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A computational and experimental analysis of the temperature stability of the enzyme Drosophila Alcohol Dehydrogenase

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Abstract

Anthropogenic climate change and its impact on ecosystems is one of the major concerns of the century. Temperature influences the rates of reactions in biochemical pathways, thus temperature adaptation among species is likely to occur in the face of this threat. The objective of this project was to analyze the ethanol metabolism pathway and the two allelic variants of the enzyme alcohol dehydrogenase (ADH) through the lens of temperature-driven evolutionary adaptations. One isozyme is found at lower latitude regions with higher temperature (ADH-S), while the fast allele is found at higher latitudes with lower temperature (ADH-F). The experimental and computational data suggest a disproportionate decrease in the stability of the two dimer forms of ADH with an increase in temperature (with the ADH-S version being more stable and thus more temperature resistant). The computational analyses of the monomer of the protein indicate no significant difference in response to the increase of temperature in the two variants. The results have led us to conclude that the structural stability of the quaternary structure of the ADH protein in the dimeric and monomeric state is altered via the mutation, with the mutation resulting in increased stability in the dimeric state and decreased stability in the monomeric state. Moreover, atomistic simulations show that there is a major difference in the Root Mean Square Fluctuations (RMSF) value of the two isozymes at the location of the mutation. This opens the possibility for future research endeavors focused on the impact of the mutation on the connection between the monomers. Such research could shed light on the biochemical basis of the temperature adaptation of ADH.

Introduction

Temperature is one of the main drivers of evolution on a micro and macro level. The impact of temperature variations on the biochemical processes of different organisms has been of significant interest to evolutionary and molecular biologists (Somero, 2010). Different biochemical pathways have adapted through various mechanisms to temperature variations such as structural changes in macromolecules (intrinsic adaptations) (Somero, 2010).

The enzyme Alcohol Dehydrogenase (ADH) is a highly conserved enzyme in many organisms, including humans, because of its versatile role in the metabolism of ethanol (Benach et al., 2005). ADH functions in a pathway, converting ethanol to acetaldehyde, which is then converted to acetate by acetaldehyde dehydrogenase (ALDH) and then into acetyl-CoA via the enzyme acetyl-CoA synthase (Montooth et al., 2006).

Individuals of the species *Drosophila melanogaster* spend most of their lifespan living in an ethanol rich environment inside fermenting fruits. Hence, the function of the ethanol metabolism pathway is integral to their subsistence (Montooth et al., 2006).

There are variations of the ADH enzyme (isozymes) in different populations as natural selection favors different alleles depending on the environmental stimuli acting upon the population (Somero, et al. 2017). ADH in *D. melanogaster* is present in two allelic forms: a slow (ADH-S) and a fast (ADH-F) form, named based on the speed at which each

allelic variant runs on a protein gel (Thatcher & Sheikh, 1981). The difference between the two isozymes is one amino acid replacement (threonine in place of lysine) in the primary sequence of the protein (Benach et al., 2005). The slow allele isozyme has a higher frequency at lower latitude regions with higher temperature, while the fast allele has a higher frequency at higher latitudes with lower temperature (David et al., 1989). This pattern suggests that temperature adaptation has shaped the evolution of this enzyme through the differing stability of the two most prominent isozymes.

The structural and biochemical difference between the ADH-S and ADH-F allelic variants has been studied in *D. melanogaster* to understand the clinal climate adaptations in the biochemical pathways of this species as temperature directly impacts the structure and function of different enzymes.

Previous work on ADH has rendered contradictory results. A study by McElfresh and McDonald (1986) investigated the structural difference in the fast and slow allele variants, showing there is no significant difference in the temperature induced structural changes between the two. However, the authors also reported that the measured Km of ADH-F was consistently higher than that of ADH-S across all tested temperatures (McElfresh & McDonald, 1986). In another study, the thermal stability of the two isozymes was shown to be different as ADH-F homodimer is less stable than the ADH-S homodimer and the heterodimer of the two alleles and the ADH-S isozyme is reported to denature at a temperature 5°C higher than the ADH-F isozyme (Thatcher & Sheikh, 1981). Moreover, the threonine to lysine substitution makes the ADH-S allelic variant

have a less negative active site domain (Benach et al., 2000). Hence, the charged residues in the active sites of these two enzyme forms behave differently, most likely resulting in structural changes to the active sites in different temperatures.

The ADH-F allele has an approximately three times higher enzymatic activity than the ADH-S allele (McElfresh & McDonald, 1986). Researchers suggest that this could be an evolutionary adaptation in organisms with the ADH-S allele at high temperatures, as the ethanol metabolism pathway has toxic products (most significantly acetaldehyde) further down the pathway (Montooth et al., 2006). However, the reasons behind this increased activity and the impact of the structural differences between the ADH-S and ADH-F variants on the reactions of this enzyme have not been discovered.

I hypothesized that the differential thermal stability of the protein will result in divergent results between the outcome of the ADH-S and ADH-F simulations in the case of the MD, Upside MD and quantum chemistry simulations. These differences were predicted to manifest in computational thermal stability analyses.

