Spin Column and Filter Binding assays. The two different protocols presented their own set of difficulties, and a concrete solution to these issues has yet to be determined. At first the main issue appeared to be human error in adjusting to a new protocol, however over time the problems persisted. I have analyzed all aspects to the filter binding assay, but have yet to determine why

the assay is not working. A different variation of spin columns are being researched in the hopes that a smaller spin column will lead to a more accurate experiment. A more in-depth analysis of both protocols is currently under investigation, and hopefully I will be able to determine the main issues at hand and fix them accordingly. If the radiation experiments prove not to be the best way of determining the binding parameters of DbpA, the next step would be to investigate the usage of fluorescence instead. This way, the filtering method can be avoided all together.

In contrast to the filter binding assays and the spin column assays, there was success with the ATPase assays and TLC plate assays. It can be verified from the results of the TLC plate assays that ATP γ S binds to DbpA and all associated mutants but does not undergo hydrolysis (Fig. 23A). The previous kinetic analysis of DbpA and the mutants suggest that arginine 334 has some connection with the ionic salt bridge that protects the ATP in its binding pocket, since the R334A mutant was the most catalytically sensitive to an increasing salt environment (Fig. 17B), and its K_m analysis alludes that there are no ionic interactions present within the binding pocket when the arginine is mutated to alanine (Fig. 16B). Therefore it can be concluded that the arginine 334 is a key amino acid residue which is responsible for the cooperative binding of DbpA, thus driving its conformational changes, allowing the protein to perform its RNA helicase activity.

Appendix A

Michaelis-Menten Kinetics and Equation Derivation

(1)

The basic Michaelis-Menten enzymatic reaction involves an enzyme (E) and its respective enzyme binding to one another to form the enzyme-substrate complex (ES). It is assumed that one enzyme binds one substrate in a reversible fashion, and that the substrate binding is very rapid. As the enzyme acts on the given substrate, the ES complex is broken apart to form the original enzyme and the product of reaction (P). Michaelis-Menten Kinetics is held under the steady state assumption, or that the [ES] is constant.

The total amount of enzyme involved in the reaction is given by:

$$[E_T] = [E] + [ES] \quad (2)$$

So the rate of formation of the [ES] complex will be:

$$v_f = k_1[E][S] = k_1([E_T] - [ES])[S] \quad (3)$$

And the rate of dissociation of the [ES] complex will be:

$$v_d = k_{-1}[ES] + k_2[ES] = (k_{-1} + k_2)[ES] \quad (4)$$

So $\frac{d[ES]}{dt} = v_f - v_d$, however since the [ES] is constant, $\frac{d[ES]}{dt} = 0$, hence

$$v_d = v_f. \quad (5)$$

$$k_1([E_T] - [ES])[S] = (k_{-1} + k_2)[ES] \quad (6)$$

$$\frac{([E_T] - [ES])[S]}{[ES]} = \frac{(k_{-1} + k_2)}{k_1} = K_m \quad (7)$$

Appendix A Cont'd

Michaelis-Menten Kinetics and Equation Derivation

K_m is referred to as the Michaelis Constant and gives insight towards the enzyme's affinity for its substrate. It is the point where half of the enzyme is bound to substrate. Therefore, the lower the K_m value, the higher the affinity of the enzyme for its substrate and the tighter it binds. From the Michaelis Constant, we can rearrange the equation to solve for $[ES]$ which will give insight towards the rate of product formation.

$$K_m = \frac{([E_T] - [ES])[S]}{[ES]} \quad (8)$$

$$[ES] = \frac{[E_T][S]}{K_m + [S]} \quad (9)$$

The rate of product formation (v) is given by $v = k_2[ES]$. Substituting in the value for $[ES]$ we get:

$$v = \frac{k_2[E_T][S]}{K_m + [S]} \quad (10)$$

Once the enzyme is saturated with substrate, it reaches a maximum rate of reaction known as V_{max} . $V_{max} = k_2[E_T]$. Substituting in V_{max} we derive the Michaelis-Menten Equation:

$v = \frac{V_{max}[S]}{K_m + [S]}$ The turnover rate of each individual enzyme is defined by its k_{cat} value:

$k_{cat} = \frac{V_{max}}{[E_T]} = k_2$ The efficiency of the enzyme can be determined by:

$$E = \frac{K_{cat}}{K_m} \quad (11)$$

Appendix B

Binding Isotherms and Equation Derivation

Binding isotherms help understand the interactions between proteins (P) and their associated ligands (L).



