2011

STRUCTURAL BASIS FOR THERMAL STABILITY OF THERMOPHILIC TRMD PROTEINS

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Acknowledgement

I would like to take this moment to thank everyone who supported me throughout my years, and my family for their consistent encouragement. I am especially grateful to have had Dr. W. Michael Holmes as my advisor and I appreciate his support and guidance. I would like to also thank my committee member's Dr. Darrell Peterson and Dr. Zendra E. Zehner for their efforts and input in assisting me with my project. I would also like to thank Dr. Diane Kellogg and the staff at the University of Richmond which provided the VP-DSC micro calorimeter. Dr. In addition, I would like to thank Maria Palesis for her knowledge and willingness to help me with my experiments. Also I would like to express gratitude to the entire Department of Biochemistry and Dr. Sarah Spiegel for their support, knowledge, experience and the opportunity to achieve this level of education.

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Abstract

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By Jamar Uzzell M.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Thermal stability of theG37 tRNA methyltransferase proteins from *Thermotoga maritima and Aquifex aeolicus* have been compared using Differential Scanning Calorimetry. It was shown that the *Thermotoga* protein is remarkably stable and is denatured at temperatures in excess of 100 degrees Centigrade. The *Aquifex aeolicus* protein was less stable, denaturing broadly at temperatures between 55^{o}C and 100^{o}C. In contrast, the mesophilic *E. coli* protein was completely denatured at 55^{o}C.

Enzymatic activity of the proteins was measured at various temperatures. Both the *Thermotoga* and *Aquifex* enzymes are active at ambient temperatures, and display a significant

decrease in activity when the temperature is raised above 50^{o}C. This may relate to subtle changes in protein structure causing an effect on the tRNA based assay.

Both enzymes contain inter subunit disulfide bonds which might contribute to thermal stability. Assays of the enzymes in the presence of high concentrations of Dithiothreitol (DTT) did not significantly reduce activity at higher temperatures, but did stimulate activity at lower temperatures.

Site directed mutagenesis of non -conserved protein sequences within *Thermotoga maritima* were initiated in order to determine what structures might confer heat stability on the protein. Alanine mutagenesis of lysine residues 103,104 led to reduced catalytic activity, but did increased activity at higher temperatures. Aspartate is the most common residue at the relative position 166 in the variable loop of most TrmD genes. It has been shown that in *E. coli* this is essential for catalytic activity and possibly the residue which carries out N1 deprotonation on residue G37 in tRNA. In *Thermotoga* glutamate is present at this position. Alanine mutagenesis of this residue did not eliminate activity suggesting another nearby residue may function in this capacity in the *Thermotoga* TrmD protein.

Background

The process of adapting and conforming to new environments through selection resulting in gradual changes which permit the survival of an organism is called evolution. Over the past few decades, scientists have been examining various extremophile genomes in an attempt to unravel structural conformations and sequences that lead to heat stability. There are two broad categories of thermophilic bacteria, including those which grow optimally at 50^{o}C – 80^{o}C and others that grow between 80^{o}C - 110^{o}C.Recently, the upper limits of growth temperatures have been extended with the discovery of a new strain of bacteria called 121 (Kashefi & Lovley, 2003). The bacterial strain 121 was given the name by its discoverers for the highest temperature at which it can grow, 121^{0}C. The previous upper limit limitation was held by another thermophilic organism *Pyrolobus funarii* which has been shown to grow at 113^{o}C (235^{o} F). The scientists discovered that Strain 121s growth range is from 85-121^{o}C while viable cell counts of *Pyrolobus fumarii* slowly decayed after an hour at the same temperature leaving only 1% of the cells viable.

"The upper temperature limit for life is a key parameter for delimiting when and where life might have evolved on a hot, early Earth; the depth to which life exists in the Earth's subsurface; and the potential for life in hot, extraterrestrial environments"(Kashefi & Lovley, 2003).

There is thought to be a limitation placed on the extremophile life that prevents growth after 140^{o}C because at this temperature cellular metabolites and amino acids become highly unstable and degrade. How the thermophiles are able to thrive in boiling water is based on the protein composition. Research has shown that there are various components of structure that allow for the stability of thermophilic proteins at higher temperatures.

Initially scientists proposed that the one adaptation thermophiles made to survive is an increase in the GC content of the DNA. This was termed the thermal adaptation hypothesis and it predicts that large positive contrasts in temperature should be matched by large positive differences in GC content. G-C base pairs offer three hydrogen bonds in comparison to the A-T base pairs which only form two hydrogen bonds. In addition base stacking is perhaps the biggest factor contributing to helical stability. The earlier data analysis of this theory seemed to prove that it had some validity, but in 2001 it was shown that high G-C content is not a direct indication that an organism would be able to withstand higher temperatures (Hurst,L.D. 2001) and that protein stability was of course an obvious factor to consider in thermostability. A protein's three dimensional structure is known to be very complex involving various inter and intra molecular bonds. The very same interactions present in mesophilic proteins can be found in thermophilic proteins. The protein is a network of amino acids whose secondary and tertiary structure is dependent on the primary sequence. The process of folding occurs in 3 known stages beginning with the unfolded protein which undergoes a hydrophobic collapse. This stage of protein folding samples 10^{16} conformations and is defined by not containing any secondary structure, exposed hydrophobic areas and being fully extended. The next stage known as the molten globule samples 10^{10} conformations contains elements of secondary structure, tends to have some degree of hidden hydrophobic areas and is more compact in comparison to the unfolded state. The native protein only has one conformational state, is catalytically active, and contains elements of secondary and tertiary structure. The process of properly folding a protein cannot be said to occur spontaneously, and it has been known since the 1980s that many proteins require the aid of molecular chaperones. Chaperones are known to act using two mechanisms, the first used by HSP70, involves maintaining the polypeptide chain in a state capable of

productive folding which occurs spontaneously after the unfolded protein is released in solution. The second mechanism used by chaperonins, which contain large cylindrical complexes that create physically isolated compartments designed for the proper folding of polypeptides and misfolded proteins (Nagradova,N.K. 2004)

In terms of protein stability under extreme conditions, the literature reveals a variety of structures found in proteins which might maintain stability at high temperatures. Interestingly, structures which might stabilize proteins under extreme conditions are found in mesophiles as well. This makes it slightly more difficult to discern exact mechanisms that thermophiles use to maintain their functionality at higher temperatures. Various methods including hydrophobic interactions, disulfide bonds, ion pairs (salt bridges), subunit-subunit interactions, hydrogen bonding, post translational modifications, metal ion bindings, and extensive packing reducing solvent accessibility to hydrophobic regions that are all seen in both mesophilic and thermophilic proteins.

Information from various publications suggests that salt bridges play a pivotal role in maintaining the stability of thermophilic proteins. A recent publication in 2004 used molecular dynamics to further explore the role of salt bridges in thermo stability and also compared the results to other interactions at higher temperatures. The simulations which measured the contact frequencies of atom pairs were performed at 25, 50, 75, and 100 °C. Results of the simulations demonstrated that the number of salt bridge contacts between the two molecules are unaffected by the temperature while hydrophobic and polar contacts are diminished (Thomas,A.S. 2004). Furthermore the group used a second study which used prototype molecules in order to further examine hydrophobic polar and salt bridge interactions and compare the results to the individual atom pair simulations. This study found that the interactions between the salt bridges actually

increase in stability at 100 °C in comparison to 25 °C (Thomas,A.S. 2004). This information is also supported by a recent publication in JBC by (Ge,M. 2008). Ge demonstrated via a double mutant cycle (DMC) that the free energy of Ssh10b, a DNA binding protein, decreases with increases in temperature while the contribution of the salt bridges to protein stability remain constant. The study also showed that the energy contribution of a salt bridge between two residues farther apart in primary sequence is higher than that of two residues which are closer in primary sequence.

Disulfide bridges, previously mentioned are believed to enhance thermal stability in proteins by providing a strong covalent interaction. This interaction is thought to restrict the protein making it more rigid and locking it into certain conformations. Looking more in depth at proteins that contain disulfide bonds the TrmD protein of *Aquifex aeolicus* contains a cys20-cys20 disulfide bond that tethers the two monomeric subunits together. In 2008, a study done by (Toyooka,T. 2008) demonstrated via SDS-PAGE that the disulfide bond of the TrmD protein found in *A. aeolicus,* can be separated by Dithiothreitol (DTT). Also, a mutant was prepared by substituting the Cys20 for a Ser preventing the formation of the disulfide bridge which links the two monomeric subunits together. The mutant was also subjected to the SDS-PAGE experiment while increasing the concentration of DTT; the results of this experiment showed that the protein never formed a dimer and migrated on the gel in its monomeric form. Studies in that paper were inconclusive with regard to the effects of the disulfide bond on enzymatic activity at increased temperatures.

Hydrophobic interactions have been studied to determine their role if any in the stability of thermophilic and hyperthermophilic proteins. Several comparative studies have been performed on both mesophilic and thermophilic proteins to look at the significance on

hydrophobic interactions and also to determine if one particular amino acid plays a major role in hydrophobic interactions. Computational analysis of proteins in 2008 by (Paiardini,Alessandro 2008) searched for hydrophobic pockets in mesophilic and thermophilic proteins to determine which amino acids, if any, played a role in thermophilic stability. The study showed that isoleucine and valine residues are shown to have preference in hydrophobic contacts. It was further stated that the residues possibly enhances thermostability of a-helices in hyperthermophilic proteins by decreasing the flexibility of those elements of secondary structure Paiardini,Alessandro 2008).

Looking further into heat stability, the subunit-subunit interactions may play a crucial role in heat stability by maintaining the quaternary structure of the proteins by using the methods of disulfide bonds, salt bridges, hydrogen bonds and hydrophobic pockets (Maugini,Elisa 2009). Maugini used a comparative approach to detect the significant structural differences between the subunits interfaces of hyperthermophilic, thermophilic and mesophilic homologous proteins. Comparative studies such as this usually suffer some statistical noise which arises from a small sample size, genetic drift and physiological adaptations, all of which must be subtracted out. The results of the study showed that the number of interface hydrogen bonds decreases in thermophiles indicating that they are not essential for maintaining the stability of the interface. This is expected because it has been shown that hydrogen bonds become weaker as the temperature increases. The role of hydrogen bonds has been a topic of immense controversy as they may only play a minor role in heat stability. Similar results were seen when looking at the ion pairs between subunits as the number of ion pairs seen did not vary significantly at the interfaces of thermophilic proteins. Though when looking at data from *Thermotoga maritima* there was a significant increase in the number of salt bridges relative to the protein length. Based

on the data of (Maugini,Elisa 2009) it was noted that extremophiles display a relative increase of interfacial ion pairs but the magnitude of the variations are not significantly larger than differences observed in homologous mesophilic proteins.

Another factor that can thermally stabilize an enzyme involves the anchoring of the loose ends of the protein. The carboxy and amino termini are commonly found anchored to the protein core in thermophilic organisms. This specific mechanism was examined when looking at *Thermotoga maritima's* ferridoxin protein, showing that the changes add to the thermostability of the protein. The study came to the conclusion that by anchoring the loop via ion pairs, hydrogen bonds and hydrophobic interactions with the protein core lead to the enhanced thermostability of the protein. Linking the ends of the protein contributes to the overall compactness of the protein, preventing the ends of the protein from moving vigorous moving when the temperature is increased.

In this study we will compare and contrast two thermophilic TrmD enzymes with that of E. coli with regard to effects of temperature on enzymatic activity and the unfolding of these proteins measured by Differential Scanning Calorimetry.

The Organisms

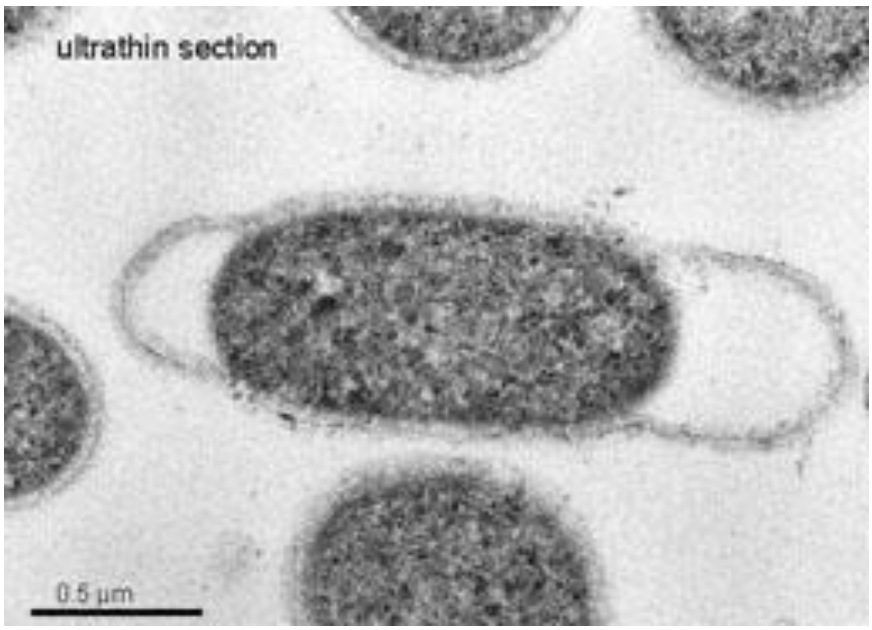
Thermotoga maritima

Thermotoga maritima, found in figure, 1 is a thermophilic organism first discovered amongst the geothermal heated sediment of Mount Vesuvius in Naples, Italy. It has an optimal growth temperature of 85^{o}C and is known to metabolize various carbohydrates both simple and complex including glucose, sucrose, starch, cellulose and xylan. Genomic analysis of *T. maritima* using the Institute of Genomic Research database indicates that the organism has a single circular chromosome consisting of 1,860,725 base pairs with a GC content o 46%.

Various articles and reviews point out that the proteins from thermophilic organisms such as *Aquifex aeolicus* and *T. maritima* have a wide range of optimal temperatures varying from50^{o}C to 120^{o}C. It has also been noted that thermophilic and hyperthermophilic enzymes do not function well below 40^{o}C (Vieille,C. 2001).

Figure 1: Ultrathin Micrograph of *Thermotoga maritima*

Figure 1 is an illustration of the *Thermotoga maritima* organism. Clearly visible in this photograph is its characteristic "Toga."



Aquifex aeolicus

Aquifex aeolicus grows in extremely hot temperatures, near volcanoes and hot springs. It has been isolated in the hot springs of Yellowstone National Park. It grows optimally at 80 ^{o}C, but can grow in temperatures up to 95 ^{o}C *A. aeolicus* is about 2.0-6.0 micrometers in length and has a diameter of 0.4-0.5 micrometers. The enzymes this organism uses for aerobic respiration are similar to the enzymes found in other aerobic bacteria. *A. aeolicus* oxidizes hydrogen gas and uses oxygen as the final electron acceptor in this process. The final product of its respiration is water, Aquifex meaning "water-maker" (Deckert,G. 1998). *A. aeolicus* can reduce and fix nitrogen and sulfur as well as hydrogen, and in sulfur and thiosulfate metabolism it produces sulfuric acid and H_2S. *A. aeolicus* has not been shown to grow anaerobically on nitrogen like *Aquifex pyrophilus;* however, it is microaerophilic, needing only 7.5 *ppm* oxygen for respiration (Deckert,G. 1998).

The circular genome of *Aquifex aeolicus* is about one third the size of *E. coli's* containing 1,551,335 base pairs with a G-C base pair content of 43.47%. About 16% of its genes originating from Archea (Deckert,G. 1998). The organism contains 1778 genes, 97.18% of which (1728) code for proteins. *Aquifex aeolicus* is a chemolithotrophic organism which uses an inorganic carbon source (carbon dioxide) for biosynthesis and as an inorganic chemical source.

The literature reveals that many of the proteins isolated and characterized from thermophilic organisms tend to show optimal activity at temperatures at 50^{o}C and has even been seen as high as 120^{o}C (Vieille,C. 2001; Vieille,C. 1996). These enzymes, more commonly referred to as thermozymes, share catalytic mechanisms with their mesophilic counterparts. When cloned and expressed in mesophilic hosts the thermophilic proteins usually retain their thermophilic properties, indicating that thermostability is genetically encoded. Sequential

analysis via amino acid comparisons, sequence alignments, and crystal structure comparisons show that thermophilic and mesophilic enzymes are very similar. In fact, there are no obvious structural or sequential features that account for thermostability, and the molecular mechanisms that provide thermostability vary from enzyme to enzyme.

tRNA N_1G^{37}methyltransferases (TrmD), extensively characterized by this laboratory, were derived from several organisms to include those from *Escherichia coli, Staphylococcus aureus, Thermotoga maritima, and Streptococcus pneumonia*. Various experiments have been used to characterize each of the proteins from enzymatic assays, crystallization and binding studies in order to gain insight into structure and function of the enzyme.

The TrmD enzyme functions as a dimeric protein in bacterial species and is observed as a monomeric protein in eukaryotic species. The enzymes function is to methylate specific tRNA molecules that have a G at position 36 and also recognize codons beginning with C such as leucine, proline and arginine (O'Dwyer,K. 2004). This is an important function because it has been shown that methylation at the G37 position prevents frame shift mutations (Qian,Q. 1997). A study performed by (Persson,B.C. 1995) demonstrated that a *trmD* deficiency caused a decrease in the rate of growth by several fold when compared to the non mutated enzyme. It was also noted that the hypomodified tRNA reads a four letter codon instead of the conventional three letter code thus resulting in the frame shift mutations that may lead to non functional proteins. The non functional proteins may be the possible cause for the decrease in the growth rate observed. Since the Guanine-37 immediately follows the anticodon loop, it has been proposed that N1 methylation prevents Watson and Crick base pairing in that position thus preventing the recognition of four lettered codons, thus preventing prevent frame shifting. The methyl donor for this reaction is S-adenosyl-methionine both *in vitro* and *in vivo*.

INTRODUCTION

In order to examine the heat stability of the *A. aeolicus* TrmD protein, experiments were designed based on previous work performed on the *T. maritima* TrmD enzyme. The *Aquifex aeolicus* TrmD protein were initially studied because it is the only thermophilic protein for which a crystal structure has been solved. Densities for The variable loop within the protein were not detected suggesting that this portion of the peptide backbone was very mobile in the structure. For these studies, a variable loop was modeled and inserted into the PDB file. Because of the similarity between the *A. aeolicus* TrmD and the *T. maritima* TrmD primary structure and because all TrmD proteins studied thus far are very similar in secondary structure, we decided to model the *T. maritima* protein to that of the *A. aeolicus*. This simulated structure was felt would be a good guide for the examination of primary structural elements that might be important for thermostability.

Comparative analyses of the crystal structures for these two enzymes, *E. coli and Aquifex,* showed that the carboxy terminal domains of the *A. aeolicus* enzyme differed markedly from that of the E. coli (Mesophilic) counterpart. Due to the fact that *Thermotoga maritima* and *Aquifex aeolicus* appear to be in closely related to each other on the evolutionary tree, it was assumed that the TrmD structures would be similar. Figure 2 displays a triple overlay of *A. aeolicus*, *T. maritima* and *E.coli* TrmD proteins. It is clear that the structures of all of the proteins are strongly conserved. Various studies using the *Escherichia coli* TrmD proteins have shown that upon deleting the final twenty amino acids catalytic activity; however, deletion of additional residues from the terminus drastically reduced or eliminated catalytic activity (Elkins,P.A. 2003). The *T. maritima* TrmD enzyme contains a disulfide bridge located in the c-terminus of the enzyme and is thought to increase the thermal stability of the protein. Cystine

245 is definitely a candidate for site directed mutagenesis in order to determine, if it in fact plays a role in heat stability of the *T. maritima* TrmD. Also bench top experiments involving the use of DTT to break the disulfide bond may be employed as an alternative.