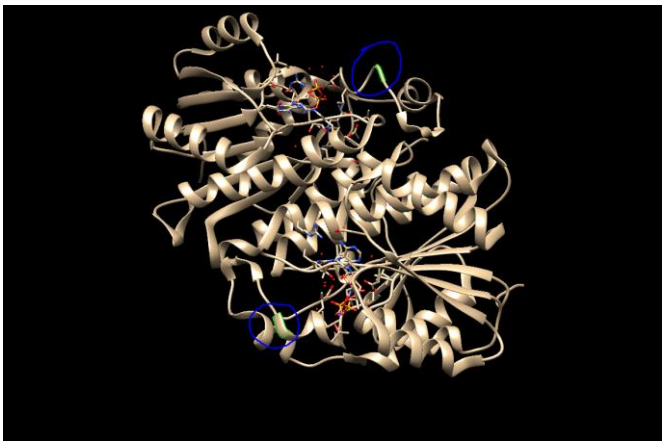

Figure 1. A picture of ADH-S produced via the program CHIMERA. The circled residue is the residue number 192 (the site of the mutation). ADH consists of two monomers bonded together through one direct covalent bond. Its active site consists of an area between the main two alpha helices of each monomer.

Methodology

Computational procedure

Molecular dynamics simulations are attempts to predict the properties of systems of atoms and/or molecules through a “bottom-up” approach. In other words, these simulations predict the properties of big physical systems through the simulation of their constituent particles (Rapaport, 2010). An inevitable result of the philosophical backbone of molecular dynamics simulations is the complete predictability of all systems that adhere to the laws of classical physics, a phenomenon predicted by physicists such as Laplace (Rapaport, 2010). MD simulations measure multiple characteristics of a given system (primarily the velocity and position of particles) over multiple timesteps through the usage of the Newtonian laws of motion . MD simulations have been used extensively for the analyses of the physical properties of various structure, including proteins and membranes. The initial system (in this case, the protein ADH) is represented by a multiplicity of vectors containing the coordinates of its structural elements (atoms and residues) and their properties (velocity, position, weight). The given vector is subsequently used to predict the motion of the system over a defined time period. The resulting changes to the initial set of vectors is used to deduce the changes of the system over the defined period.

In this study, MD simulations were utilized to analyze the general molecular interactions between the substrates (ethanol and acetaldehyde) of the two reactions and ADH. The program GROMACS was used for the MD simulations (Van Der Spoel et al., 2005).

At the beginning of the experiment, a total of 6 normal atomistic MD simulations were performed. The simulations included the two allelic variants of the protein, two different protonation states of the protein and cases with and without the substrate and each simulation ran for a total of 100 ns. Due to the computational expense of the simulations (over 60 GPU hours per simulation), the Upside MD method was chosen as an alternative in the later stages of the study due to being significantly lower in computational cost and having comparable accuracy to the atomistic simulations in terms of the general data.

A set of 2 quantum chemistry simulations were performed with the program IQ-mol, yet the structural complexity of the ADH protein made correct inferences implausible as the maximum number atoms covered in the simulations (approximately 300) did not cover the entire protein. Moreover, the physical distance between the active site and the point of mutation (residue 192) rendered quantum chemistry simulations focused on the electron flow at the active site practically ineffective.

The recently published model named Upside MD (Jumper et al., 2016 and 2018) was utilized as an alternative to both the normal MD simulations and the IQ-mol simulations to generate a total of 64 simulations categorized in 4 groups, wildtype monomer, mutated monomer, wildtype dimer, and mutated dimer. This program was chosen as it provides a much computationally less-expensive method in comparison to normal MD simulations and is comprehensive by including all particles of the protein as opposed to the quantum model. This program utilizes a novel approach to the traditional accuracy and computational trade-off in biomolecular simulations through utilizing less complex force fields that are able to perform the energy calculations of the system with less

computational resources and with no major decline in accuracy. Nevertheless, this approach has its limitations as it does not provide a comprehensive particle-by-particle simulation of the structure and hence cannot be utilized for computational purposes outside of general energy analyses.

The Upside MD program was modified to include the dimeric connection between the two monomers of the protein (both dimeric and monomeric variations of the simulations were run). Each group contained 16 simulations at temperatures ranging from 0.9 to 1.2 spaced out by 0.2 temperature units. It is noteworthy to mention that the units of Upside simulations are theoretical in the computational analyses and are not representative of SI units (approximately 1 measure of the unit is equivalent to 300 degrees kelvin). The range of the temperature was chosen based on previous simulations ran from 0.5 to 2 spaced out evenly every 0.1 units which indicated that the major configurational transition happens in the 0.9 to 1.2 range.

Each simulation ran for 50,000 timesteps with data being taken at every 10^{th} frame to increase the computational efficiency of the procedure. Each simulation took a total of approximately 9 GPU hours on the central GPUs utilized at the Vanegas lab. The were 3 categories of data in the simulations, number of hydrogen bonds, radius of gyration and energy of the system. All data categories were chosen as indicative proxies for protein stability, with decreased hydrogen bonds, increased radius of gyration and increased net energy of the system indicating less stability respectively.

The radius of gyration of a body about the axis of rotation is defined as the radial distance to the center of mass, a point which would have a moment of inertia the same as the body's actual distribution of mass, if the total mass of the body were concentrated

there. Mathematically the radius of gyration is the root mean square distance of the object's parts from either its center of mass or a given axis.

$$R_g^2 = (r_1^2 + r_2^2 + \cdots + r_n^2)/n$$

The radius of gyration can be used as a proxy for the stability of a given protein, as functional proteins can operate at very limited bandwidths of this value depending on their structure (Fixman, 1962).