Therefore, the equilibrium constant for protein-ligand dissociation will be:

$$K_d = \frac{[P][L]}{[PL]} \quad (2)$$

Since this constant is representative of the dissociation of the ligand from the protein, it is often referred to as the dissociation constant (K_D). Let the fraction of protein bound to ligand (f) be defined as the fraction of bound protein divided by the total protein:

$$f = \frac{\text{Protein Bound to Ligand}}{\text{Total Protein}} = \frac{B}{T} = \frac{[PL]}{[P] + [PL]} \quad (3)$$

By rearranging K_{eq} to solve for $[P]$ and substituting into the above equation, we see:

$$f = \frac{[PL]}{\frac{K_d[PL]}{[L]} + [PL]} \quad (4)$$

$$f = \frac{[PL]}{\frac{K_d[PL] + [L][PL]}{[L]}} \quad (5)$$

$$f = \frac{[PL][L]}{[PL](K_d + [L])} \quad (6)$$

$$f = \frac{[L]}{K_d + [L]} \quad (7)$$

Appendix B Cont'd*Binding Isotherms and Equation Derivation*

By substituting in $\frac{B}{F}$ from (3) into f and solving for $[L]$ we get:

$$\left(\frac{B}{T}\right) ([L] + K_d) = [L] \quad (8)$$

$$\left(\frac{B}{T}\right) [L] + \left(\frac{B}{T}\right) (K_d) = [L] \quad (9)$$

$$\left(\frac{B}{T}\right) [L] - [L] = -\left(\frac{B}{T}\right) (K_d) \quad (10)$$

$$\left(\frac{B}{T} - 1\right) [L] = -\left(\frac{B}{T}\right) (K_d) \quad (11)$$

$$\frac{-\left(\frac{B}{T}\right)}{\left(\frac{B}{T}-1\right)} = \frac{1}{K_d} [L] \quad (12)$$

Now we have $[L]$ on the x-axis, with a slope of $\frac{1}{K_d}$.

$$1 = \frac{B}{T} + \frac{F}{T} \text{ Where F is the fraction of ligand free, so } \frac{B}{T} = 1 - \frac{F}{T}$$

$$\frac{-(1-\frac{F}{T})}{(1-\frac{F}{T}-1)} = \frac{1}{K_d} [L] \quad (13)$$

$$\frac{-(1-\frac{F}{T})}{-\frac{F}{T}} = \frac{1}{K_d} [L] \quad (14)$$

$$\left(1 - \frac{F}{T}\right) \frac{T}{F} = \frac{1}{K_d} [L] \quad (15)$$

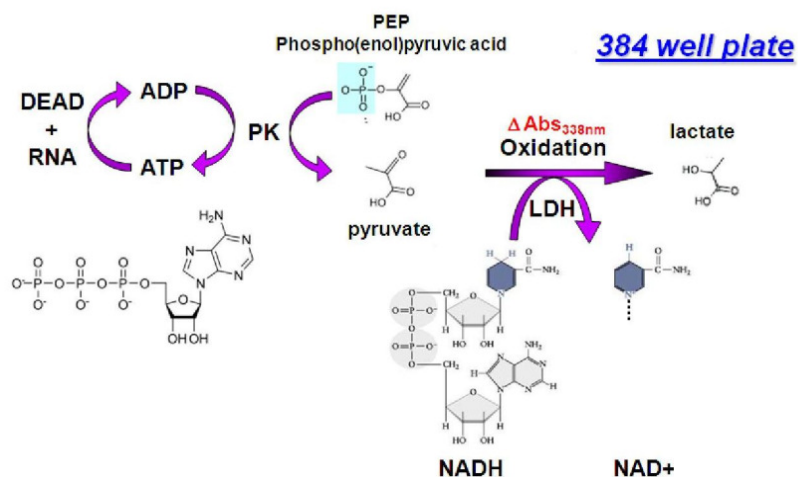
$$\frac{T}{F} - 1 = \frac{1}{K_d} [L] \quad (16)$$

And we know the fraction of ligand free is $\frac{Free}{Total}$, therefore:

$$\underbrace{\frac{1}{\text{Fraction Free}} - 1}_{\text{Y-Axis}} = \underbrace{\frac{1}{K_d}}_{\text{Slope}} [L] \quad \swarrow \text{X-Axis}$$

Appendix C

Conceptual Background of the ATPase Assays



ATPase Assay Detection Pathway. PK is pyruvate kinase which converts PEP to pyruvate. LDH is lactate dehydrogenase which reduces pyruvate to lactate. NADH the reduced state of nicotinamide adenine dinucleotide, while NAD⁺ is its oxidized state.