To select other candidate residues for mutagenesis the *Expasy.org* Swiss model program was employed (Arnold,K. 2006; 37 Kiefer,F. 2009). A comparison of TrmD primary structures, figure 3, reveals two lysine residues which were found only in two thermophiles *A. aeolicus* and *T. maritime.* But not in either other Mesophilic sequences such as those of *E. coli* or the *Staphylococcus* TrmD enzymes. These residues (lysines, 103 and 104) of the *T. maritima* enzyme are located on the surface of the amino terminus and therefore may to be capable of interacting with surrounding water molecules.

Figure 2: Model(Chimera 3-D) of a Triple overlay of *A. aeolicus* subunits A (white)B (purple), E. coli chain A (turquoise) and B (yellow), and the modeled, hypothetical structure of *T. maritima* chains A and B (both in red).

A comparison of the three dimensional structures for *Thermotoga maritima*, *Aquifex aeolicus* (figure 4) and *Escherichia coli* (figure 5) demonstrates that the distribution of hydrophobic regions is different. A common hydrophobic region found in all TrmD proteins is located at the dimer interface. The hydrophobic region of *Thermotoga maritima* (figure 6) and *Aquifex aeolicus* (figure 7) contain a greater hydrophobic density when compared to that of *E. coli* (Figure 8). It has been proposed that an increase in hydrophobicity within a protein can contribute to the stability of the protein. Therefore, disruption of the hydrophobic region located at the dimer interface via mutagenesis might reduce thermostability. For example, Phenylalanine is known to be a very hydrophobic amino acid and it is located within the dimer interface of the *T. maritima,* these characteristics made it a candidate for mutagenesis(Maugini,Elisa 2009; 25 Paiardini,Alessandro 2008).

Salt bridges play a key role in the maintenance of protein stability and/or thermal stability. In order to determine the significance of salt bridges within the protein, select residues involved in these bridges in *A. aeolicus* and *T. maritima* could be mutated to determine if the thermal stability is affected.

In a previous study from this laboratory it was shown that an Aspartate residue in the variable loop of the E. coli enzyme was probably required for deprotonation of the G37 residue. Aspartate 169 of *T. maritima* was selected as a possible residue for mutagenesis to determine if it might play a similar role in that organism.

Fig 3 Sequence comparison of various TrmD proteins displaying the strong homology between the various protein sequences

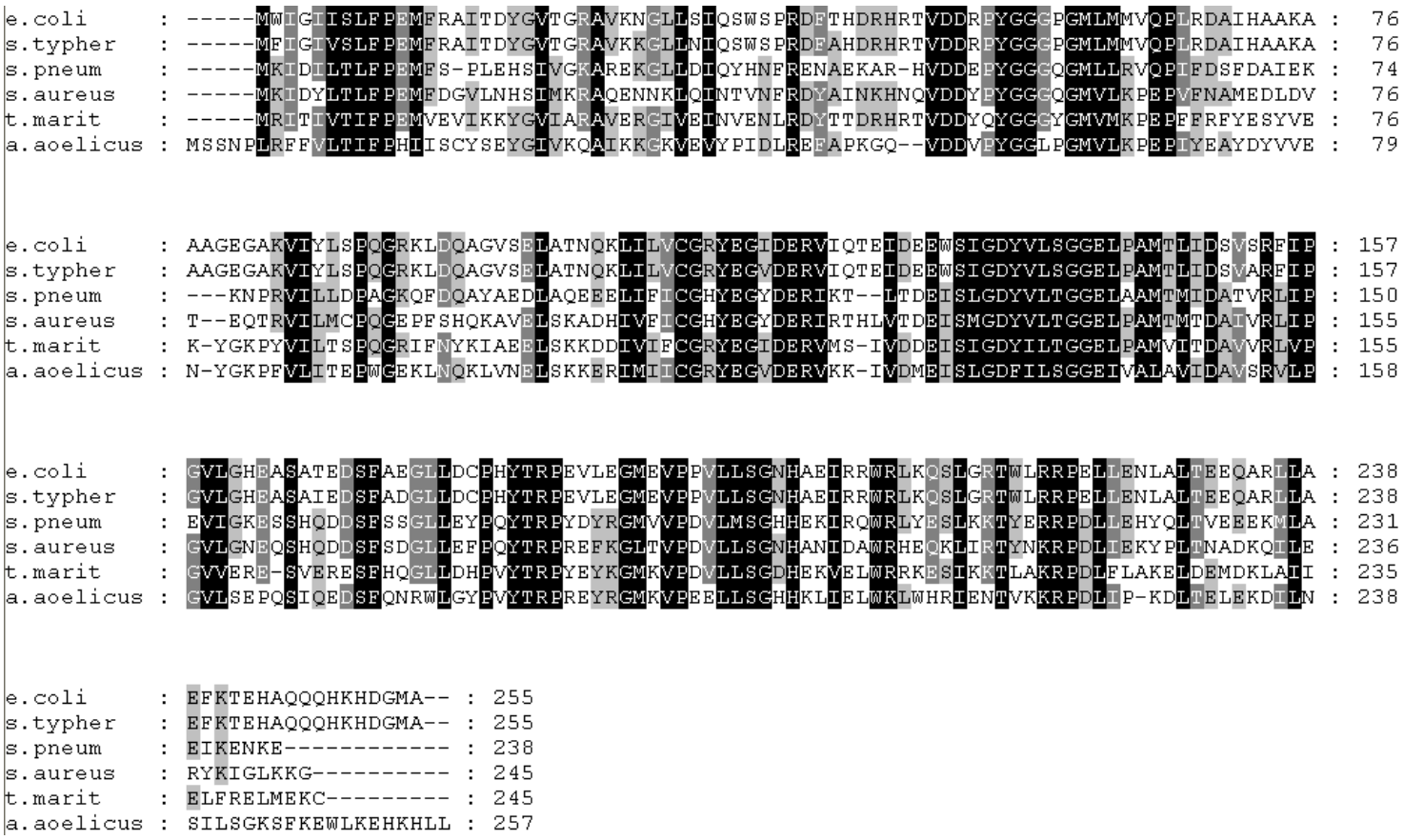


Figure 4. *A. aeolicus* Crystallographic structure. The unstructured region shown has been modeled to provide a more complete model of the protein, highlighting key structures to provide reference points

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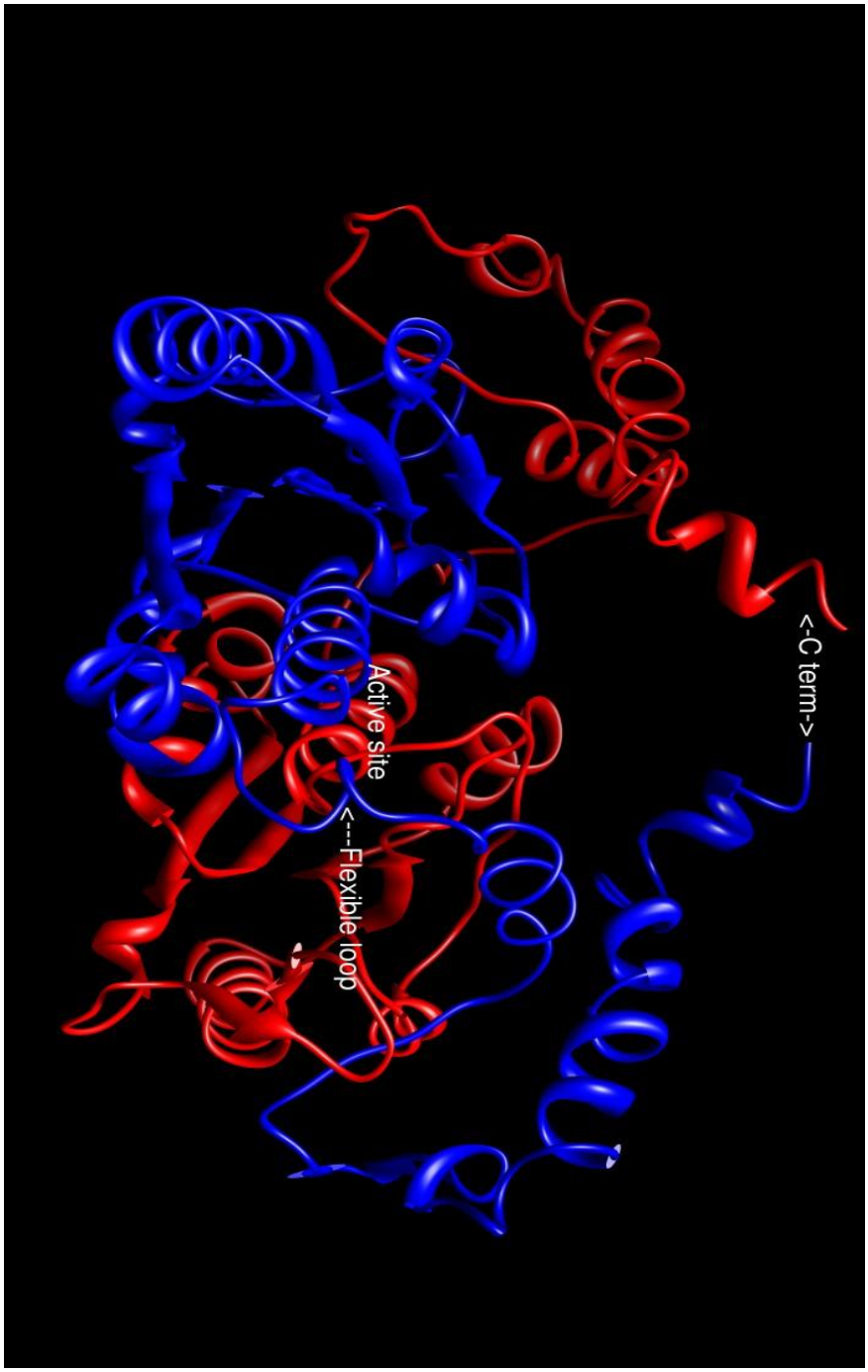


Figure 5 *E. coli* Crystallographic structure obtained from NCBI's database provides a means to compare various TrmD structures

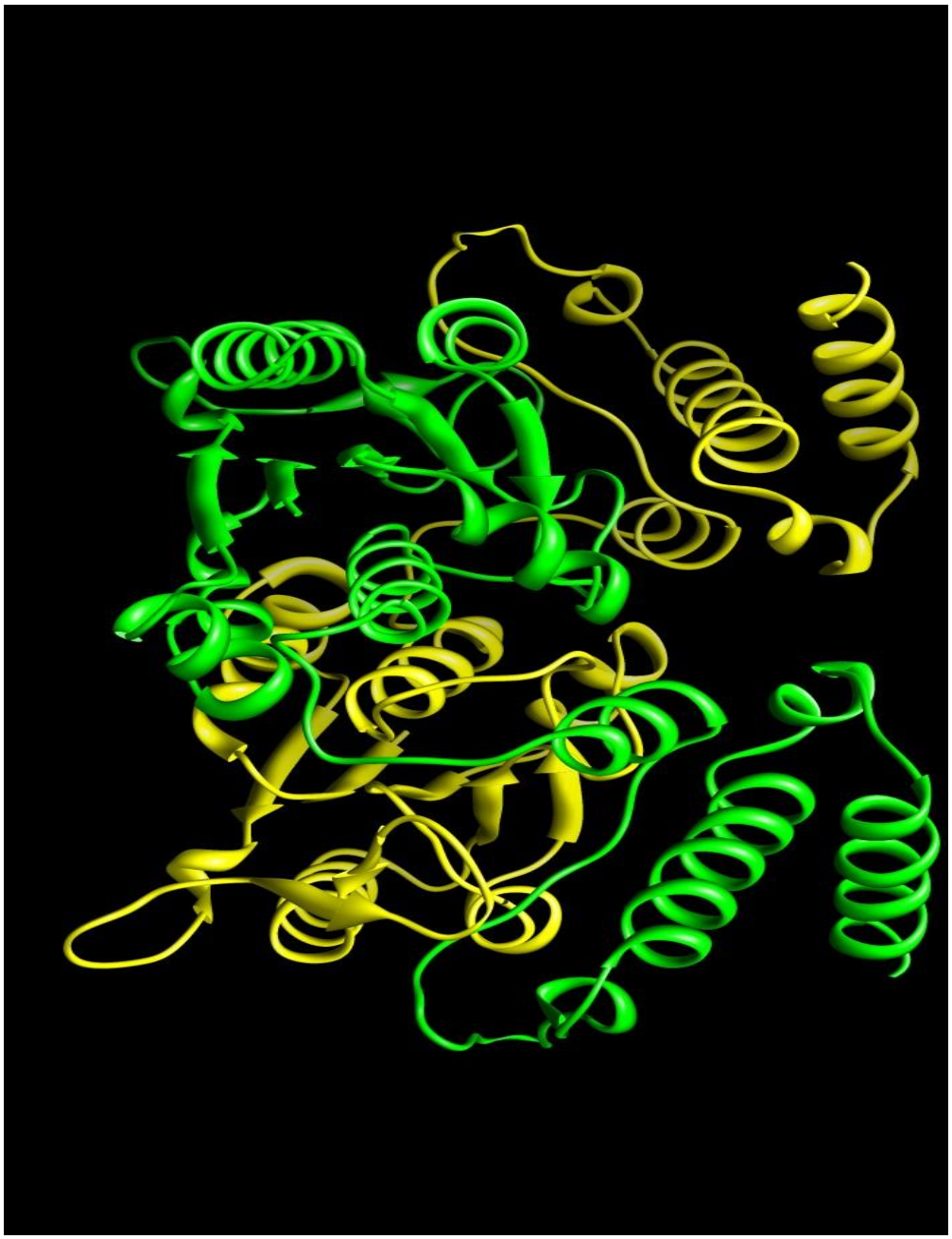


Figure 6.*T. maritima* structure illustrating hydrophobic regions done using Chimera program (UCSF). The globular areas display areas of hydrophobicity within the core of the protein

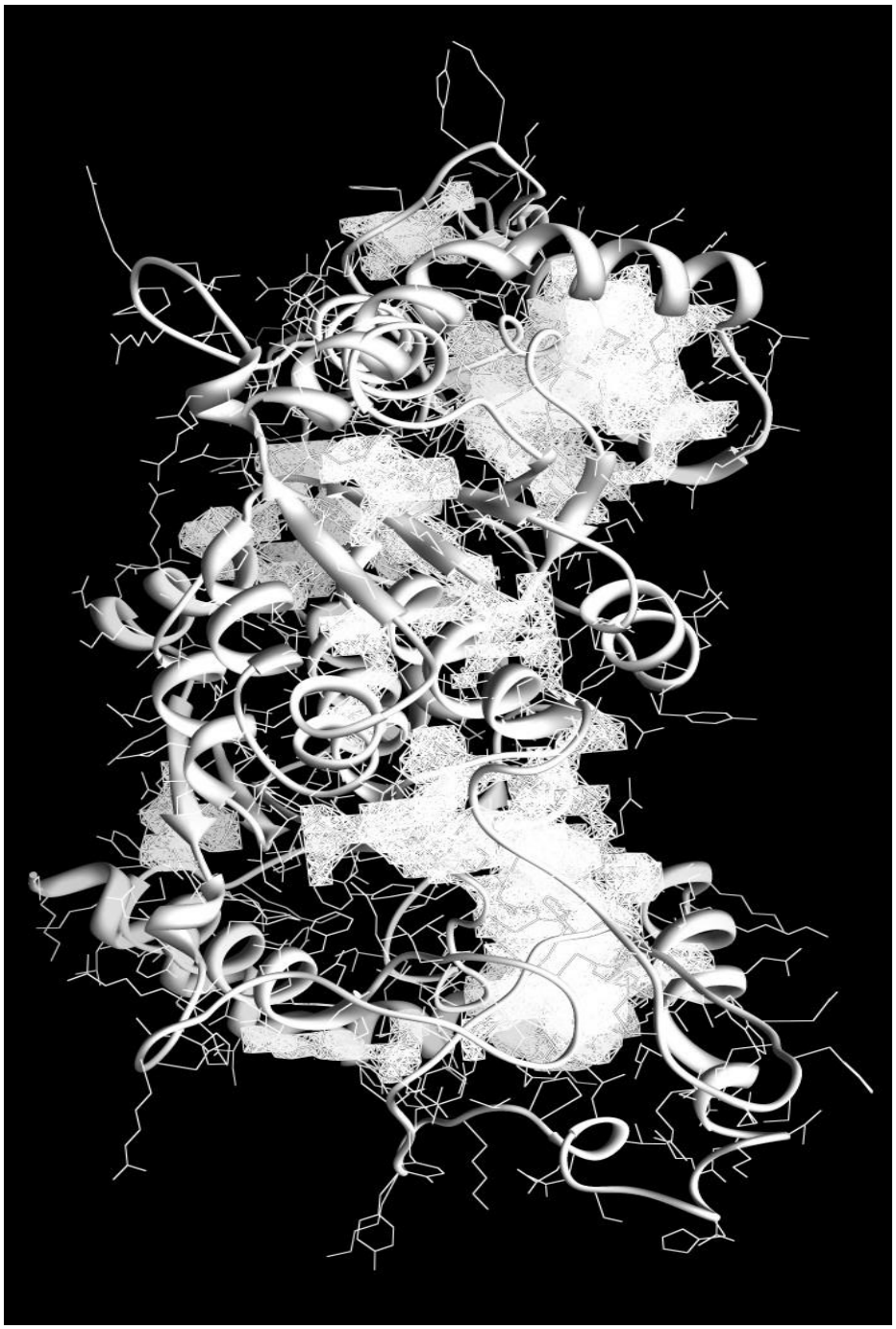


Figure 7. Hydrophobic regions of the *A. aeolicus* TrmD protein performed by using Chimera program (UCSF). The globular areas display areas of hydrophobicity within the core of the protein