The statistical differences between the two variations of ADH was demonstrated through a sigmoidal curve fitted to the radius of gyration curves and the estimated error bar of the temperature of transition. The curve was fitted using the Scipy curvefit module in python. The sigmoid curve followed the following equation:

$$f(x) = \frac{L}{1 + e^{-k(T-\mathrm{T0})}}$$

Where T0 is the value of the midpoint (also known as the transition temperature), L is the curve’s maximum value, and k is the logistic growth rate of the curve.

Root-Mean-squared Fluctuations (RMSF) analyses were utilized to analyze the structural changes of the atomistic simulations over time. RMSF analyses are a measure of the difference between the position of a particle and a reference position (in the case of this study, the available crystal structure of ADH-S) calculated through the following formula:

$$\mathrm{RMSF}_i = \left[\frac{1}{T} \sum_{t_j=1}^{T} |\mathbf{r}_i(t_j) - \mathbf{r}_i^{\mathrm{ref}}|^2 \right]^{1/2}$$

where T is the total time of the simulation r_i^{ref} is the reference position of particle i.

Results

The computational results are three-fold, the data acquired through the atomistic MD analyses and the upside MD analyses of the ADH-F and ADH-S monomers and dimers.

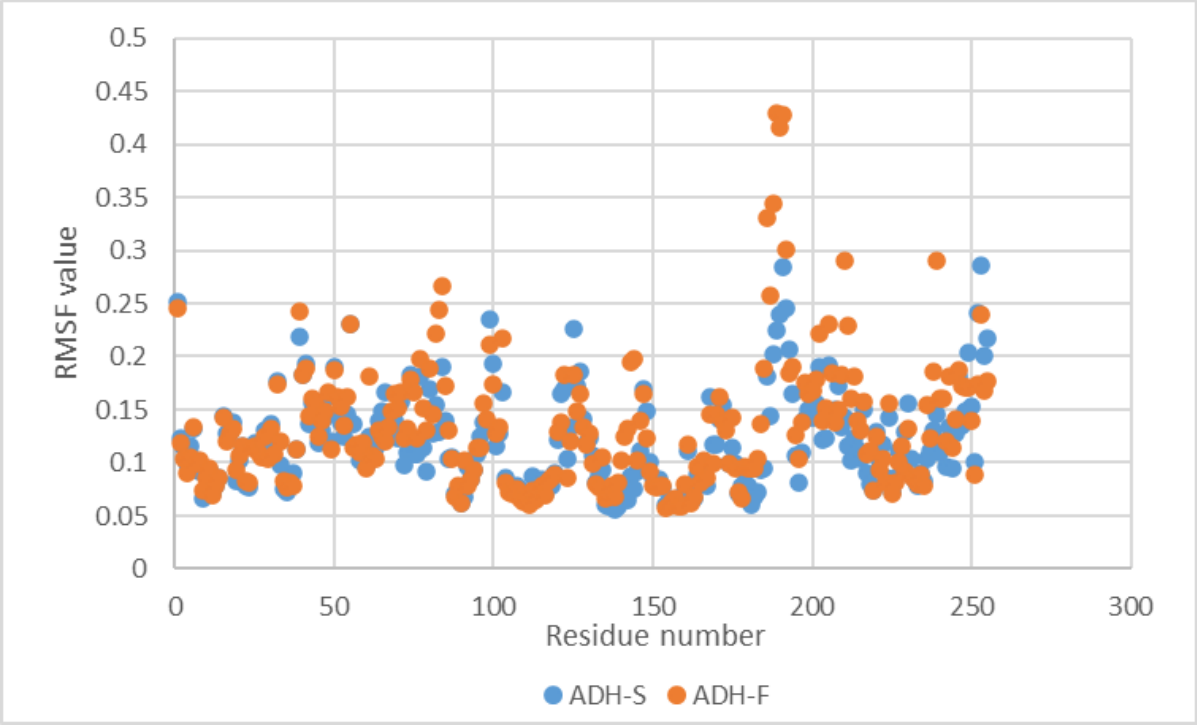

Figure 2. RMSF analysis of the dimeric variations of the ADH-F and ADH-S enzymes across each residue. The mutational difference between the mutant and the wildtype is located at residue 192.

The RMSF analysis indicates a generally similar trend for the positional fluctuations of the two dimeric variants. Notable differences in positional fluctuations between the ADH-F and ADH-S variants can be observed at residues 188,189,190,191 and 192 with the ADH-F variant showing more divergence from the basic crystal structure (which is based on the ADH-S variant).