The protein in question (DbpA) is completely saturated with one substrate. Upon its release from the protein, ADP and phospho(enol)pyruvic acid (PEP) bind to pyruvate kinase (PK) which dephosphorylates PEP, producing pyruvate and regenerating ATP. The oxidation of nicotinamide adenine dinucleotide (NADH to NAD⁺) results from the reduction of pyruvate to lactate via lactate dehydrogenase (LDH). The oxidation of NADH can be detected spectroscopically at 338 nm. The decrease in absorbance is directly proportional to the rate of hydrolysis, which can be plotted as a function of substrate concentration (Appendix D). The resulting curve was fit using the Michaelis-Menten model (Appendix A).

Appendix D

Derivation of Kinetic Parameters from ATPase Assays

The spectrophotometer gave a numerical value for the slope of the decrease in absorbance as a function of time, which needed to be corrected for a 1 cm pathlength. The background was then subtracted to yield:

$$\frac{A_{338}}{\text{min}} = \left(\frac{-\text{slope}}{\text{pathlength}} \right) - \text{background} \quad (1)$$

Next, the obtained value for $\frac{A_{338}}{\text{min}}$ was substituted into the following equation:

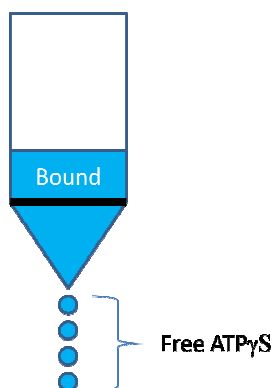
$$\boxed{\frac{38}{\text{in}} \times \frac{M(\text{NADH})}{6220 A_{338}} \times \frac{1M(\text{ATP})}{1M(\text{NADH})} \times \frac{1}{M(\text{Enzyme})}} \quad (2)$$

$\frac{ATP}{\text{min}^{-1} \text{enzyme}^{-1}}$ was plotted as a function of substrate concentration, and graphs were fit using the

Michaelis-Menten equation (Appendix A).

Appendix E

Conceptual Background of the Spin Column Binding Assays and Data Analysis



Cartoon representation of a spin column. The bound ATP γ S stays trapped above the filter while the free ATP γ S flows through.

Reaction binding mixture is placed above the filter in a spin column. The spin column possesses a filter which prevents protein from flowing through. Therefore, any ATP γ S which is bound to a protein remains above the filter, and any ATP γ S that is not bound to a protein is allowed to flow through. The intensity of The radioactive counts of ATP γ S bound is divided by the total radioactive counts of ATP γ S to yield the fraction of ATP γ S that is bound to protein. The fraction bound can be plotted as a function of substrate concentration and used in accordance with the Binding Isotherm Model (Appendix B)⁶. This method is tedious and time consuming, but it is highly accurate. Therefore, only one round of spin column assays was to be completed to serve as a reference point when completing the filter binding assays.

Appendix F

Conceptual Background of the Filter Binding Assay and Data Analysis

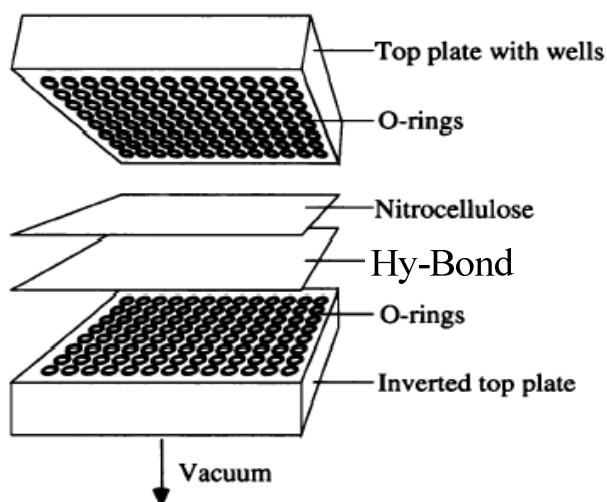


Figure 3. Basic set up for a double filter binding assay. The nitrocellulose filter traps any bound ATP γ S while the Hy-bond filter traps any free ATP γ S⁶⁸.

A reaction mixture similar to that of the spin column assay is filtered through both a nitrocellulose and a hy-bond filter. Due to its hydrophobic nature, the top nitrocellulose filter will bind any protein and any bound ATP γ S. The hy-bond filter is composed mainly of positively charged amine groups, and thus will bind any free ATP γ S⁵. The phosphorescence of the phosphor-imaging screen was detected and gave the intensity used in data analysis. The intensity of the nitrocellulose filter served as the amount of bound ATP γ S, while the intensity of the TLC spot served as a measure of the total ATP γ S.