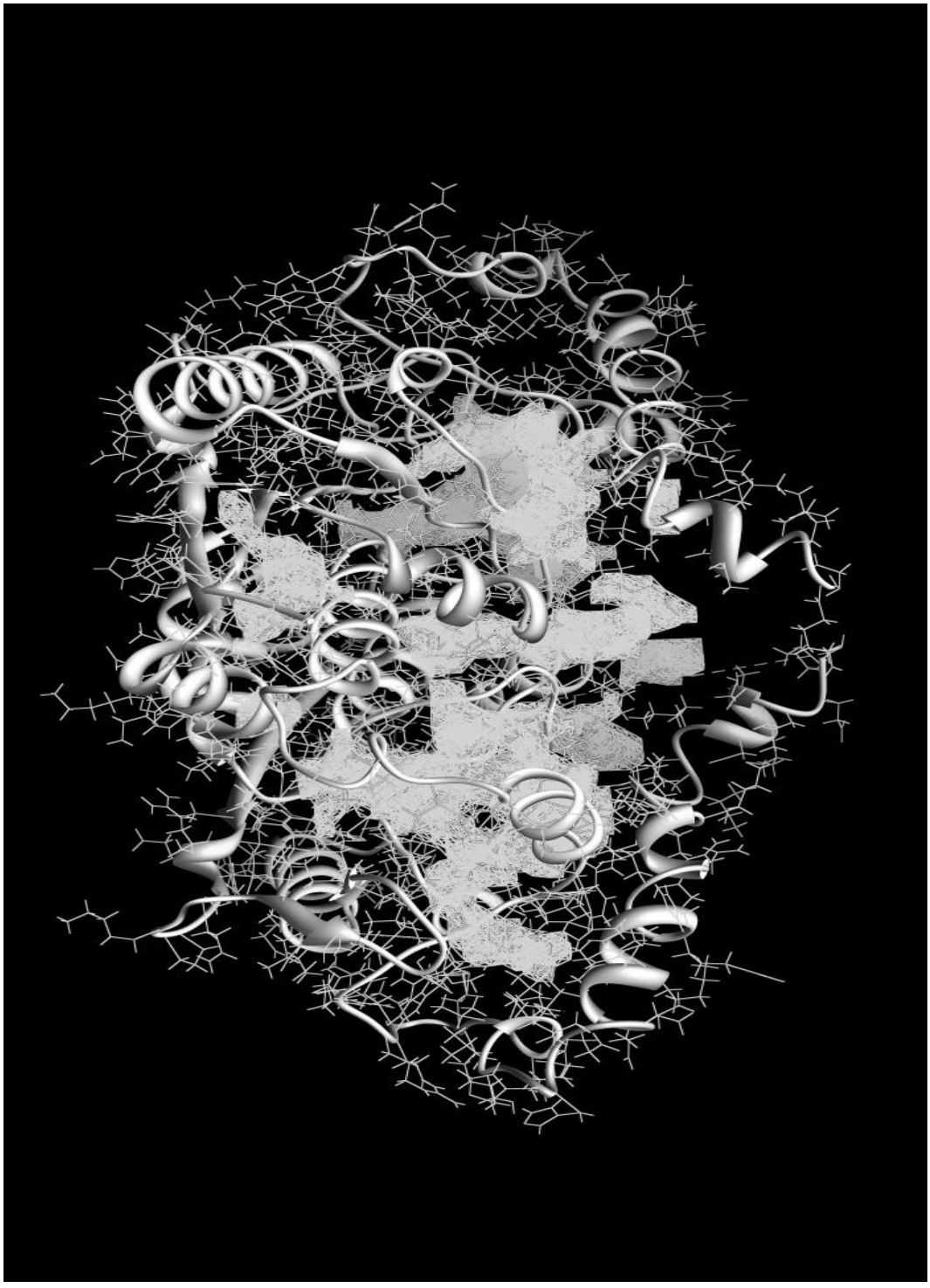
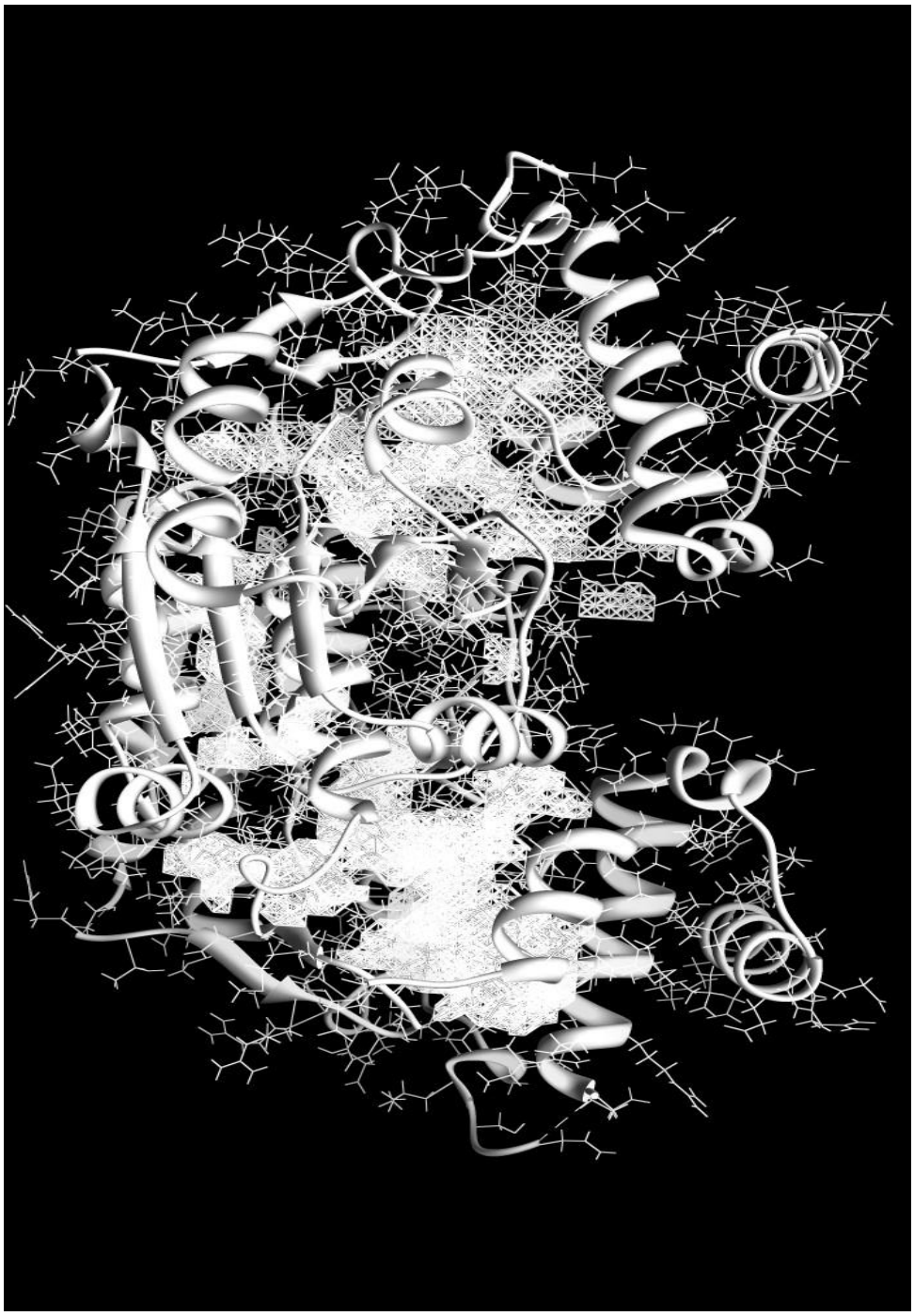


Figure 8 *E. coli* hydrophobic model constructed using the Chimera program from UCSF. The globular areas display areas of hydrophobicity within the core of the protein



Disulfide bonds were observed in both of the thermophilic enzymes are located in different positions; in *A. aeolicus* the bond is located at the subunit interface while in *T. maritima* it is located at the c-terminal end of the protein. Experimental analysis has shown that by increasing the amount of DTT (Dithiothreitol), one can separate the protein into its individual subunits which can be seen via native gel electrophoresis (Bruggeman, 2004). The creation of a Disulfide bond via mutagenesis at the c-terminus of the *A. aeolicus* enzyme, might link the subunits in two places, one at the subunit interface and the other at the c-terminus. This cross linking might increase the amount of disulfide bonds and test the theory that increasing the disulfide bonds increased the thermostability of the protein.

Previous evidence suggested that the *T. maritima* TrmD enzyme was active at higher temperatures of 60 and 70 ^{o}C (Brueggemann, 2004). In addition it was reported that the enzyme was much less active at ambient temperatures. Because *Aquifex aeolicus* is also a thermophile, we wished to determine if the TrmD enzyme there from enzyme might have a similar thermophilic profile. Given these considerations experiments were designed to compare and contrast these enzymes with regard to thermostability.

Materials and Methods for TrmD Characterization

Bacterial Growth and culture preparation

Each liter of broth was prepared by adding 25g of granular LB-Broth from Fisher to 1 liter of deionized water. The media was then autoclaved and ampicillin was added at a final concentration of 50ug/ml in the case of *T. maritima* and in reference to *A. aeolicus*50ug/ml of Kanamyacin was added. Usually 25 -30 ml of this medium was used to make a starter culture by overnight inoculation with the desired strain. On average, four liters were grown at a time in the case of *A. aeolicus* in order to obtain a good yield. On the other hand, 32 liters of *T. maritima* protein was prepared in a large fermentor separated into aliquots and placed in the-80 degree freezer until needed.

All cultures were grown to an optical density between 0.6 - 0.8 at a wavelength of 600nm, then induced with 1mM isopropyl β-D-thiogalactopyranoside (IPTG) overnight with continuous shaking. The resulting cells were harvested by centrifugation at 5000g for 10 minutes which resulted in a pellet. The cellular pellets were resuspended in the lysis buffer, which contained 300mM NaCl, 50mM pH 7.6 phosphate buffer and 10mM Imidazole. Other pellets were stored in the -80 freezer until needed

Purification

The cellular pellets were then lysed using a French Press, followed by another centrifugation at 8500 rpm for 30 min. to separate the cellular debris from the crude cellular extract. In order to isolate our proteins from those in the crude extract, they contain a His-tag so we are able to selectively bind the protein using a Ni-NTA nickel column from Qiagen. The column is washed with wash buffer containing 50mM NaCl, 50mM pH 7.6 phosphate buffer and

10mM imidazole, to remove any non specific binding. The absorbance at 280 nm was monitored and allowed to reach an OD of .01 before the desired protein was eluted in order to ensure sample purity. The protein is eluted with a buffer consisting of 50mM NaCl, 50mM pH 7.6 phosphate buffer and 250mM imidazole. Fractions of the elutant were collected and the protein concentration was measured using UV absorbance at 280nm. Because imidazole can interfere with the UV reading it is removed by means of dialysis, the dialysis buffer consists of 50mM Sodium Phosphate buffer, 50mM NaCl, .5mM Dithiothreitol (DTT) and 10% glycerol. Glycerol and DTT were used to help stabilize the protein and decrease the degradation of the protein after it was made. The purity of the protein was determined via sodium dodecyl sulfate polyacrylamide gel electrophoresis.

SDS Polyacrylamide Gel Electrophoresis (SDS Page)

The SDS Page gels initially corresponded to a protocol designed by laemmli which consisted of a 12.5% acrylamide separating gel and a 4.1% stacking gel. This protocol was altered slightly by our lab to consist of a 12.5% acrylamide separating gel and a 10% stacking gel. The laboratory uses "the petit gel" protocol, which generates a gel that fits into a 10x10cm gel rack with a thickness of .75mm. The protein samples to be analyzed are pretreated by adding the appropriate amount of 2X SDS loading buffer consisting of 100mM Tris pH6.8, 20% glycerol, 4% SDS, 200mM DTT and .1% Bromophenol Blue. The protein was then denatured by placing the sample into boiling water for 5-7 minutes. The SDS PAGE running buffer used in the experiments contained 25mM Tris, 190mM glycine, and .02% SDS. 15- 30 ul of the denatured sample in the sample buffer is loaded into a selected well and run at a constant voltage of ~80mV at room temperature. The bromophenol blue added to the sample buffer is used as a marker to determine when to stop the gel. Once the dye forms a band at the bottom of the gel the

electrophoresis was stopped by decreasing the voltage to 0. The gel is then stained with a coomassie brilliant blue solution that consists of 50% H2O, 40% Methanol, 10% acetic acid, and .05% coomassie brilliant blue R250. Following this step the gel is destained with a solution containing 70% water, 20% methanol, and 10% acetic acid removing the coomassie brilliant blue from the gel.

Plasmid Isolation

Plasmid DNA from both the wild type enzymes and mutants were isolated using the Qiagen mini and midi Kit along with the reagents and protocols necessary. The kits generate between 20ug and 100ug of DNA, sufficient for sequencing and transformation and mutation reactions. Upon completion of the protocol the resulting plasmid isolated was eluted with dH2O and stored at -20^{o}C. The plasmid that was isolated from both the *A. aeolicus* and *T. maritima* protein was sequenced by DNA CORE laboratory to ensure validity for future experiments and mutagenesis.

Site Directed Mutagenesis

Mutations were carried out using the Stratagene QuikChange kit II, and the primers were designed using the guidelines provided in the Quick Change protocol. The Stratagene site provided a free primer design program that automatically designed the mutation desired based on the requirements of the protocol which include proper melting temperatures, limited stem loop formations and ideal GC content. The derived primers created were used in a Polymerase Chain Reaction (PCR) protocol. The 3' and 5' primers containing the mutation were obtained from STRATAGENE. The site directed mutagenesis protocol was edited to increase the elongation phase to 2 min/kB. After the mutagenesis protocol was complete DPN-1 was added to the sample

in order to degrade the parental strands of DNA. DPN-1 recognizes the methylated DNA of the parental strands and degrading it, the suggested amount of DPN-1 was 1 ul but better results were obtained using 3 uls. The suggested incubation time of an hour was not altered and following the digestion the plasmid DNA was stored at –20^{o}C for further experimentation such as transformation reactions.

Transformation Reaction

Plasmids generated from the site directed mutagenesis reaction were transformed into XL-1 competent cells. Since the XL-1 cells do not express protein, plasmid isolated from the XL-1 cells was transformed into chloroamphenicol resistant Rosetta® cells encoding rare tRNA species. To ensure the validity of the mutagenesis, plasmids isolated from the XL-1 super competent cells were sequenced. Subsequently, plasmids were placed in the Rosetta cells for protein production.

Enzyme Activity Assay

Enzyme activity was determined using a modification of the method developed by of Hjalmarsson et al.(31). 50 µl of reaction mixture consists of 50 µM cold SAM, 1.8 µl of hot ^{3}H-SAM (specific activity 85 Ci/mmol) , buffer (0.1 M Tris-HCl pH 8.5, 1 mM DTT, 0.1 mM EDTA, 6mM $MgCl_2$, 24 mM NH_4Cl and ~20 ug of enzyme and variable amounts of bulk tRNA, *E. coli* or *T. maritima* tRNA transcripts. The assay for the *Thermotoga maritima* TrmD protein was conducted at 60^{o}C or 70^{o}C. The enzyme reaction was allowed to proceed for four minutes. Following four minutes, the reaction was stopped by adding 200ul of ice cold tri-chloro-acetic acid (TCA) and placed on ice for 15 minutes. This treatment ensured that all of the tRNA is completely precipitated out. The reaction mixture was loaded onto a filter paper and vacuum

filtration performed with TCA washes to remove excess labeled AdoMet from the GF-A Whatman filter paper. The filter paper was then dried with 100% ethanol and activity measured. The filter paper retains only radioactively labeled tRNA and thus can be counted via a scintillation counter. The machine used in this lab is the Packard Tri-Carb 1500 Liquid Scintillation Analyzer.

Heat Inactivation Experiments

This following experimental procedure was used to determine the relative heat stability of both wild type TrmD protein and the mutant proteins that were created. In order to generate a heat inactivation curve, we placed the TrmD protein into a water bath at various temperatures to determine at which temperature is the protein stable, and at various time points enzyme aliquots were removed for assay at the assay determined optimal temperature or each wild type enzyme. Aliquots of proteins were removed in 15 min intervals of0 minutes, 15 minutes, 30 minutes, 45 minutes, 60 minutes, and a control was also used to determine baseline readings which contained no enzyme.

Differential Scanning Calorimetry

This method was utilized to determine the melting temperature of the TrmD proteins. Differential Scanning Calorimetry (DSC) is a method commonly used to determine differences in melting temperatures under a variety of conditions. The method was first introduced by Privalov and Filimonov whose research was used to prepare an appropriate buffer system and used as a guide for our studies. The samples were all placed into a 100mM NaCl and 50mM Phosphate pH 7.5 solution. DSC machines compare the heat capacities of two liquid containing cells and plot the amount of energy needed to heat the cell to a specified temperature. Buffer was

added to the reference cell to eliminate the background noise and the sample chamber contained protein suspended in the same buffer. The machine then is able to determine a difference in heat that needs to be added to maintain both cells at the same heat level. At the time of protein denaturation, heat energy is lost due to the breakage of bonds. Thus, the machine itself is not required to put in more heat. This comparative analysis is then visualized in a heat capacity graph. The concentrations of protein used are typically 1-2mg/ml, therefore a volume of 0.7ml of the protein solution or buffer was placed into the appropriate cells after degassing. Degassing is critical since bubble formation and evaporation at higher temperatures interfere with the reading of the true heat values. Typically the sample or reference buffers were degassed for 15 minutes. The samples were subsequently loaded and pressure was applied to additionally reduce any bubble formation and evaporation. The temperature range was set between 25-120^{o}C and the calorimeter was allowed to increase the temperature of the samples at a rate of 1^{o}C per minute.

Results

Studies were initiated with the tRNA N^1G_{37} methyl transferase protein of *Aquifex aeolicus* and purified protein was produced as outlined in materials and methods. Also the mesophilic *Escherichia coli* protein was produced and analyzed as well as the *Thermotoga maritima* TrmD's protein. All three of the proteins produced contained a 6 member His-tag group at the amino terminus. The his-tag allowed the protein to bind to the Ni-NTA column acquired from Qiagen. TrmD proteins were eluted using increasing amounts of imidazole. SDS PAGE gels in figures 9 and 10 demonstrate the degree of purity of a typical preparation of TrmD proteins of *A. aeolicus* and *T. maritima* respectively. When the proteins are prepared in the presence of a reducing agent such as beta-mercaptoethanol or DTT, and further electrophoresed,

the molecular size is observed to be approximately 28kD for all three TrmD enzymes. In the absence of a reducing agent the molecular size is approximately 60kD for both the *T. maritima* and *A. aeolicus* enzymes. This confirms that the subunits are joined by disulfide bonds. This is not the case for the E. coli enzyme.(data not shown)

Pure protein samples are important for DSC and ITC experimentation due to the sensitivity of the experiments. In the case of DSC which monitors protein unfolding, increased amounts of non specific protein create distortions in the data leading to altered melting temperatures over a wide range.

To begin characterization of these enzymes, the activity of these enzymes was determined as a function of temperature. Initially the enzyme assay developed by Hjalmarsson et al was utilized for all TrmD proteins; this assay was modified slightly to improve assay conditions. Because *A. aeolicus* and are both thermophilic enzymes and are very closely related based on the phylogenetic tree (figure 11) it was postulated that the activity profile as a function of temperature might be similar to the data published on *Thermotoga maritima* previously carried out in this laboratory.