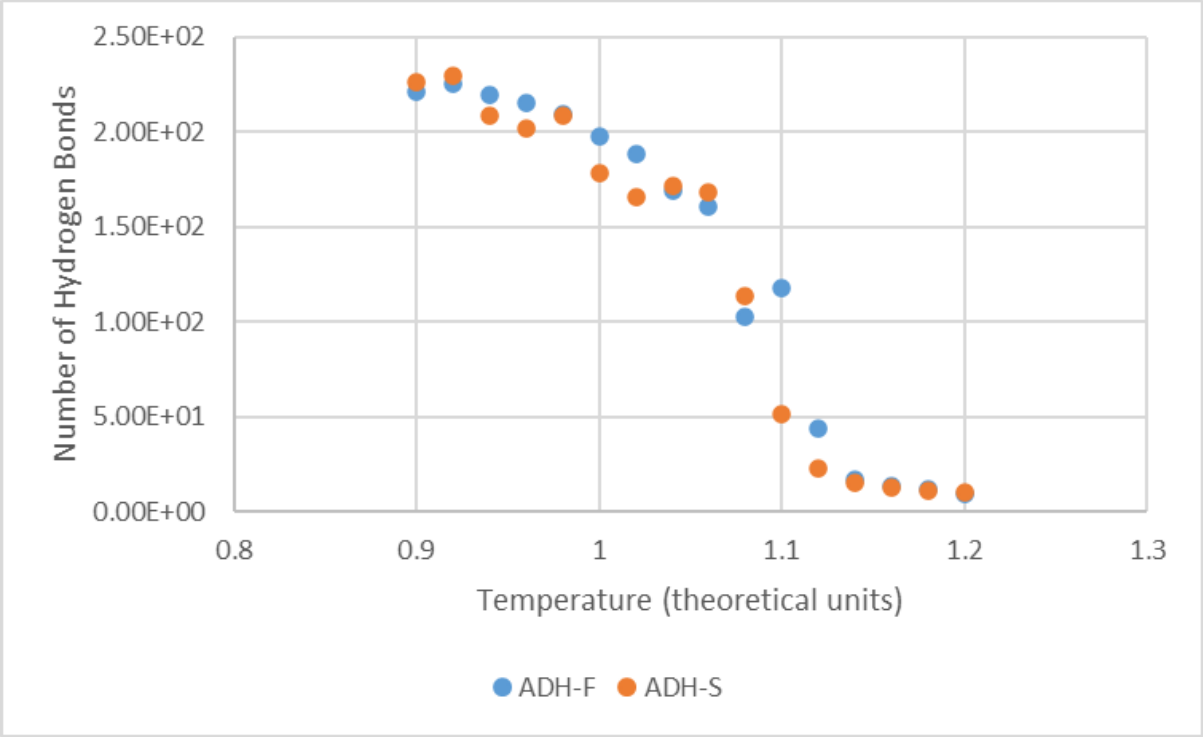

Figure 3. Number of hydrogen bonds of the monomer simulations across temperatures varying from 0.8 to 1.2 units. WT denotes the wildtype version of the enzyme (ADH-S) and Mut denotes the mutated version of the enzyme (ADH-F).

The number of hydrogen bonds in a given protein has an inverse relationship with its stability in most cases (Pace et al., 2014). A general decrease in the number of hydrogen bonds is observed with the increase of temperature, with no discernable difference between the ADH-S and ADH-F monomers (Figure 3). This is indicative of the relative ineffectiveness of the mutated residue (residue 192) on the hydrogen bonding of each of the monomers of the protein separately. This results indicates that the polarity of threonine (compared to lysine) is sufficient to replace any intra-monomeric hydrogen bonds.

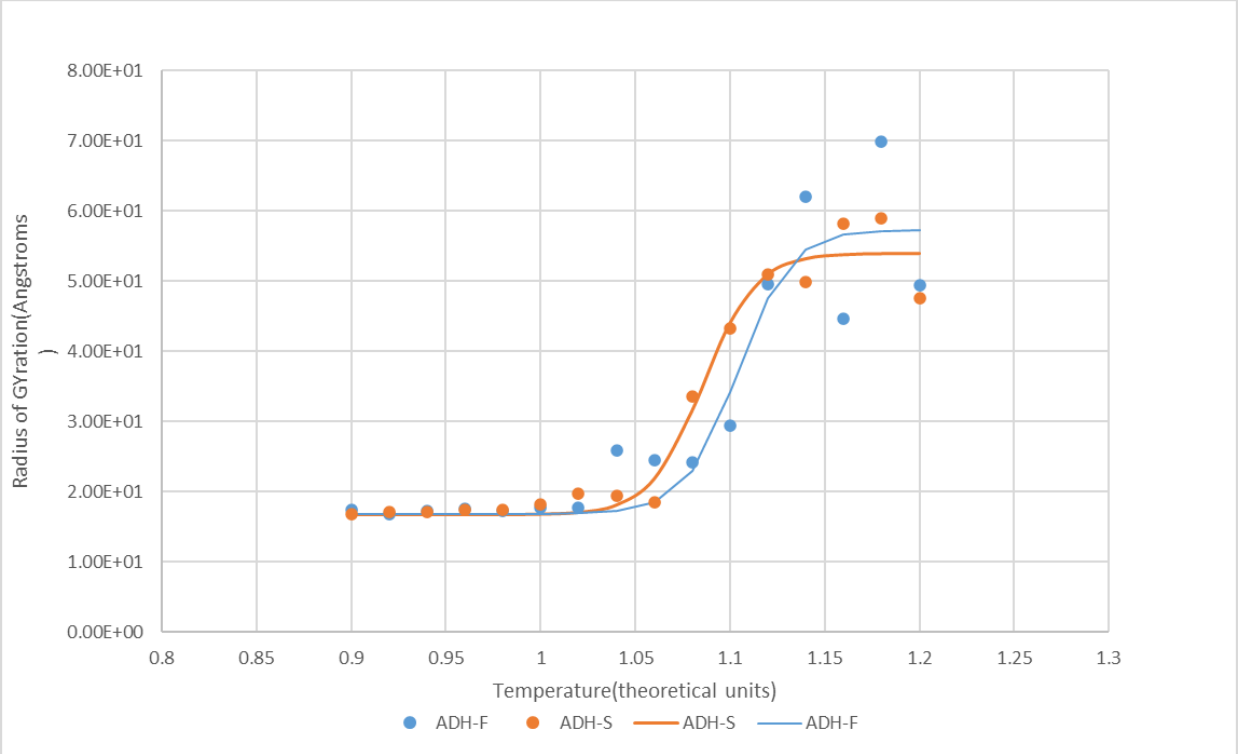

Figure 4. Radius of Gyration of the monomer simulations across temperatures varying from 0.8 to 1.2 units. WT denotes the wildtype version of the enzyme (ADH-S) and Mut denotes the mutated version of the enzyme (ADH-F).