Appendix F Cont'd*Conceptual Background of the Filter Binding Assay and Data Analysis*

The fraction of free ATP γ S was calculated as follows:

$$fraction\ bound = \frac{I_{Nitrocellulose}}{25 \times I_{TLC}} \quad (1)$$

$$fraction\ free = 1 - fraction\ bound \quad (2)$$

The intensity of the TLC plate was multiplied by 25 since 25 μ L of the reaction is filtered through the membranes. In accordance with the Binding Isotherm Model (Appendix B),

$\frac{1}{fraction\ free} - 1$ was plotted as a function of protein concentration. The slope of the line is

approximately $\frac{1}{K_d}$ and can be compared to previous studies.

References

1. Schroeder R, Barta A, Semrad K. Strategies for RNA folding and assembly. (2004). *Nature*. 5. 908-919.
2. Tanner KN, Linder P. DExD/H Box RNA helicases: from generic motors to specific dissociation functions. (2001). *Mol Cell*. 8. 251-262.
3. Treiber DK, Williamson JR. Exposing the kinetic traps in RNA folding. (1999). *Curr opin Struct Biol*. 9. 339-345.
4. Rogers GW, Richter NJ, Lima WF, Merrick WC. Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F*. *J Biol Chem*. (2001). 276(3). 30914-30922.
5. Wong I, Lohman LM. A double-filter method for nitrocellulose-filter binding: Application to protein-nucleic acid interactions. (1993). *Proc Natl Acad Sci USA*. 90. 5428-5432.
6. Polach KJ, Uhlenbeck OC. Cooperative binding of ATP and RNA substrates to the DEAD/H protein DbpA. (2002). *Biochemistry-US*. 41. 3693-3702.
7. Karginov FV, Uhlenbeck OC. Interaction of *Escherichia coli* DbpA with 23S rRNA in different functional states of the enzyme. (2004). *Nucleic Acids Res*. 32(10). 3028-3032.
8. Wyatt JR, Tinoco I. RNA structural elements and RNA function. (1993). *The RNA World*. 465-496.
9. Herschlag D. RNA chaperones and the RNA folding problem*. (1995). *J Biol Chem*. 270(36). 20871-20874.
10. El Hage A, Tollervey D. Why is ribosome assembly so much more complicated in eukaryotes than bacteria? *RNA Biol*. 1(10). 10-15.
11. Nierhaus KH, Dohme F. Total reconstitution of functionally active 50S ribosomal subunits from *Escherichia coli*. (1974). *Proc Nat Acad Sci USA*. 71(12). 4713-4717.
12. Rocak S, Linder P. DEAD-box proteins: the driving forces behind RNA metabolism. (2004). *Nature Rev Mol Cell Biol*. 5. 232-241.
13. Rajkowitsch L, Chen D, Stampfl S, Semrad K, Waldsich C, Mayer O, Jantsch MF, Konrat R, Blasi U, Schroeder R. RNA chaperones, RNA annealers, and RNA helicases. (2007). *RNA Biol*. 4(3). 118-130.
14. Bleichert F, Baserga SJ. The long unwinding road of RNA helicases. (2007) *Mol Cell*. 339-352.
15. Draper DE. Themes in RNA-Protein recognition. (1999). *J Mol Biol*. 293. 255-270.
16. Cordin O, Banroques J, Tanner NK, Linder P. The DEAD-box protein family of RNA helicases. (2005). *Gene*. 367. 17-37.
17. Varani G. RNA-protein intermolecular recognition. (1997). *Accounts Chem Res*. 30(7). 189-195.
18. Sengoku T, Nureki O, Nakamura A, Kobayashi S, Yokoyama S. Structural basis for RNA unwinding by the DEAD-box protein *Drosophila* Vasa. (2006). *Cell*. 125. 287-300.
19. Cordin O, Tanner NK, Doere M, Linder P, Banroques J. The newly discovered Q motif of DEAD-box RNA helicases generates RNA-binding and helicase activity. (2004). *EMBO J*. 23. 2478-2487.
20. Tanner NK, Cordin O, Banroques J, Doere M, Linder P. The Q motif: a newly identified motif in DEAD box helicases may regulate ATP binding and hydrolysis. (2003). *Mol Cell*. 11. 127-138.
21. Tanner NK. The newly identified Q motif of DEAD box helicases is involved in adenine recognition. *Cell Cycle*. 2(1). 16-17.
22. Xing X, Bell CE. Crystal structures of *Escherichia coli* RecA in complex with MgADP and MnAMP-PNP. (2004). 43. 16142-16152.
23. Elles LM, Uhlenbeck OC. Mutation of the arginine finger in the active site of *Escherichia coli* DbpA abolishes ATPase and helicase activity and confers a dominant slow growth phenotype. (2007). *Nucleic Acids Res*. 36(1). 41-50.
24. Iost I, Dreyfus M. DEAD-box RNA helicases in *Escherichia coli*. (2006). *Nucleic Acids Res*. 34(15). 4189-4197.
25. Garcia I, Uhlenbeck OC. Differential ATP-dependent ATPase activities of four rRNA processing Yeast DEAD-box proteins. (2008). *Biochemistry*. 47. 12562-12573.