Figure 9. *A. aeolicus* TrmD protein analyzed by means of SDS-PAGE analysis to determine the purity of the enzyme obtained

A. aeolicus his tag protein *A. aeolicus* protein (after heating)

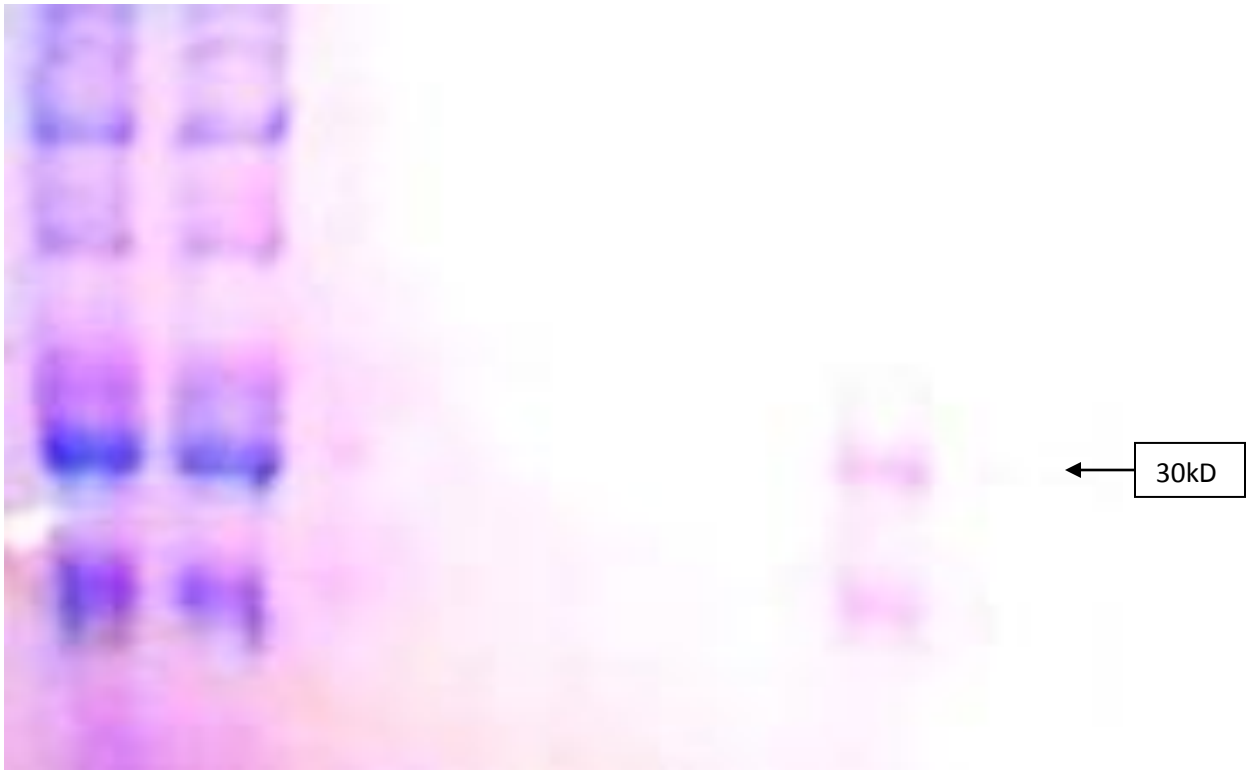
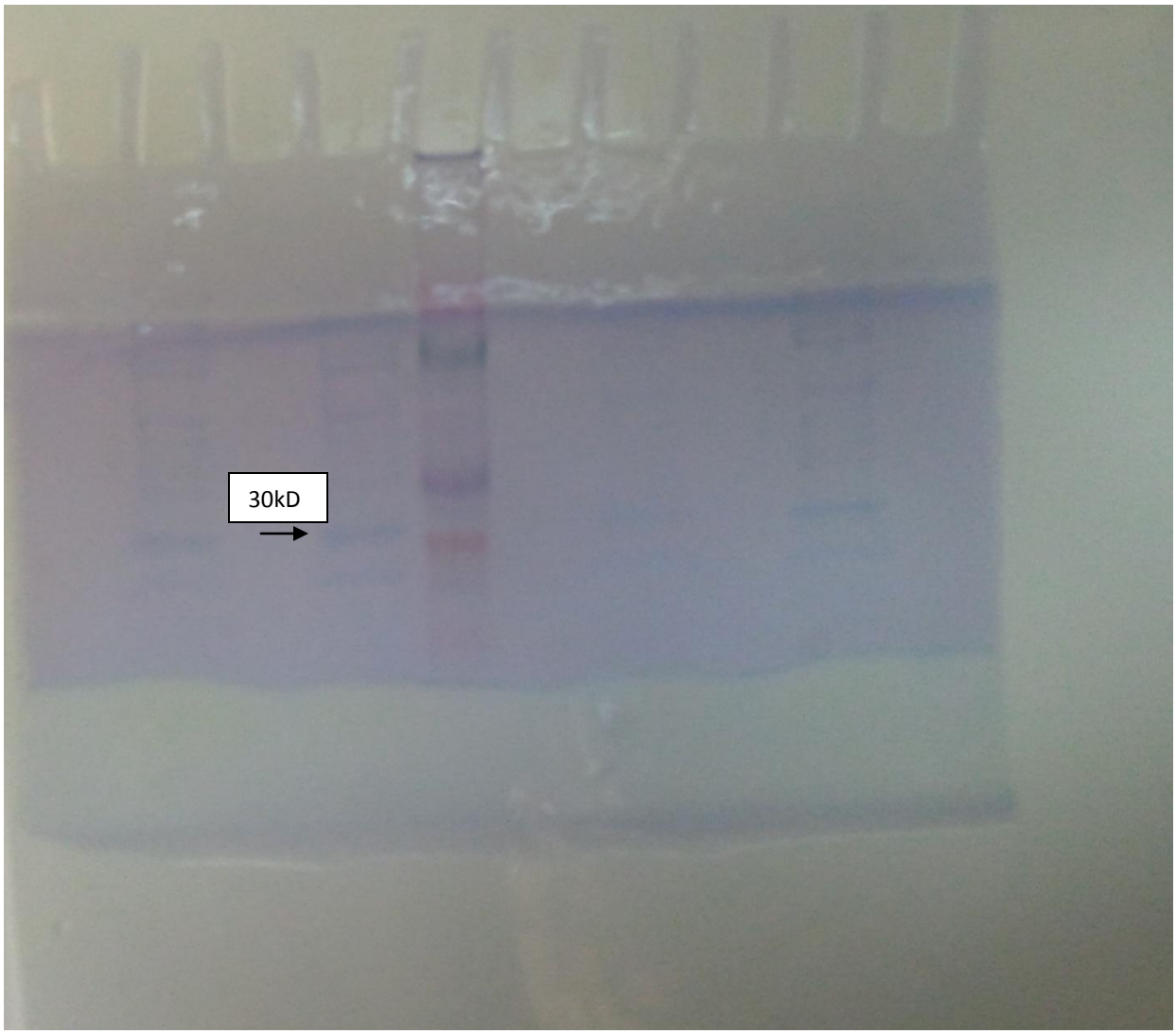


Figure 10.Purified his tagged *T. maritima* TrmD protein analyzed by means of SDS PAGE

T. maritima protein MW markers *T. maritima* protein



Fortunately, the crystal structure of *A. aeolicus* was solved (Figure 4). This provided us the unique opportunity to examine the amino acid interactions within the 3-D structure which might contribute to the increased heat stability of the enzyme. Initially, the isolated expression vector which contained the *A. aeolicus trmD* gene was sequenced to ensure the validity of the *trmD* sequence (Figure 12) the same was also done for *T. maritima*'s *trmD* (figure 13). An activity profile of the *Aquifex* enzyme was carried out at various temperatures (figure 14). The results observed show that the *A. aeolicus* protein is somewhat heat stabile during assay as compared to the E. coli protein which shows little activity at 50 degrees (data not shown). Next, *A. aeolicus* enzyme was incubated at 40 degrees Celsius in buffer, and aliquots assayed at 15 minute intervals at 25 degrees Celsius. These results demonstrate that at 40 degrees Celsius the enzymatic activity drops gradually to about 50 percent activity after an hour (figure 15).

Figure 11. A Phylogenetic tree of major phyla displaying the divergence from common ancestors

Phylogenetic Tree of Life

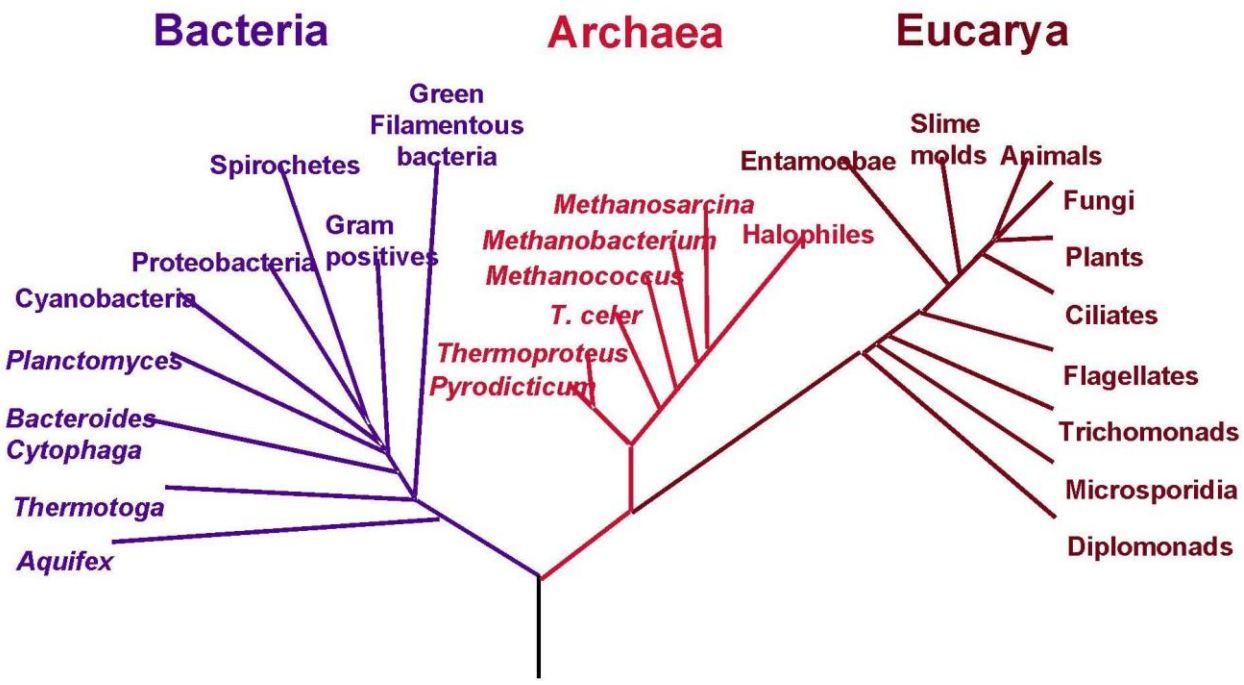


Figure 12. A Confirmation of the plasmid sequence for *A. aeolicus*. The isolated *A. aeolicus* plasmid was sequenced and compared to NCBIs BLAST to ensure that the correct species of TrmD was isolated.

>gb|AE000657.1| Aquifexaeolicus VF5, complete genome

Length=1551335

Features in this part of subject sequence:
tRNA guanine-N1 methyltransferase

Score = 1212 bits (656), Expect = 0.0
Identities = 687/704 (98%), Gaps = 6/704 (0%)
Strand=Plus/Plus

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Figure 13: BLAST analysis of *T. maritima* plasmid sequence

Thermotoga maritima MSB8, complete genome

Length=1860725

Features in this part of subject sequence:
tRNA guanine-N1 methyltransferase

Score = 1264 bits (684), Expect = 0.0
Identities = 703/711 (99%), Gaps = 5/711 (0%)
Strand=Plus/Minus

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Sbjct  1557370  CTTCTTTATAACTTCGACCATTTCAGGGAATATAGTCACTATTGTGATTCT  1557320
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Figure 14. *A. aeolicus* Activity as a function of assay temperature . Each data point is average of two assays and values shown are corrected for background counts.

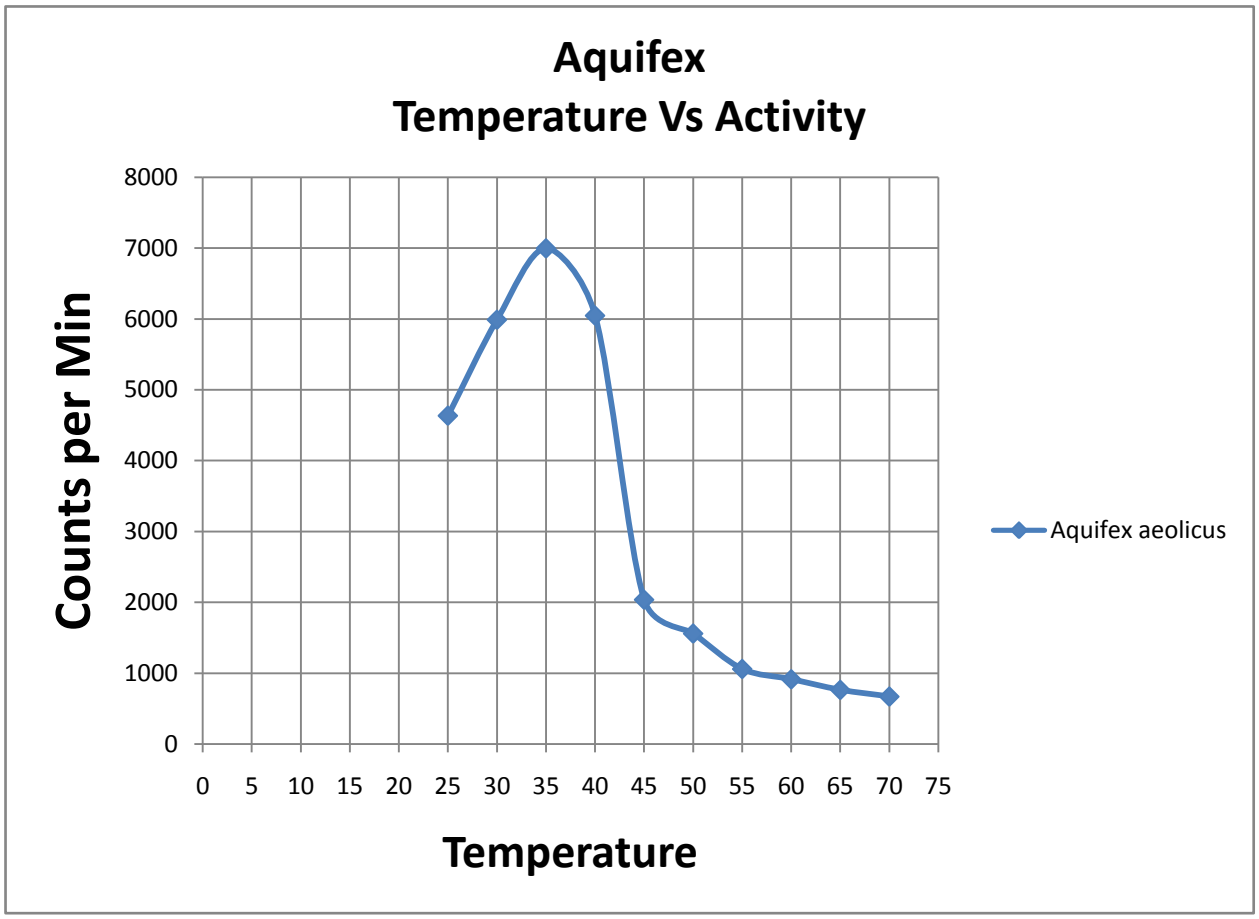
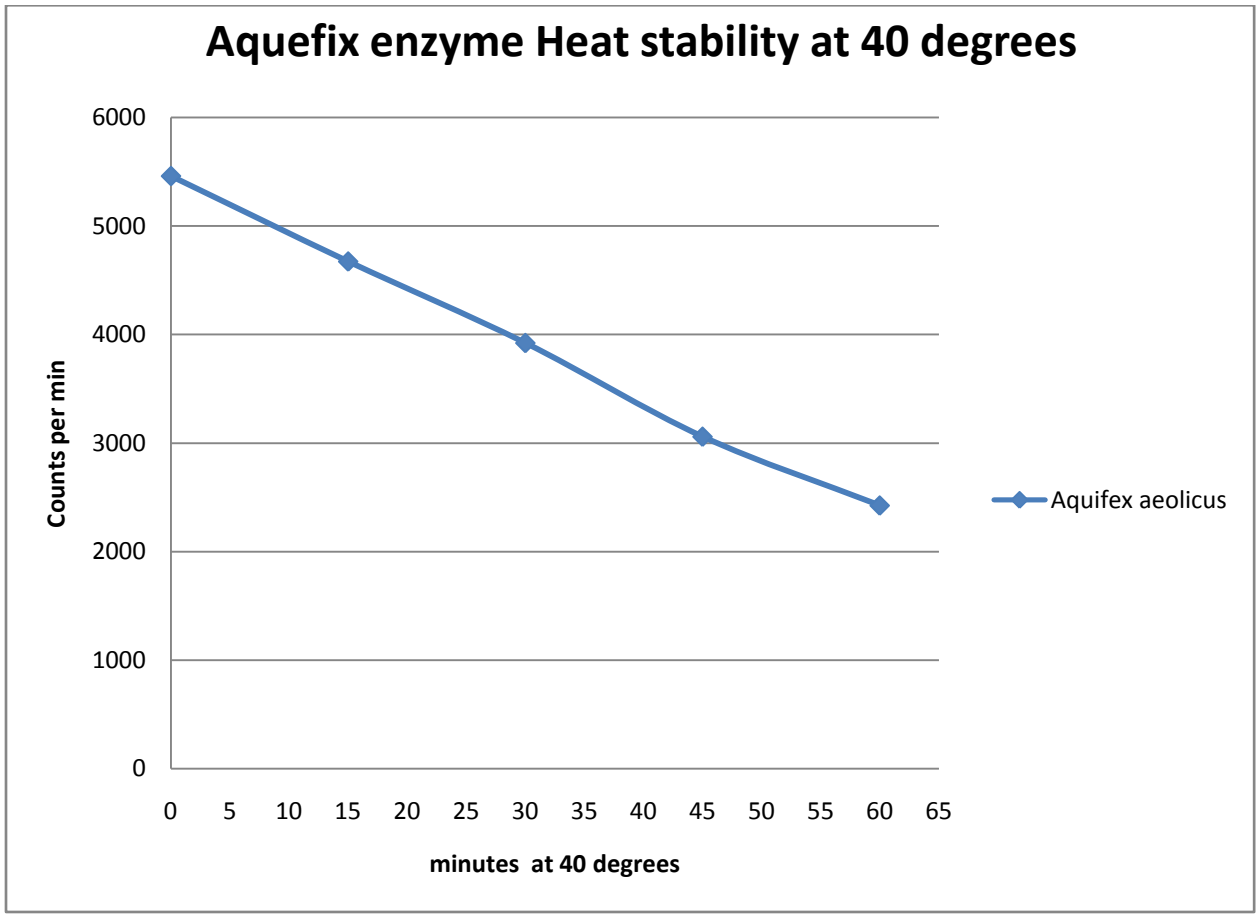


Figure 15. Heat Stability of *A. aeolicus* protein at 40 ^{o}C using normal Assay conditions.