The overall trend suggests that there is no significant difference in the radius of gyration of the two monomers (Figure 4). Nevertheless, the response of radius of gyration follows the predicted response with temperature.

Table 1. Summary of the statistics of the logistic curve fitted to the Radius of Gyration of the monomers.

Type	L	k	T0(in kelvin)
ADH-S	37.1 +/- 1.6	71.2 +/- 17	325.8 +/- 1.2
ADH-F	40.5 +/- 4	71.6 +/- 34	331.2 +/- 2.4

The fitted sigmoidal curve shows a statistically significant difference between the transition temperature of the two monomeric variants as the ADH-F variant has a statically significant higher transition temperature (T0) (Table 1).

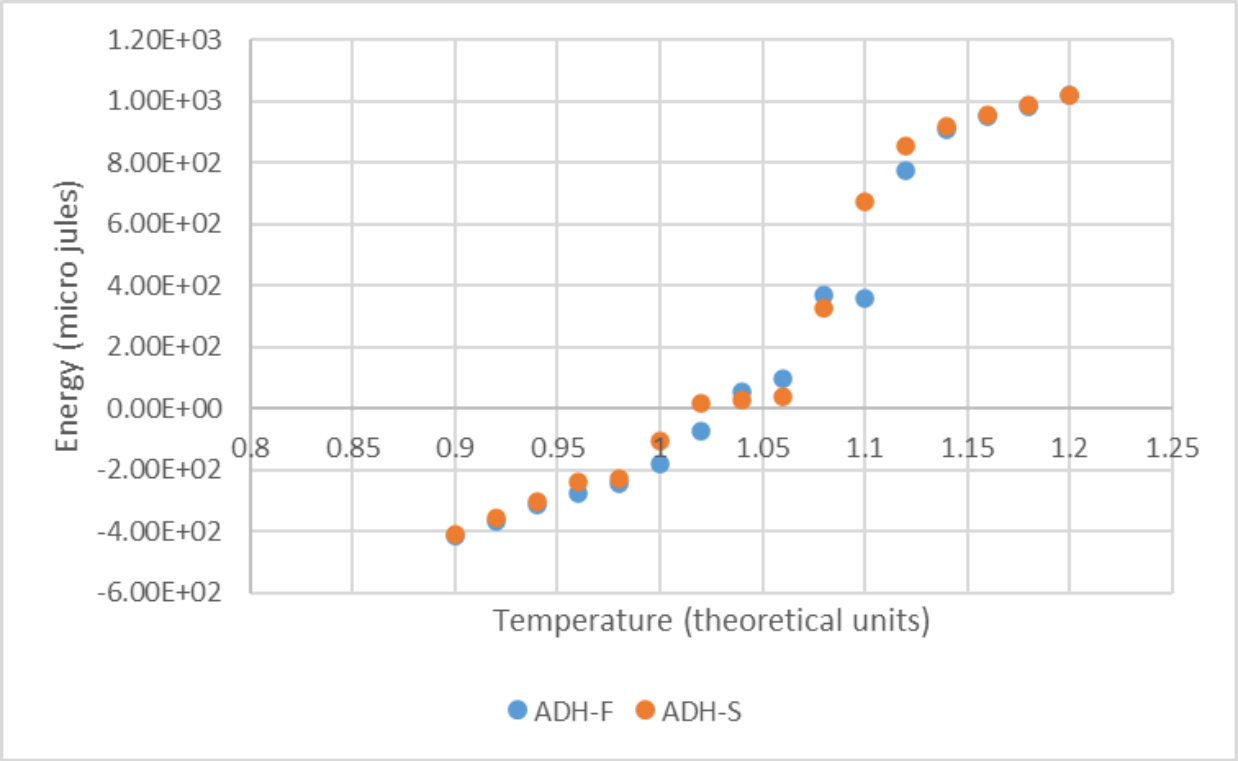

Figure 5. Energy of the monomer simulations across temperatures varying from 0.8 to 1.2 units. WT denotes the wildtype version of the enzyme (ADH-S) and Mut denotes the mutated version of the enzyme (ADH-F).

The protein stability curve denotes the free energy of the protein across different temperatures. The lower the free energy, the more stable the protein and the closer it is to optimal functioning in most cases (Beckel, 1987). There appears to be no significant difference between the protein stability curve of the two monomers (Figure 5). This further supports the trends observed with the number of hydrogen bonds and the radius of gyration of the two monomers. Moreover, the graph suggests that the thermal denaturation of the enzyme occurs at approximately 1.03 temperature units (309 kelvin) (Figure 5).