26. Lorsch JR, Herschlag D. The DEAD protein eIF4A. 1. A minimal kinetic and thermodynamic framework reveals coupled binding of RNA and nucleotide. (1998). *Biochemistry*. 37. 2180-2193.
27. Iost I, Dreyfus M, Linder P. Ded1p, a DEAD-box protein required for translation initiation in *Saccharomyces cerevisiae*, is an RNA helicase*. (1999). *J Biol Chem*. 274(25). 17677-17683.
28. Lorsch JR, Herschlag D. The DEAD-box protein eIF4A. 2. A cycle of nucleotide and RNA-dependent conformational changes. (1998). *Biochemistry*. 37. 2194-2206.
29. Karamanou S, Gouridis G, Papanikou E, Sianidis G, Gelis I, Keramisanou D, Vrontou E, Kalodimos CG, Economou A. Preprotein-controlled catalysis in the helicase motor of SecA. (2007). *EMBO J*. 26. 2904-2914.
30. Rogers GWJ, Lima WF, Merrick WC. Further characterization of the helicase activity of eIF4A. Substrate specificity. (2001). *J Biol Chem*. 276. 12598-12608.
31. Wilson DN, Nierhaus NH. The weird and wonderful world of bacterial ribosome regulation. (2007). *Cri Rev Biochem Mol*. 42. 189-219.
32. Diges CM, Uhlenbeck OC. *Escherichia coli* DbpA is an RNA helicase that requires hairpin 92 of the 23S rRNA. (2001). *EMBO J*. 20(19). 5503-5512.
33. Nicol SM, Fuller-Pace FV. The "DEAD-box" protein DbpA interacts specifically with the peptidyltransferase center in 23S rRNA. (1995). *Proc Natl Acad Sci USA*. 92. 11681-11685.
34. Bizebard T, Ferlenghi I. Studies on three *E. coli* DEAD-box helicases point to an unwinding mechanism different from that of model DNA helicases. (2004). *Biochemistry*. 43. 7857-7866.
35. Tsu CA, Kossen K, Uhlenbeck OC. The *Escherichia coli* protein DbpA recognizes a small RNA hairpin in 23S rRNA. (2001). *RNA*. 7. 702-709.
36. Sharpe Elles LM, Sykes MT, Williamson JR, Uhlenbeck OC. A dominant negative mutant of the *E. coli* RNA helicase DbpA blocks assembly of the 50S ribosomal subunit. (2009). *Nucleic Acid Res*. 37(19). 6503-6514.
37. Tsu CA, Uhlenbeck OC. Kinetic analysis of the RNA-dependent adenosinetriphosphatase activity of DbpA, an *Escherichia coli* DEAD protein specific for 23S ribosomal RNA. (1998). *Biochemistry*. 16989-16996.
38. Baici A. Enzyme kinetics: the velocity of reactions. (2006). *Biochem J*. 1-3.
39. Record, M. J., Anderson, C., and Lohman, T. (1978) *Q. Rev. Biophys*. 11, 103-178.
40. Carey J, Uhlenbeck OC. Kinetic and thermodynamic characterization of the R17 coat protein-ribonucleic acid interaction. (1983). *Biochemistry*. 22. 2610-2615.
41. . Peck ML, Herschlag D. Adenosine 5'-O-(3-thio)triphosphate (ATP γ S) is a substrate for the nucleotide hydrolysis and RNA unwinding activities of eukaryotic translation initiation factor eIF4A. (2003). 9. 1180-1187.
42. Caruthers JM, Johnson ER, McKay DB. Crystal structure of yeast initiation factor 4A, a DEAD-box RNA helicase. (2000). *PNAS*. 24(97). 103080-103085.
43. Caruthers JM, McKay DB. Helicase structure and mechanism. (2002). *Curr Opin Struc Biol*. 12. 123-133.