It is known that the thermophilic proteins of *A. aeolicus* and *T. maritima* contain disulfide bonds which might stabilize subunits at higher temperatures. Dithiothreitol, a known reducing agent commonly employed to reduce disulfide bonds, was employed to determine if disulfide bonds found within both of the thermophilic organisms were important for thermal stability. In addition, the DTT was removed from the assays in order to determine the catalytic effects if any of the reducing agent on the enzyme. Also DTT was added to the reaction in excess in an attempt to sever the disulfide bond and evaluate the activity of the protein. Figure 16 again shows the effects of temperature on enzyme activity but now in the presence of 125 mM DTT. These results show little or no effect on enzyme activity at several temperatures. Next, the effects of elevated DTT at two different temperatures were assessed as a function of time figure 17. At 25 degrees DTT had little effect on activity as a function of time. However, it can be seen that at 50 degrees there is lower activity than at ambient temperature. After 4 minutes it can be seen that activity increases to higher levels.

Next, to determine if longer times and higher concentrations of DTT affect activity, the incubation time was increased to 30 minutes in the presence of 250 mM DTT (Figure 18).Again no significant effects of DTT were seen even at these higher concentrations and extended time.

Figure 16.Effects of elevated DTT on enzyme activity. *A. aeolicus* DTT Temperature vs. Activity in the presence of a high concentration of DDT (125mM). A control assay in the absence of added DTT is shown as preformed at 25 degrees.

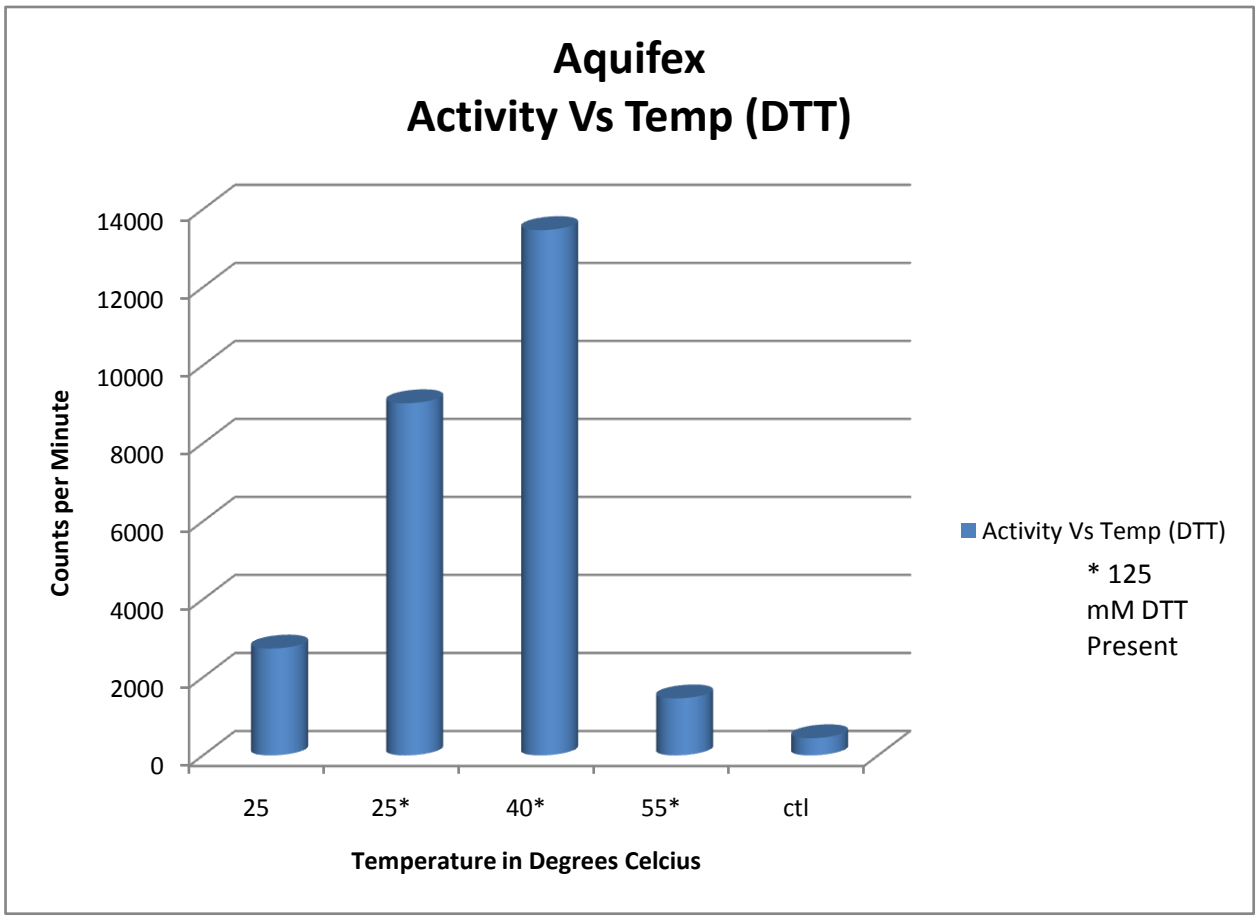


Figure 17.Effects of elevated DTT (250mM) on *A. aeolicus* TrmD activity at 40 ^{o}C and room temperature.

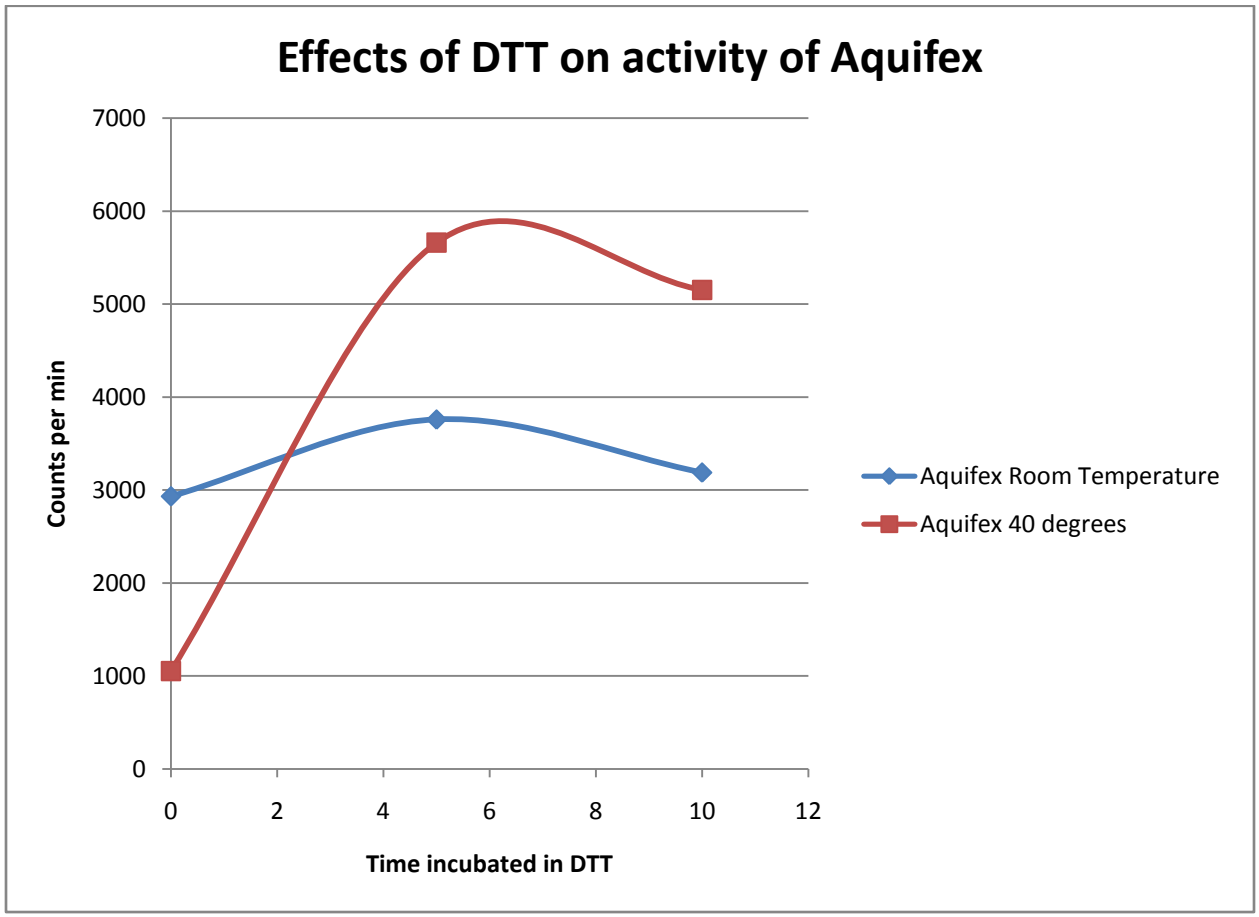


Figure 18. *A. aeolicus* in the presence of DTT assay performed at 37 ^{o}C incubation time in DTT was 30 minutes

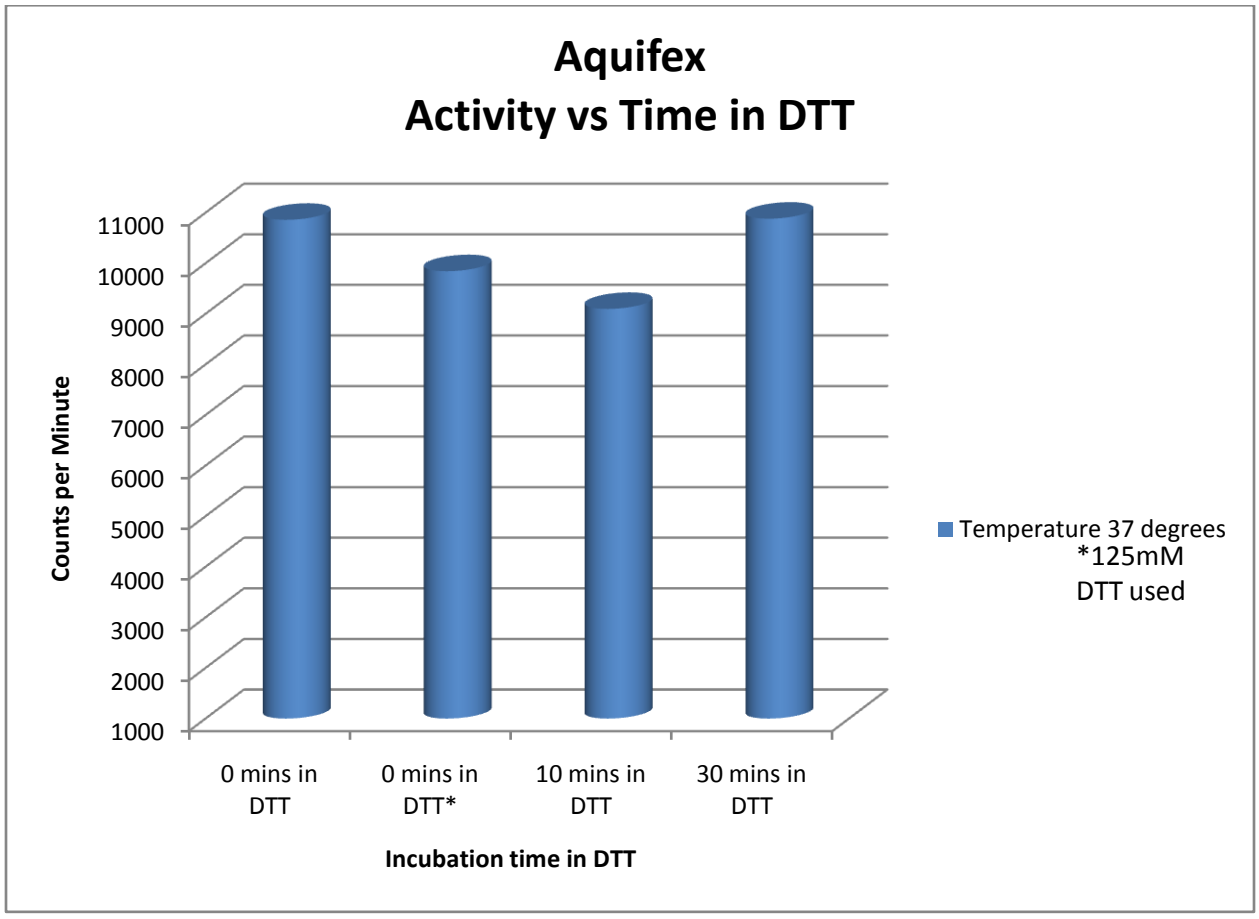
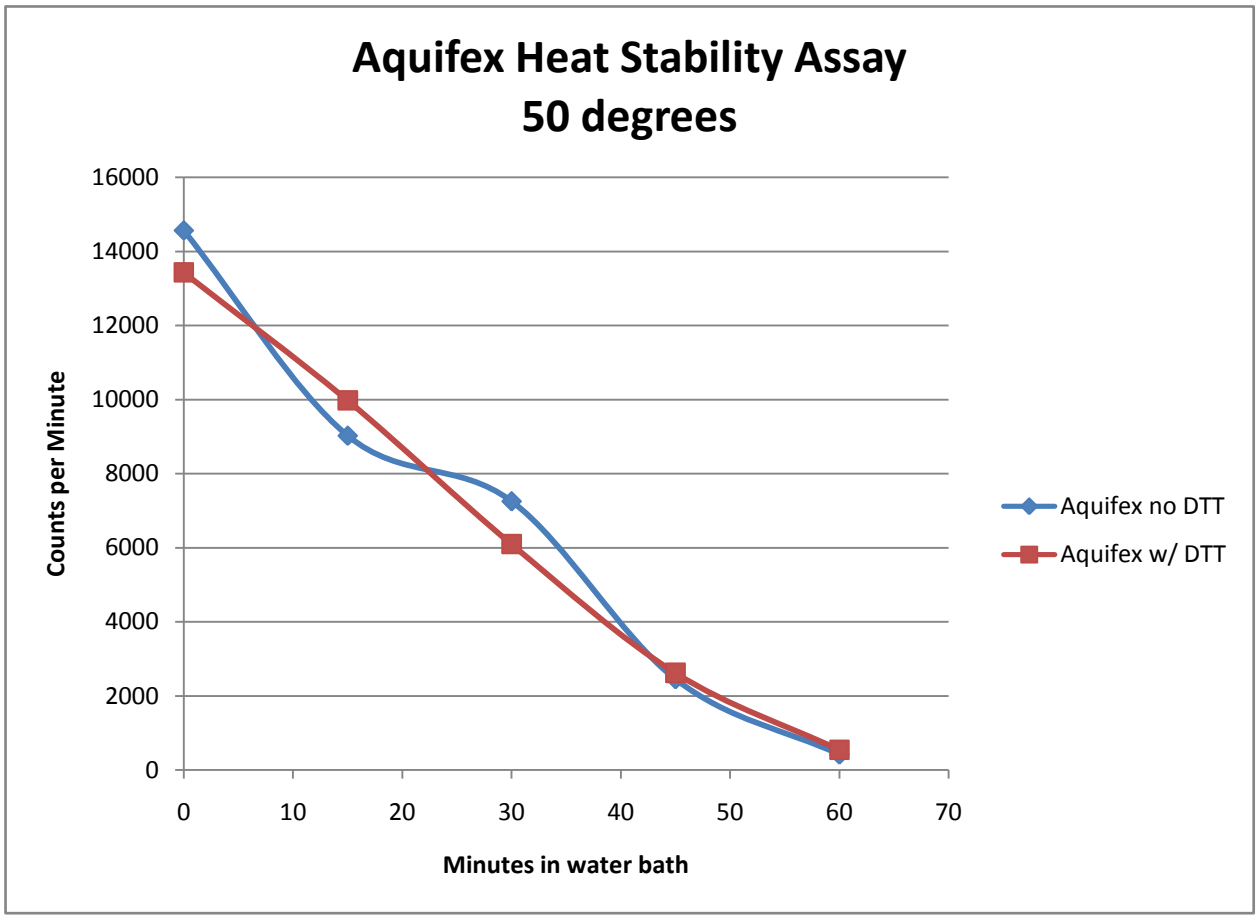


Figure 19 Heat stability assay at 50 ^{o}C in the presence of normal concentrations of DTT (blue) and excess concentrations of DTT 125mM (red)



Next, the stability of the *Aquifex* enzyme was assessed at an even higher temperature (50^{o}C) in the presence then absence of 125 mM DTT. In this experiment samples were removed at 15 minute intervals and assayed at 25^{o}C. It can be seen in figure 19 that in both the presence and absence of DTT the half life of the enzyme was approximately 30 minutes. This indicates that very high DTT concentrations have little effect on the heat stability of the enzyme.

Heat stability of the *Thermotoga* enzyme

Next, the activity of the T. maritima enzyme was examined as a function of temperature. As can be seen figure 20, this enzyme is optimally active at 37 degrees and maintains some activity at temperatures as high as 60 degrees. The *E. coli* enzyme is totally inactive at this temperature. However prolonged incubation of the enzyme up to 90 minutes led to considerable loss of activity.(Figure 21)

These results were surprising because this is a thermophilic protein which survives at temperatures above 70 degrees. Nevertheless, the *T. maritima* enzyme retains significant activity at temperatures over 60 degrees under these assay conditions.