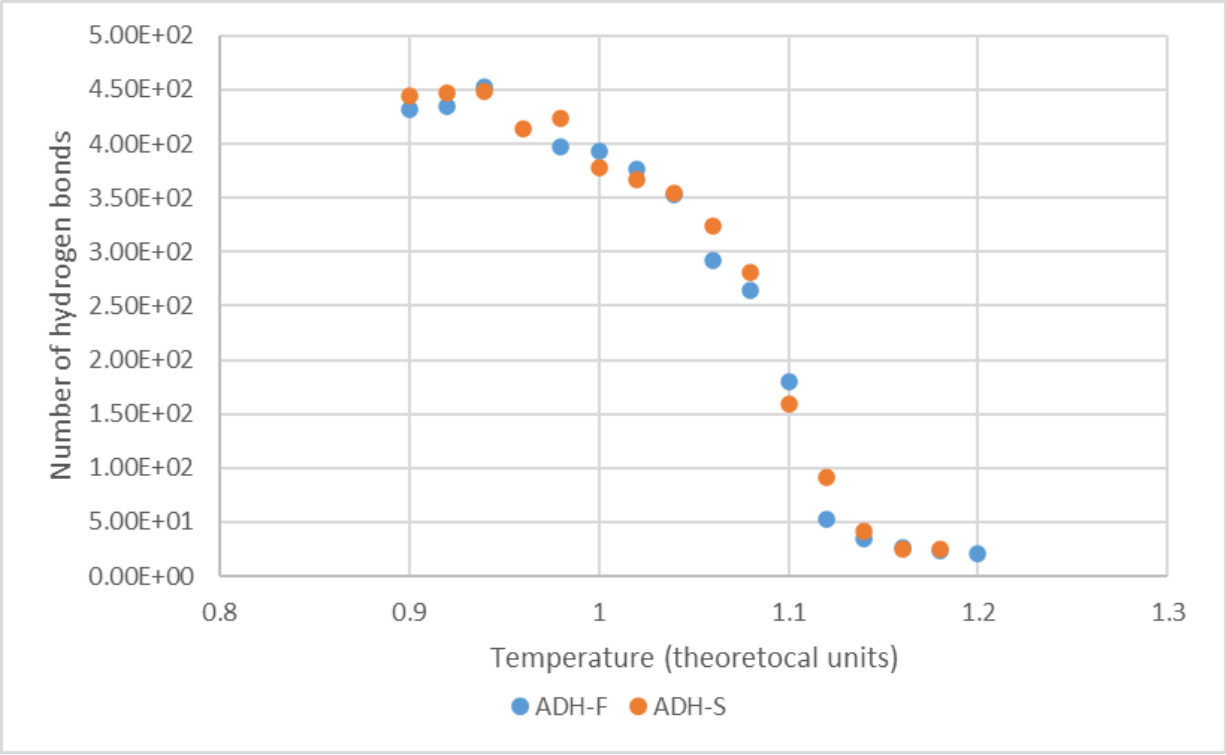

Figure 6. Number of hydrogen bonds of the dimer simulations across temperatures varying from 0.8 to 1.2 units. WT denotes the wildtype version of the enzyme (ADH-S) and Mut denotes the mutated version of the enzyme (ADH-F).

The number of hydrogen bonds between the two dimeric variations of the ADH enzyme indicate a similar decrease of the number of hydrogen bonds in both variants (Figure 6).