Next, both enzymes were compared with respect to effects of high levels of DTT on enzyme activity and stability. Similar results for both were observed indicating no decrease in activity in the presence of elevated concentrations of DTT. (Data not shown)

Figure 20: Activity Vs temperature Assay for *T. maritima* outlining the temperatures at which the enzyme displays optimal activity in response to increased temperatures

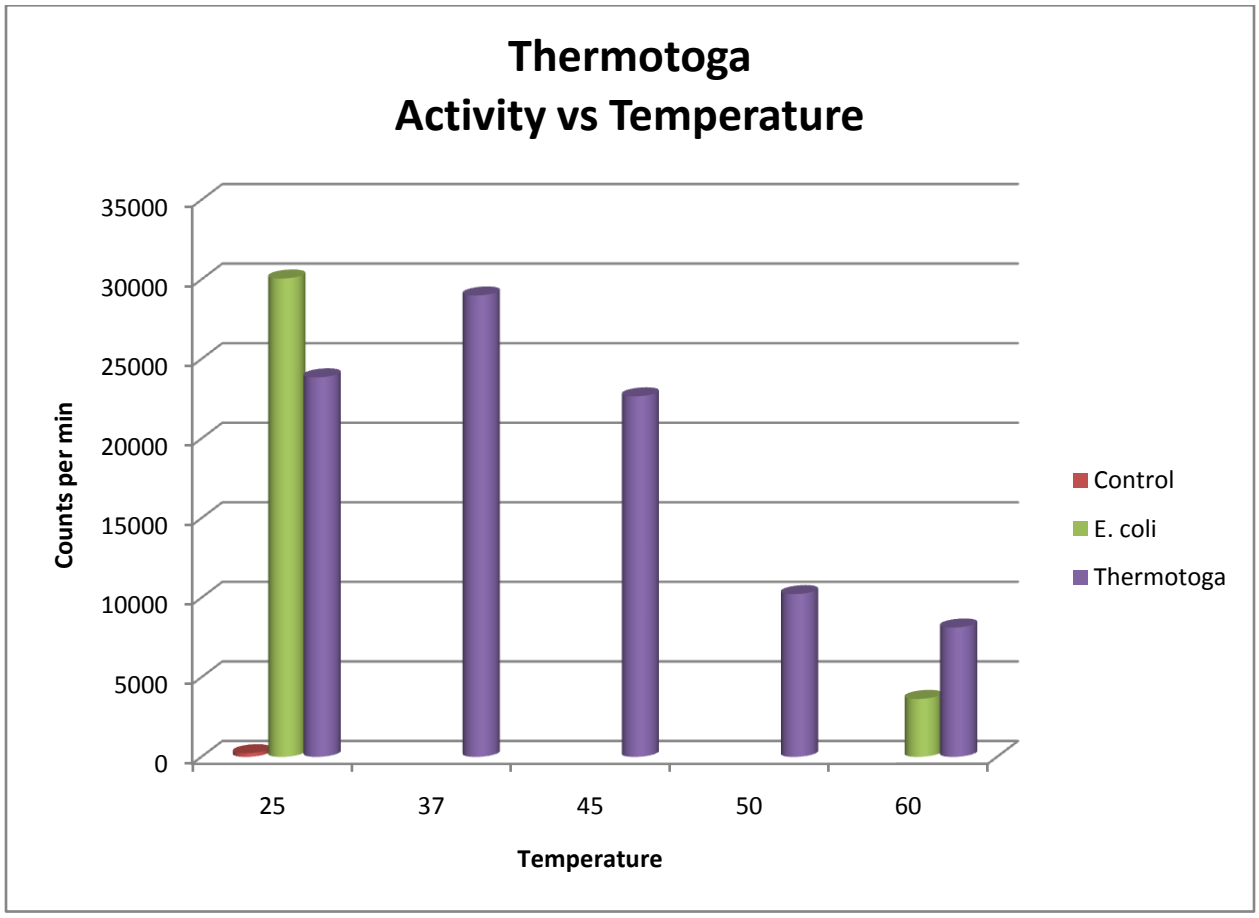
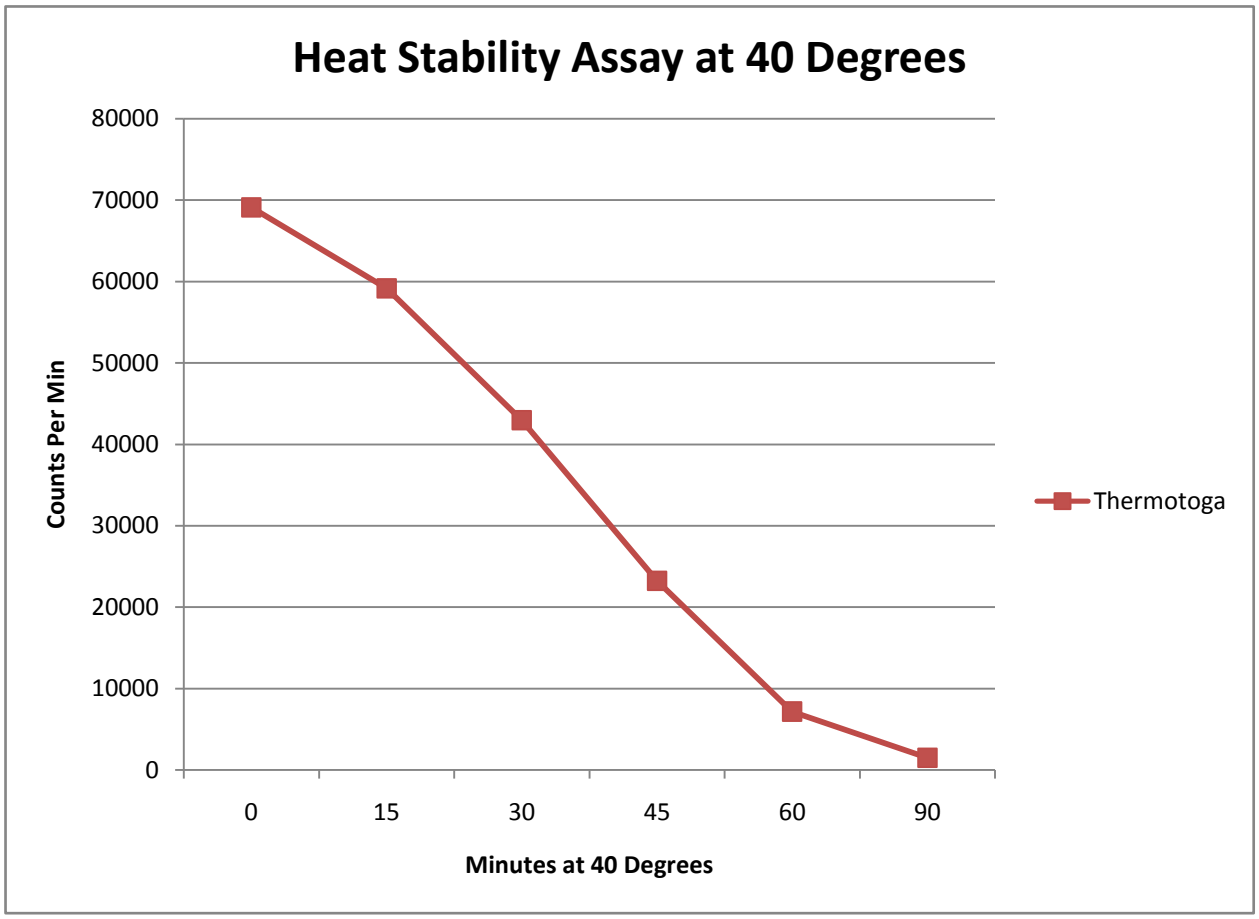


Figure 21: *T. maritima* TrmD protein Heat Stability Assay. The protein was incubated at 40 ^{o}C and assayed at 40 ^{o}C to determine if prolonged periods at elevated temperatures had an effect on activity.



Mutagenesis experiments

Given the above results, mutations on both the *A. aeolicus* and *T. maritima* enzyme were attempted in order to determine if amino acid changes can be identified which might alter the stability and function of the enzymes. In the *Aquifex aeolicus* enzyme, two sites were selected based on the positions within the crystal structure. The first site, E235 located at the C terminus of the subunit, was chosen to be mutated to a cysteine creating a disulfide bond at the C terminal position, possibly increasing the stability of the protein at higher temperatures. The second site would be to eliminate the C20 disulfide bond.

In the *T. maritima* enzyme mutation of the C245 residue was attempted. This mutation was not successfully obtained. However, this mutant had previously been prepared in this laboratory and did have subtle effects on heat stability of the *Thermotoga* enzyme. (Data not shown).

The second site of interest within the *A. aeolicus* enzyme was C20 which constitutes the internal disulfide bond that links the two subunits together (Figure 4). It has been proposed that loss of this bond reduces the heat stability of the enzyme. Unfortunately, this mutation was not obtained. However, another group has reported that mutations eliminating this bond did result in a less heat stabile protein as judged by denaturing gel analyses (Toyooka,T. 2008).

Previous data performed on the *E. coli* enzymes suggest that an aspartate amino acid performs the critical deprotonation of the G37 N1 position, and when changed to alanine results in an inactive enzyme. (Christian,T. 2006, Christian,T. 2010) . In that study, placing Glutamic acid at the position (E169) resulted in a recovery of activity, indicating that a similar charged amino acid can work at this position. *T. maritima* has a Glutamic acid (Glutamate) at position

E166 which may carry out the same function as Asp (169) in E. coli and Asp (170) in *Aquifex aeolicus*. Therefore this residue was chosen for alanine mutagenesis.

Lysine residues 103 and 104, are residues which were present in the two thermophilic *trmD* genes but not in any of the other mesophilic *trmD* orthologes, and were therefore selected for mutagenesis. These residues are located near the surface of the TrmD protein and may provide surface charges which may be critical in thermostabilization through interacting with surrounding water molecules. Mutating lysines naturally found at the site to an alanine might disrupt the normal packing of the molecule with water causing the destabilization of the enzyme.

The Stratagene QuikChange® II Site-Directed Mutagenesis Kit was used to prepare all mutants. Unfortunately, all of the proposed mutants were not successfully produced as indicated above. However, two *T. maritima* mutant TrmD proteins were produced. Both plasmids were isolated and sequenced before being transfected into new host cell lines for expression. Figure 22 illustrates activity of the *T. maritima* KK enzyme as a function of temperature. The results of the double K mutant experiments indicate that the enzyme is able to function at room temperature, is more active at 37 degrees, and loses little activity at 50 degrees. Figure 23 contains a heat stability assay at 70 ^{o}C where the protein maintained its activity even after incubating for 60 minutes. Thus it is clear that the *Thermotoga* enzyme functions quite well at elevated temperatures.

Figure 22.*T. maritima* Double lysine Mutant Activity Vs Temperature assay displaying the temperatures at which the protein optimally functions

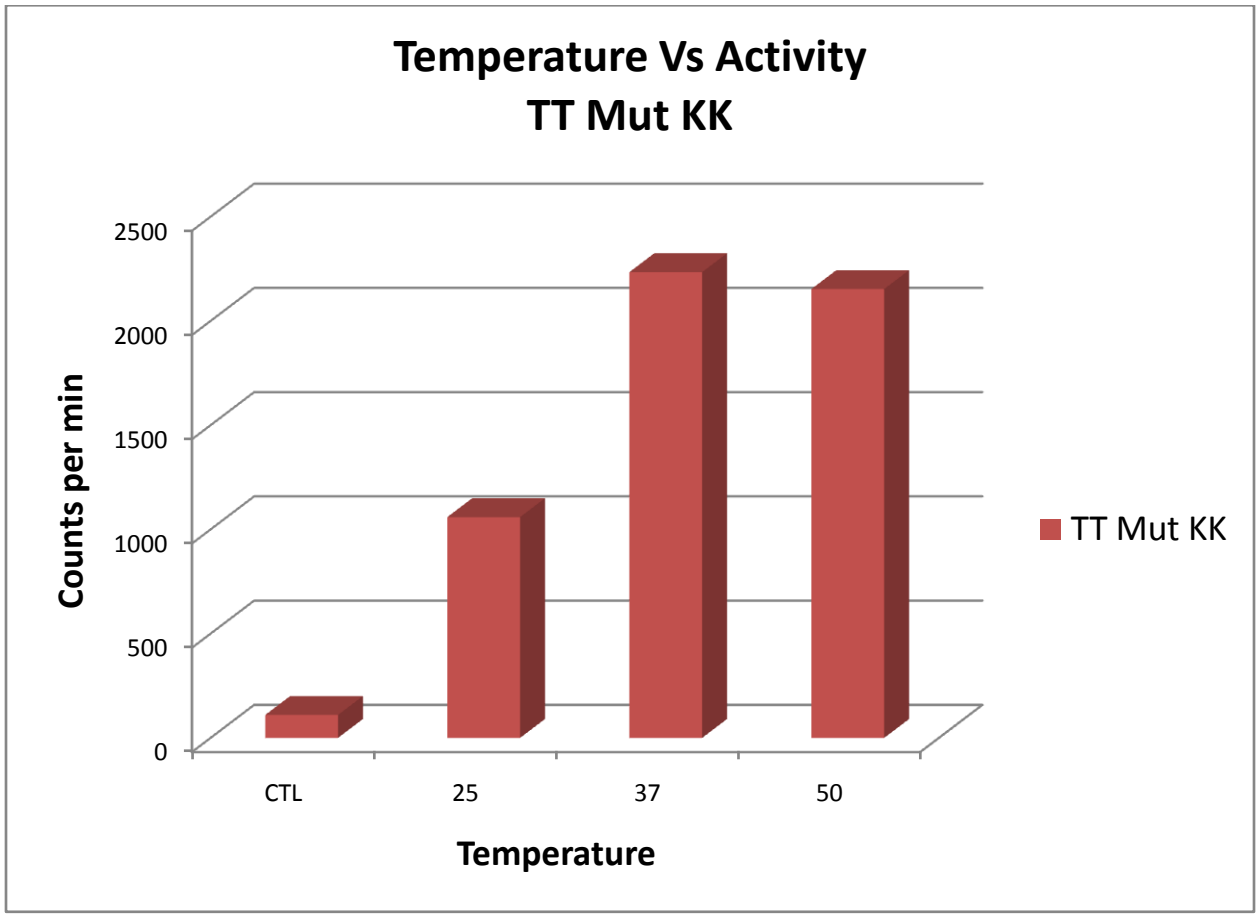
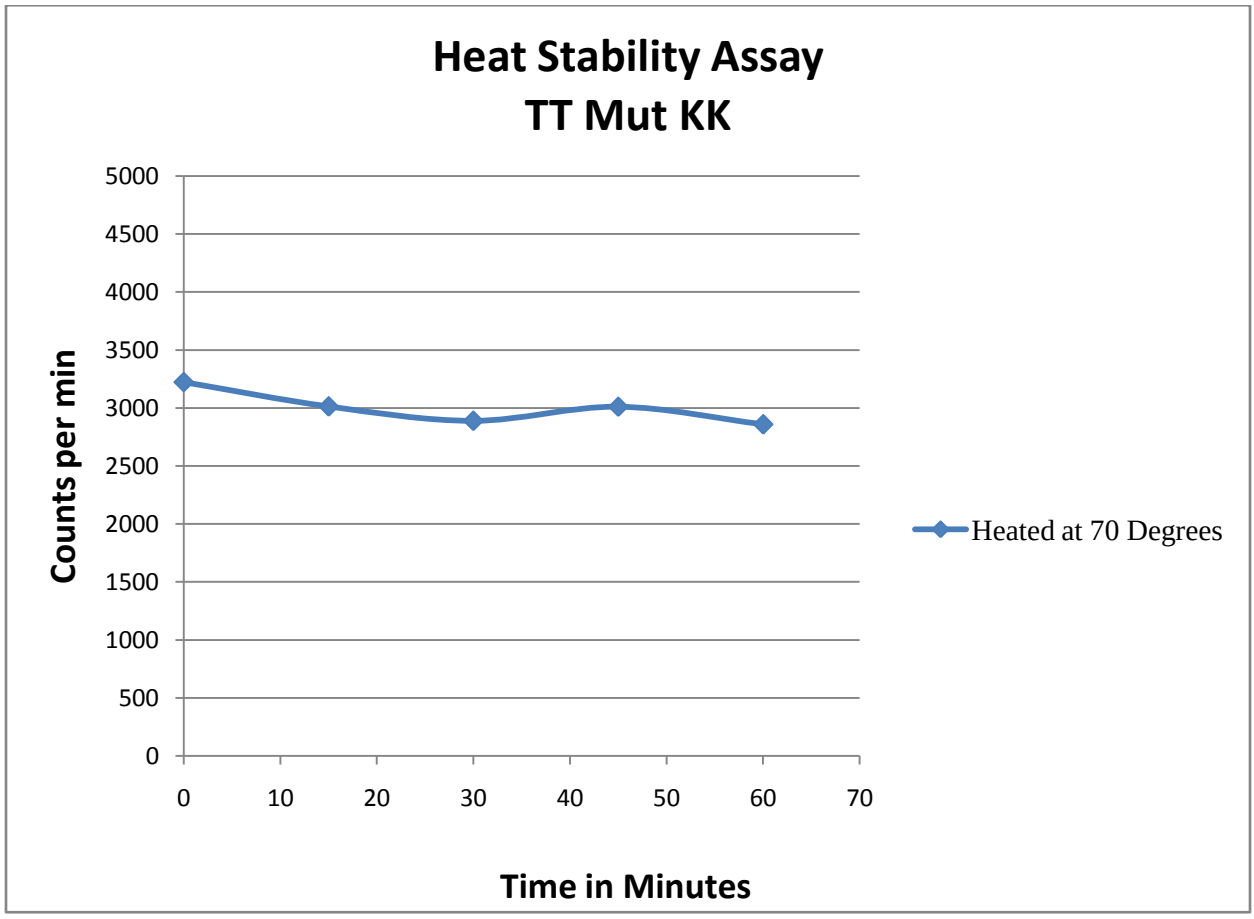


Figure 23 *Thermotoga* double K mutant heat stability assay performed at 70 degrees and assayed at room temperature to determine if prolonged periods at elevated temperatures had an effect on activity.



To ensure that the reaction was linear during the time course of the assay used in these experiments ,preliminary time trials were performed in which the reaction was stopped at 1, 2, 3, 4, and 6 minutes. Figure 24 contains the results of the experiment indicting its continuous linearity well past the normal 4 min termination point we selected.

What might the key residue be that deprotonates the G37 N1 position?

The second mutant studied was the *T. maritima* E166A mutant, created to test the theory that glutamic acid is key in performing the deprotination of the N1 residue in G37. If this was the case, all activity of the TrmD protein would be lost. Because the enzyme retained activity (figure 25) it was determined that the glutamate residue was not essential for catalytic activity. Also, further experiments were performed on this *Thermotoga* mutant protein to determine if the mutation caused any changes in thermostability. Figure 26 demonstrates that the protein is substantially less activity at 70 ^{o}C, It is concluded that this residue is not detectably important for thermostability.