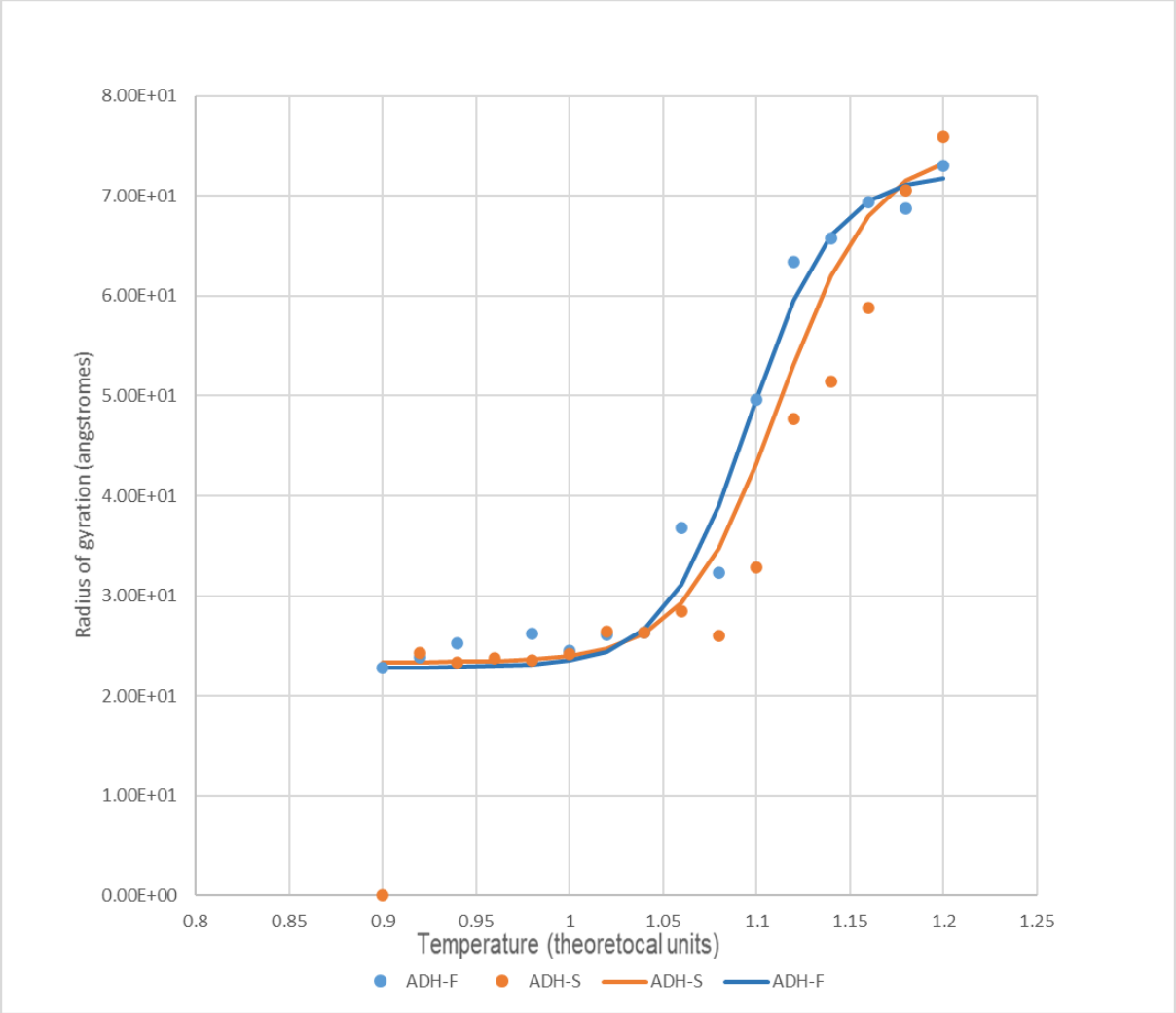

Figure 7. Radius of Gyration of the dimer simulations across temperatures varying from 0.8 to 1.2 units. WT denotes the wildtype version of the enzyme (ADH-S) and Mut denotes the mutated version of the enzyme (ADH-F).

The radius of gyration of the two dimeric variations of the ADH enzyme indicate a more significant increase in the case of the ADH-F variation (Figure 7). This suggests that dimeric ADH-F has denatures more rapidly and is less stable.

Table 2. Summary of the statistics of the logistic curve fitted to the Radius of Gyration of the dimers.

Type	L	k	T0 (in Kelvin)
ADH-S	51.5 +/- 3	39 +/- 6	333.6 +/- 1.5
ADH-F	49.5 +/- 2.4	44.4 +/- 7.4	328.8 +/- 1.5

The above table highlights the statistics resulted from fitting a sigmoid curve to the model. The ADH-S variant has a statically significant higher transition temperature (T0) whereas the ADH-F variant has a steeper slope.