Differential Scanning Calorimetry is a method used to examine the unfolding of proteins, by measuring the caloric difference between a sample cell and a reference cell while applying heat, recoding the uptake or release of heat in real time. The remaining figures 27-30 are DSC graphs obtained from *A. aeolicus*, *E. coli*, *T. maritima*, and *T. maritima* Mutant Double K mutant respectively. Unfortunately the DSC results obtained from the E166A mutation were inconclusive and not reported here. As can be seen, the *E. coli* enzyme is extremely heat sensitive and is essentially completely unfolded by 55 ^{o}C (figure 28). The *Thermotoga* enzyme shows remarkable stability and requires temperatures in

Figure 24.*T. maritima* Double K Mutant activity as a function of time. Performed to ensure that the assay remained within the linear range

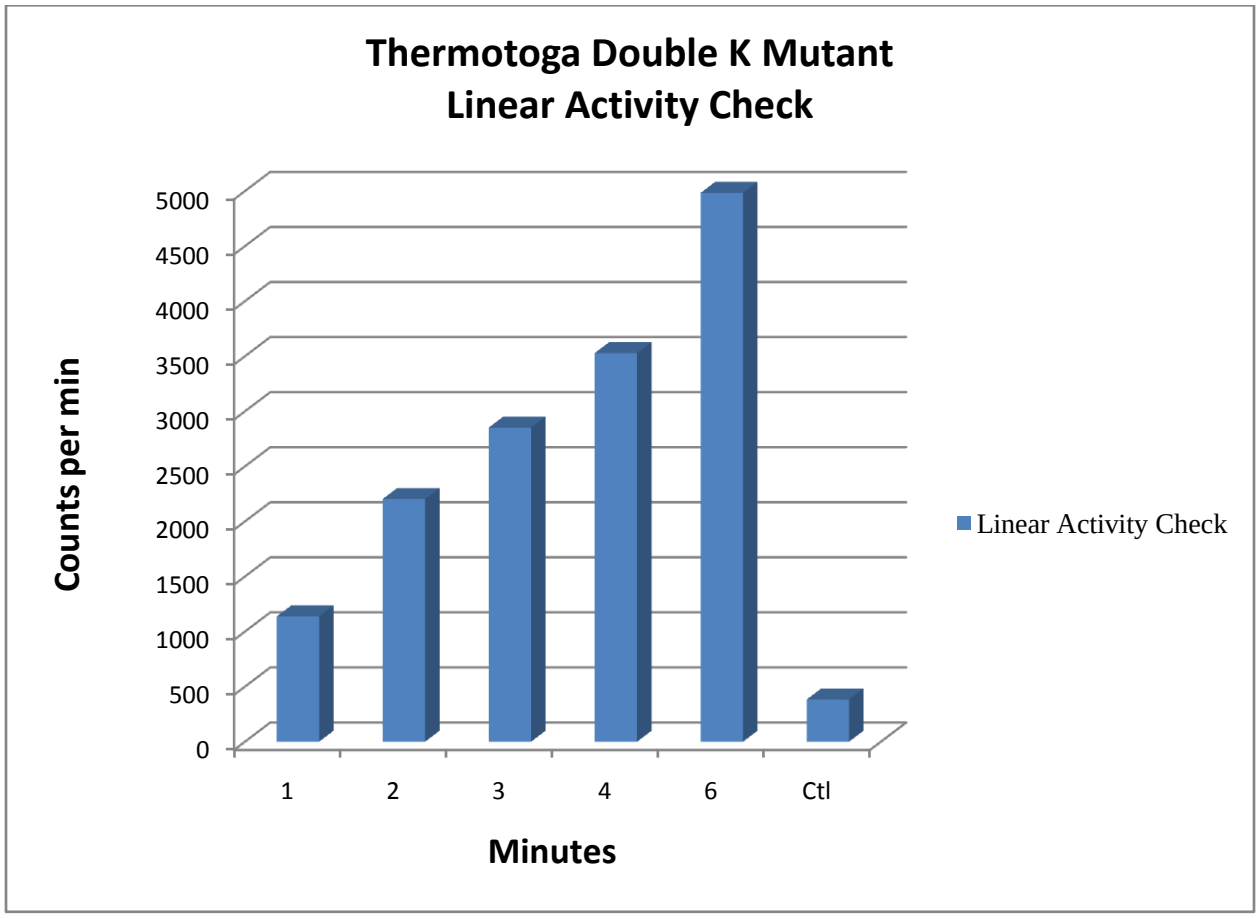


Figure 25. *T. maritima* E166A mutation activity VS Temperature graph which outlines the optimal temperature at which the protein functions

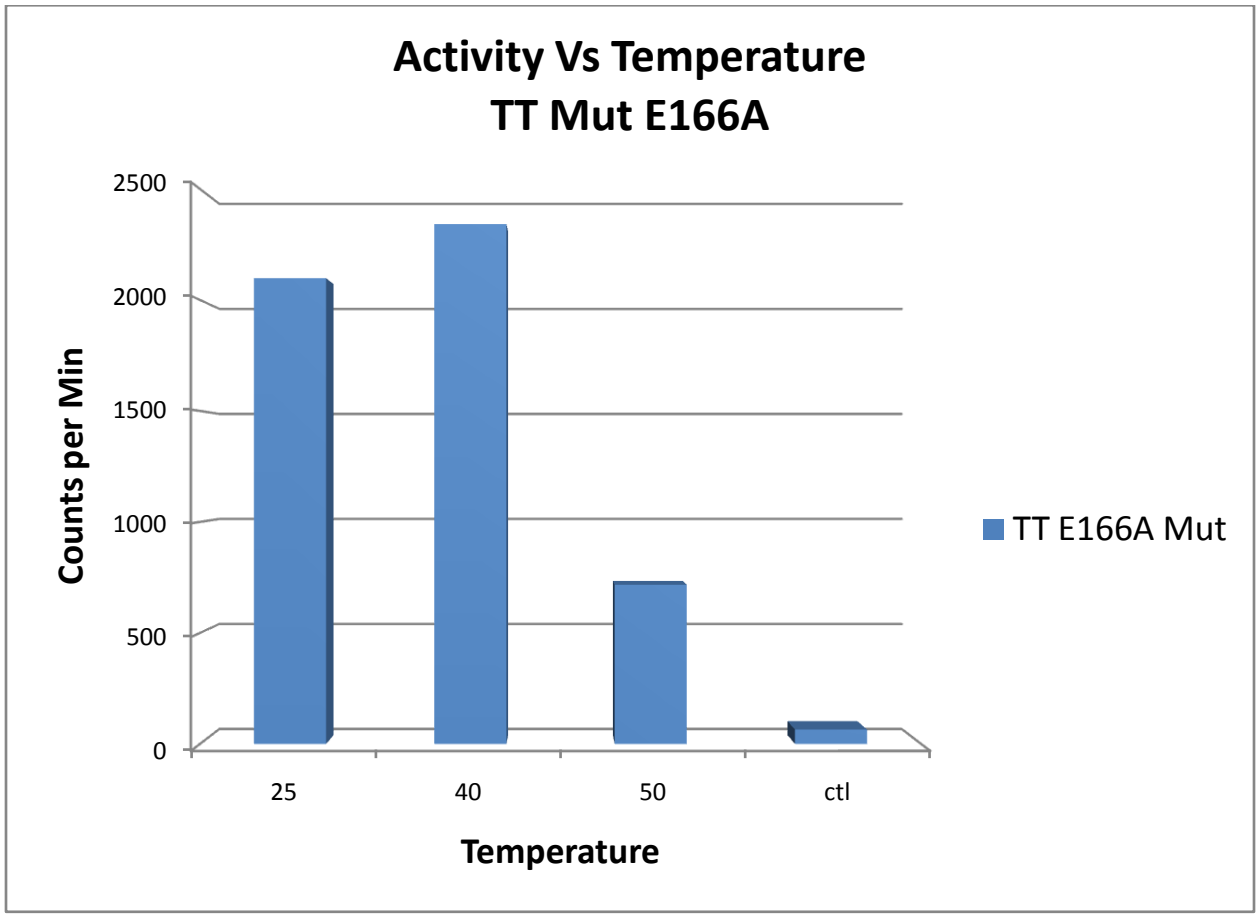


Figure 26. E166A Heat Stability Assay heated at 70 ^{o}C and assayed at 25 ^{o}C to determine the resilience at 70 ^{o}C and the effects if any on the performance at 25 ^{o}C

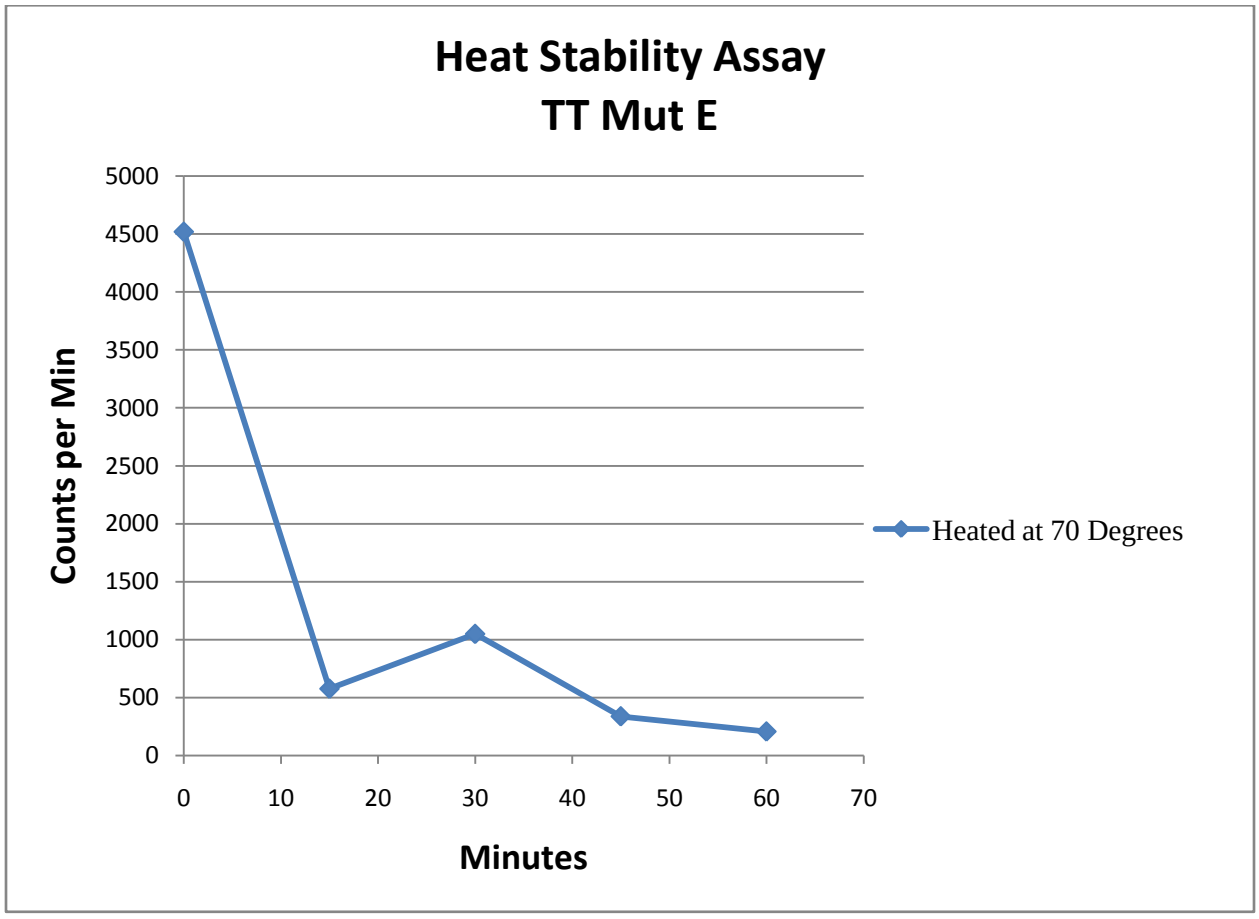


Figure 27. *Aquifex aeolicus* TrmD protein DSC analysis (25 though 100 ^{o}C) to determine the denaturing pattern of the protein. The mutli peak graph is characteristic of a multi domain protein

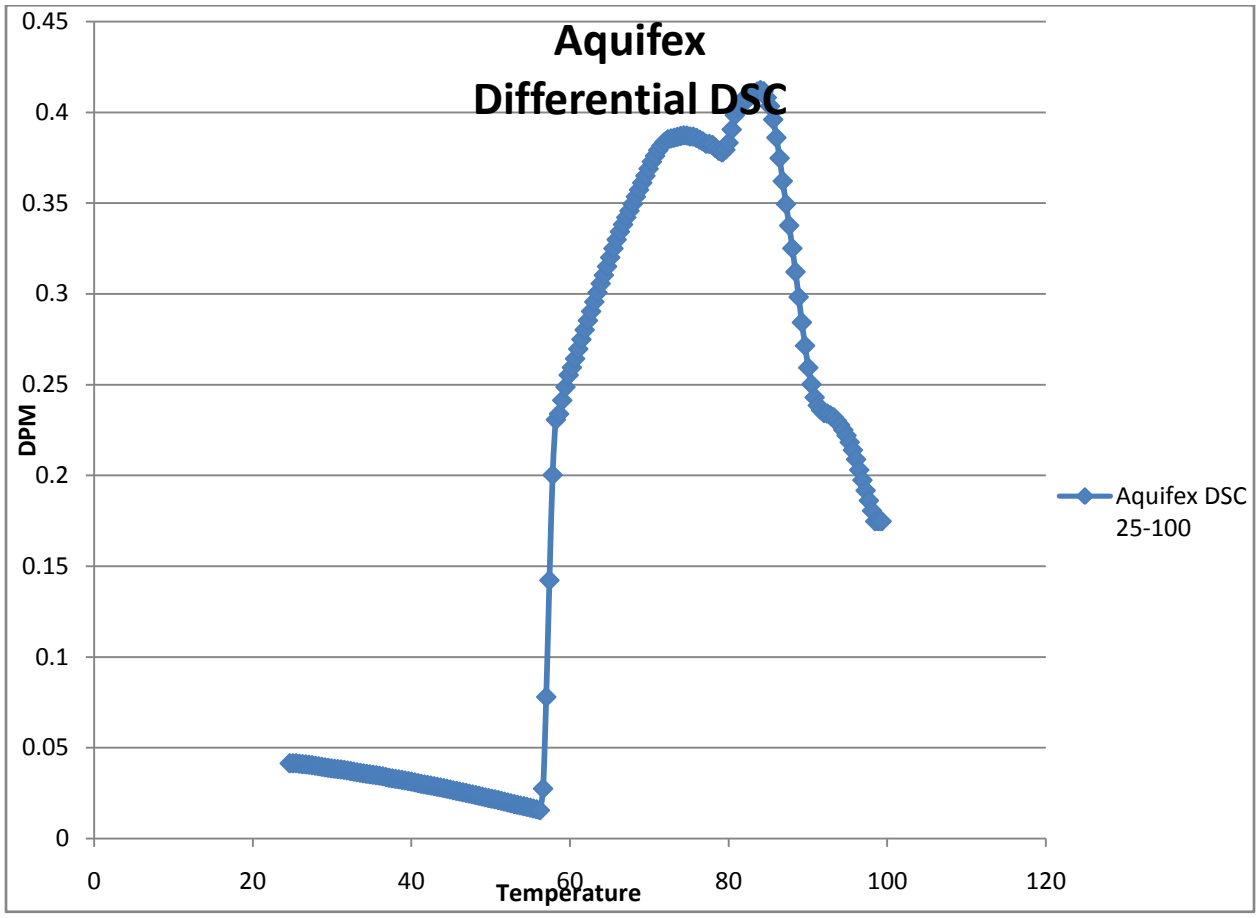


Figure 28.*E. coli* DSC (25-100^{o}C) analysis to determine the denaturing pattern of the protein. The sharp peak is indicative of a cooperatively unfolding protein showing little resistance to unfolding as the temperature is increased

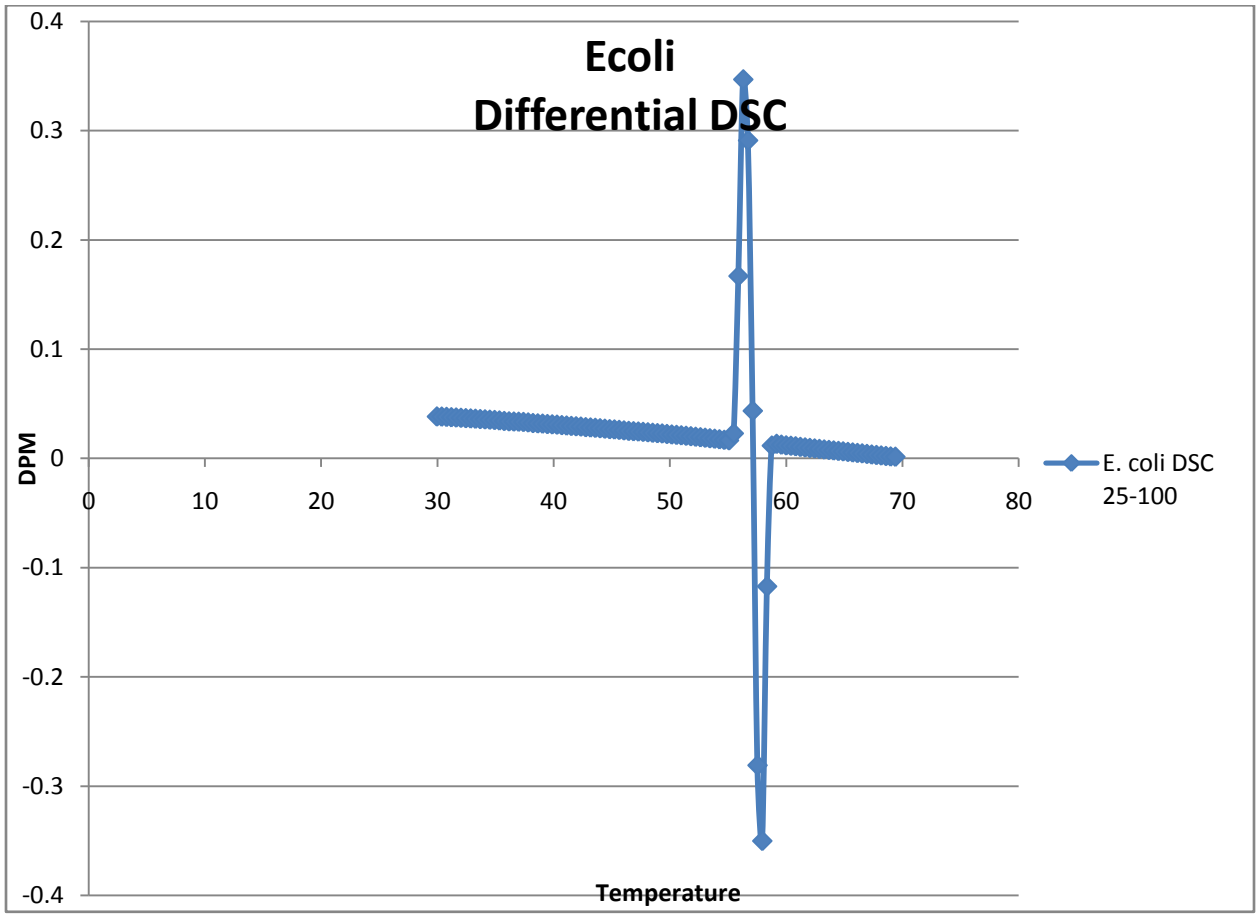


Figure 29 *T. maritima* TrmD protein DSC analysis (25 though 120 ^{o}C) to determine the denaturing pattern of the protein

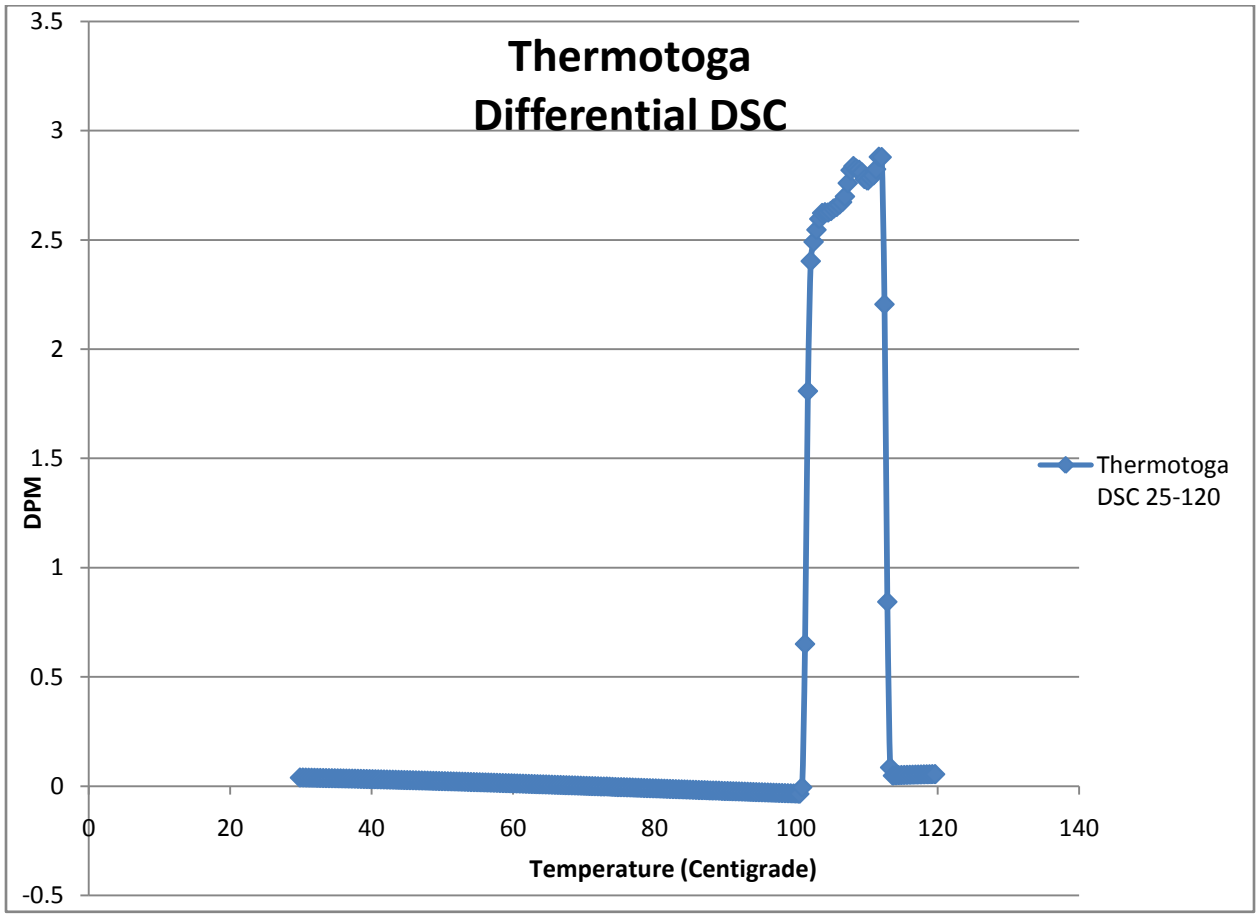
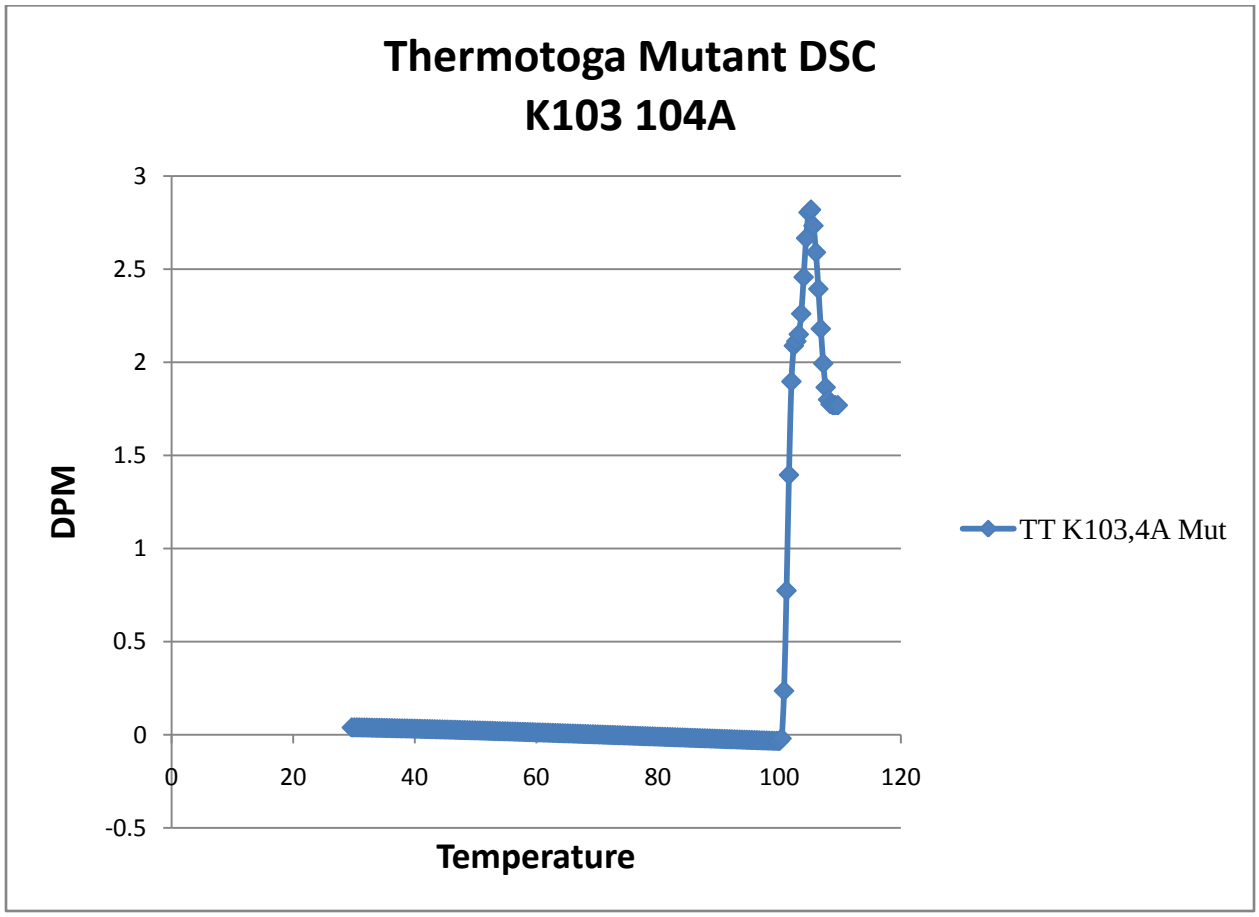


Figure 30: *T. maritima* K103A K104A Mutant DSC displaying the melting temperature of the enzyme in response to extreme temperatures.



excess of 100 ^{o}C for complete unfolding. The *A. aeolicus* enzyme shows intermediate heat stability and is not completely unfolded until about 90^{o}C. Interesting it can be seen that multiple phases of unfolding can be seen figure 27. In comparison to the native protein, the mutant enzyme of *T. maritima* (K, 103,104, A) displayed little if any changes in thermal stability.

Discussion

In general, results presented here have shown that TrmD proteins are all somewhat active at room temperature and increase in methylation activity at higher temperatures before losing activity as a result of denaturation. In addition to enzymatic assay experiments, DSC experiments were conducted to determine the temperature at which unfolding occurs for various TrmD proteins. It was thought that these two measurements might show some correlation.

A comparison of the thermophilic proteins from *A. aeolicus* and *T. maritima* with the mesophilic TrmD protein from *E. coli* revealed interesting facts about the structural components of thermostability. No major differences were observed when examining the primary sequence of the various TrmD proteins. Upon careful analysis of the primary sequences of *A. aeolicus* and *T. maritima* genes there are some subtle differences that may contribute to thermostability. For example, unlike the E. coli gene there is a clear increase in proline residues within both *A. aeolicus* and *T. maritima* but not observed in *E. coli.* Studies performed on the alcohol dehydrogenase of *Clostridium beijerinckii* show an increase in proline content contributing to heat stability by means of decreasing the entropy of the unfolded state (Bogin,O. 1998). Also, after comparing the thermophilic sequences with the E. coli, there is an increase in the intermolecular ionic interactions. The importance of the intermolecular ionic interactions may be detrimental to the overall structural stability of the thermophilic proteins. Analysis of the primary

sequences shows a significant increase in the amount of lysine residues. Also there are two separate consecutive lysine residues present within both of the thermophilic proteins residues 17, 18 and 103, 104 of *T. maritima* and residues 32, 33 and 106, 107 of *A. aeolicus*.

Initially it had been shown in this laboratory that the *T. maritima* enzyme displayed low activity at room temperature. The studies here show that both enzymes show some activity at ambient temperatures. It is not clear at this point why these results are different. It may relate to subtle differences in assay techniques. What is clear is that both the *A. aeolicus* and *T. maritima* enzymes are more thermally stabile as judged by Differential Scanning Calorimetry. This could have been achieved by increasing the rigidity of the structure via the addition of more salt bridges and disulfide bonds for example.

It was also proposed that the positioning of the disulfide bonds contribute to the enzymes ability to function at higher temperatures. One instance where the subunits are linked via disulphide bonds which partially contribute to heat stability can be seen in the archaeal *Pyrococcus abyssi* tRNA m^1A57/58 methyltransferase. This protein is a homo tetramer that contains 4 intermolecular disulfide bonds that when mutated to serines decrease the Tm by 16.5 degrees (Guelorget,A. 2010).The structures of *A. aeolicus* and *T. maritima* both thermophilic enzymes contain inter-subunit disulfide bonds but the positioning of the bonds are different within each protein.

In *T. maritima* the c-terminal domains contain Cysteine residues which link the ends of the two subunits together via a disulfide bond. On the other hand the subunits of *A. aeolicus* are joined internally at the base of the subunits. This becomes significant when comparing the two enzymes. Because of the position of the disulfide bond the *A. aeolicus* enzyme is subject to

something we might call the "playful cat." If one can imagine the water molecules that surround the enzyme vigorously interacting with the structure as a cat would a ball of yarn, once an end is exposed it makes it easier to unravel the remainder of the structure with continued thermal input. If the ends of the protein were linked together preventing separation, then the remainder of the structure would be less affected at higher temperatures thus preventing further denaturation. *Aquifex aeolicus* c-terminal domains of the enzyme are not linked together, making it theoretically easier to separate and denature the structure. On the other hand, *Thermotoga maritima's* TrmD contains disulfide bonds connecting the two subunits at the c-terminal domains theoretically adding additional thermal and structural stability to the protein. This enzyme was studied extensively by Toyooka (Toyooka,T. 2008)who observed incubated the protein at 85 degrees then performed an SDSPAGE to determine how much of the protein precipitated in a given amount of time. The difference between the assay performed within our study and the one performed by Toyooka is the test of functionality. The gel demonstrates the structural stability of the protein at high temperature but not the overall activity of the protein at that temperature. Toyooka demonstrated via SDS PAGE that the protein is stable at high temperatures by means of a disulfide bond formed between the two subunits.

We have compared various TrmD's from different organisms with regard to optimal assay temperatures. The assay shown in Figure 14 was designed to test the ability of *A. aeolicus* to operate at room temperature. Most thermophilic proteins have a minimum operating temperature where below a specific temperature the protein is inactive. Both *T. Maritima* and *A. aeolicus*, which are very closely related in terms of the primary sequence having over 70% homology, are both quite active at room temperature as shown in figures 14 and 20. Also another surprising fact that was discovered while performing the heat stability assays is that both the *T.*

maritima and *A. aeolicus* TrmD proteins both lose activity at 40 ^{o}C which is shown in figures 15 and 21. These facts raised questions about the mechanism of the reaction, the fact that two thermophilic proteins are losing activity in a heat stability assay at 40 ^{o}C over a period of an hour is unexpected. It is possible that these differences relate more to the stability of the tRNA substrate and it is possible that these enzymes might protect the tRNA molecule from unfolding at higher temperatures. We have previously shown within this lab via Circular Dichroism that the tRNA displays a Tm of 52 degrees Celsius. Also with the use of co-incubation studies using TrmD proteins and Elongation Factor – TU (EF-TU) with tRNA which binds the acceptor stem of the tRNA it has been shown to prevent the unfolding of tRNA thus increasing the melting temperature of tRNA. Finally, it is difficult to completely reconcile results seen *in vitro* as compared to the *in vivo* condition.

Given these considerations, DSC was performed to determine the kinetics of unfolding as a function of temperature for each of the TrmD proteins. The first enzyme to be examined was the *A. aeolicus* TrmD protein, which displayed a unique multi peak curve which is shown in figure 27. Multiple peaks within the graph are indicative of multiple domains unfolding. Also the melting range for the protein is very broad which leads to the conclusion that there are some portions of *A. aeolicus* that are not as stable as the remainder of the enzyme. At the conclusion of the experiment, when the sample was removed from the well there was no precipitation meaning that the structural changes observed did not completely denature the protein. It is possible that the changes made to the protein could have rendered it inactive or reduced the activity of the protein which is reflected within the assay. The E. coli DSC, located in figure 28 has a very sharp peak at 55 ^{o}C followed by a negative peak of equal magnitude. This may be attributed to the complete unfolding of the protein which had precipitated within the sample well upon the

completion of the experiment. The result from *Thermotoga maritima*'s DSC shown in Figure 29 the story becomes even more interesting. The *T. maritima* TrmD is remarkably stable displaying a Tm of 110 ^{o}C, which is obtained by selecting the median of the peak observed. The observed Tm adds another level of complexity when comparing the data to the heat stability assays which began to lose activity at approximately 50 degrees Celsius. Small changes to the protein structure can affect the active site of the protein possibly reducing the activity within the assay.

In reference to the standard assays performed that compared the activity to temperature, it is possible that the binding of the tRNA caused a decrease in the activity due to the RNA denaturing before being consumed in the methyl transferase reaction. This leads to the possibility that the tRNA is beginning to denature before it can bind to the thermophilic proteins of *T. maritima* and *A. aeolicus* which should protect the tRNA at high temperatures. Unfortunately, the DSC of tRNA was not clearly defined, and a curve was not extrapolated from the results. A tRNA DSC graph would have provided key information about the Tm, proving that the binding of tRNA to the TrmD enzyme protects is from the unfolding. However, other CD studies in this lab show that tRNA melts quite readily by 50 degrees and can be stabilized by the *T. maritima* enzyme (Brueggemann, 2004) as well as by EfTu. It could be that the *A. aeolicus* enzyme may not effectively do this in comparison to the *T. maritima* enzyme.

Regrettably, neither of the *A. aeolicus* mutations were obtained after several efforts, but the two *T. maritima* mutations E166A and K103A, K104A (double mutation) was created. The E166A mutation was proposed based on the primary amino acid sequence along with the hypothetical crystallographic overlay. The purpose of the mutation was to knock out activity within the protein by inhibiting the deprotonation step within the reaction. The *E. coli* TrmD protein has been shown to use aspartic acid to aid in this step, but based on the hypothetical

structure, the aspartic acid seemed to be out of range for the reaction to take place. By changing the Glutamic acid to an Alanine with the goal to inhibit activity of the enzyme and confirm the role of glutamate within *T. maritima* as opposed to Aspartate. Unfortunately, that was not the case as it displayed activity (figure 25) similar to the other experiments performed and it also decreased in activity at 50^{o}C. The DSC results of the E166A mutant were unfortunately, inconclusive. This was an unexpected result as we hoped to determine the role of the glutamate residue in reference to the stability to the protein.

The lysine 103 104 double mutant showed impressive stability at the higher temperatures. When assayed at 50^{o}Cit maintained the same activity displayed at 40 ^{o}C, which is a first amongst the TrmD proteins that have done so. The results raised questions about the rate of the reaction and the possibility that the reaction was going to completion providing an inaccurate activity curve. Figure 24 shows the progressive linear increase in the activity from one minute to 6 minutes, proving that the reaction is not going to completion. The heat stability assay performed on the enzyme displayed activity at 70 ^{o}C which differs from all of the other TrmD assays. Based on this info, it was concluded that the active site of the double lysine mutant is better protected in that conformation when compared to all of the other TrmD proteins, allowing the reaction to proceed under normal conditions. The mutations made on the *T. maritima* protein were done so without a crystal structure with the intention of destabilizing the thermophilic stability. Figure 30 contains the DSC of a double lysine mutant, revealing the incredible resilience at the upper temperature extremes with what appears to be an incomplete biphasic melting curve. Because the melting curve is incomplete the Tm is unable to be determined.

The upper limits of the DSC were restricted to a maximum of 120 ^{o}C because of instrument limitations; an additional adapter is needed to accurately determine mcal/min. To be

completely sure about the accuracy of the instrument the experiments were set not to exceed 110-115^{o}C. The same limitation is also applied to the lower temperature settings in which the graphs began at approximately 25^{o}C.

Because both of the thermophilic TrmD's contain disulfide bonds and it was proposed that the addition of excess DTT to the assay buffer should reduce the activity of the enzyme at higher temperatures. To begin this process *A. aeolicus* enzyme was isolated in the absence of DTT. The assay conducted in figure 16 was conducted in the presence and absence of DTT at 25^{o}C. The removal of DTT from the assay decreased the activity of the enzyme approximately 50%, but in the presence of DTT the remaining results were similar to previously conducted experiments. This is consistent with previously published results (O'Dwyer,K. 2004). The next experiment shown in figure 17 was carried out in order to determine the effects of DTT as a function of time on the disulfide bonds within the protein which might affect thermal stability. The pre-incubation of protein with DTT for a set time was proposed to determine if the disulfide bonds could be dissociated more completely. Initially time points of 5 and 10 minutes were selected but there was no notable difference in enzyme activity. Figure 18 displays the results of three separate incubation time points of 0, 10, and 30 minutes in 250mM DTT. Based on the results there was no significant differences in activity through 30 minutes in 250mMDTT. In addition to this experiment a heat stability assay in the presence and absence of excess DTT was conducted as shown in figure 19. These results demonstrate that the protein can function in the presence of high concentrations of DTT. This suggests that the disulfide bond is not absolutely critical for thermal stability. However, subtle effects here might be additive and important in vivo. This effect was also seen in previous studies within our lab in which TrmD was incubated in the presence of DTT and observed via circular dichroism. This experiment produced a result

in which there was no change in elipticity in the presence of an increased concentration of DTT. Results of enzymatic activity assays also confirm the fact that DTT does not dramatically affect the activity of the enzyme. Heat stability assays were conducted in the presence and absence of excess DTT which resulted in minimal differences in enzymatic activity.

Future studies of the TrmD enzymes will look into more mutations of amino acid residues. Observing the effects of mutating the non conserved proline residues located within the thermophilic proteins of *A. aeolicus* and *T. maritima* will be the first of many mutations. Proline residues have been proposed to reduce the entropy of the unfolded state (Bogin,O. 1998). This is significant because proline residues are usually located within alpha helixes and beta turns because of their unique structural conformation they permit the looping of helical structures.

A comparative analysis of both double lysine mutations 103,104 in *T. maritima* and 106, 107 in *A. aeolicus* would be key in determining the particular residues that are important in maintaining thermostability. If the results are comparable to that of *T. maritima* it may be proposed that those residues have no effect on thermostability. Ideally the mutations made would not affect the activity of the protein at ambient temperatures but as the temperature increases the effects are observed, which corresponds to a DSC graph.

Additional mutations that can be performed would be the addition of disulfide bonds within *E. coli* in an attempt to mimic the thermophilic proteins. This would demonstrate the significance of the disulfide bonds in reference to thermostability. Each mutation performed on *A. aeolicus* and *T. maritima* can be mimicked and reversed *E. coli.* Mutating certain proline residues within E. coli that are only present within the thermophilic organisms should provide insights into the importance of proline residues on thermostability.

Overall, it has been shown that the *Aquifex* and *Thermotoga* enzymes are quite heat stabile as judged by DSC and that the *Thermotoga* enzyme is more stable than the *Aquifex* enzyme. The effect of temperature on enzyme activity shows much less thermostability. It is clear that more experiments need to be performed in order to determine why these results don't correlate well with thermal denaturation profiles. It will be interesting to determine if the presence of ligands alter the heat stability assays as well as specific assay conditions. Finally, a more extensive mutagenesis study will have to be done to better identify structural features involved in thermal stability.

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