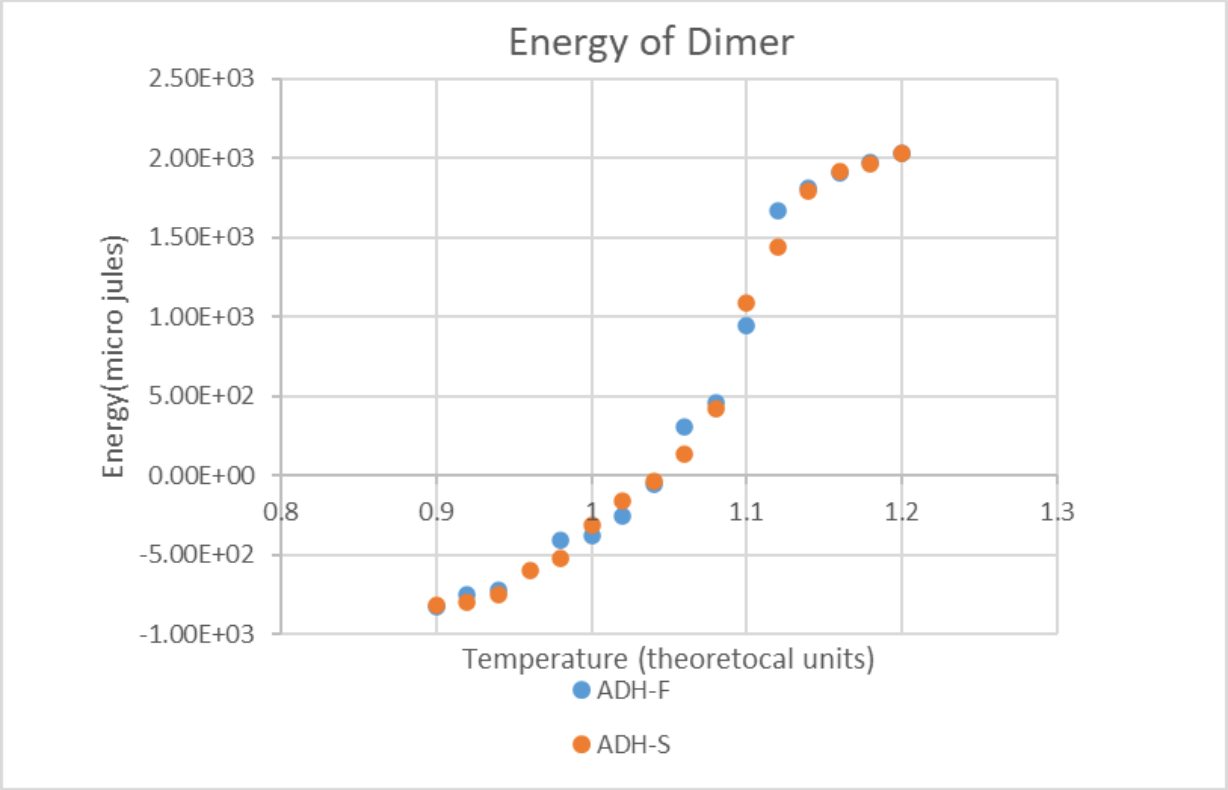

Figure 8. Energy of the dimer simulations across temperatures varying from 0.8 to 1.2 units. WT denotes the wildtype version of the enzyme (ADH-S) and Mut denotes the mutated version of the enzyme (ADH-F).

The protein stability curve of the two dimeric variations of the ADH enzyme indicate a similar increase in the overall free energy state of the protein(Figure 8). Moreover, the protein approximately passes enters the realm of positive free energy at approximately

1.04 temperature units (approximately 312 degrees of Kelvin) which is slightly higher than that of the monomeric variations (Figure 5 and 8).

The protein stability curves of the monomeric and dimeric variations of ADH indicate that the most stable configuration of the dimer is at a lower temperature than that of the monomer (0.8 vs approximately 1) (Figures 5 and 8). This is most likely due to the complementary quaternary structure exhibited in the dimer.

The differential stability of the dimeric variations of ADH indicates that the role of the mutation is primarily focused on promoting structural integrity. Based on the preliminary quantum chemistry simulations that did not include the mutated residue in the chemical reaction and the distance of the location of the mutation (residue 192) to the active site, it can be further hypothesized that the role of the mutation is mostly structural with minimal impact on the catalyzed reactions in the ethanol metabolism pathway.

Discussion

As previously shown experimentally (Benach et al., 2000), the trends observed in the results section of this study indicate that a steady increase in temperature leads to a faster denaturation of the dimeric ADH-F variant of the protein (Figure 7, Table 1). This result is statistically significant as the temperature of denaturation is shown to be much higher in the ADH-S variation of the enzyme (333.6 for the ADH-S variant in comparison to 328.8 for the ADH-F variant) (the difference is more than three times as the error bar that is estimated by the least squares method while performing the regression). The approximate 4.8° K difference (Table 2) is consistent with the 6 ° K difference shown in experimental trials where the melting temperature of the ADH-S variant was reported to be 38 ° C (311.15° K) and 32 ° C (305.15 ° K) for the ADH-F variant (Thatcher and Sheikh,1981). This data was not replicated in the monomeric analyses as the monomeric ADH-F variant was shown to be more thermally stable (Table 1).

It is noteworthy to examine the differences between global and local analyses of this protein. The offered Upside MD data in this study are a general global examination of the behavior of the protein and do not provide comprehensive insight into the local changes resulting from mutation. For example, both in the case of the number of hydrogen bonds and the free energy states of the protein, there might be slight local differences in the values that could result in important adaptations, whereas the data provided only covers the sum of all hydrogen bonds and the total free energy state of the protein. Nevertheless, the data presented on the monomeric and dimeric fluctuations of the radius of gyration indicates that the local mutation has exerted a global impact on the functionality of the protein. This ties into the comparison between

the local and global natural selection acting on this enzyme, as the specific nature of the mutation, and the dimeric differences indicate that both types of selection have happened. Unfortunately, the performed quantum simulations did not add any meaningful insight into the local adaptations as they failed to replicate the entire structure of the active site effectively due to computational inefficiency.

The atomistic MD simulation provided a more local analysis of the fluctuations of the protein structure of the two dimeric variants over time. The most significant changes between the dimeric structure of the ADH-S and ADH-F variants over the course of the 100ns atomistic simulation was shown to be in the vicinity of the mutated residue (residue 192) (Figure 2). This supports the idea that a singular local mutation in this protein has led to both local and global differences in the behavior and stability of the protein.

The approximate 20-residue distance of the site of the mutation (residue 192) and the active site, and its outward orientation in each of the monomers (facing the surrounding area in close proximity to the space between the monomers), rise multiple possible explanations for the impact of this mutation on overall protein function. The possibility exists that the added steric hindrance of Threonine in comparison to Lysine due to the more complex structure of Threonine is responsible for the relative instability of the ADH-F variant. Nevertheless, this does not explain the sharp contrast between the impact of the mutation between the dimeric and monomeric analysis, as it was shown that the mutation stabilizes the ADH-S variant in the dimeric state, and the ADH-F variant in the monomeric state.

Another possibility is the role of hydrophobicity in the differences between the two variants. While the Upside MD simulations did not cover water molecules, the inclusion of water molecules in the atomistic simulation and the radical change in the behavior of the residues close to the point of mutation (residue 192) indicates that the hydrophobic interactions could also play a role in the impact of the mutation on the enzyme.

The difference in the denaturation temperature of the two side chains between the experimental and computational analysis could be a result of computational inefficiencies and oversimplification of the forcefield utilized in Upside MD simulations. Nevertheless, the findings in this project provide a proof of concept for the usage of computational methodologies for the analysis of subtle biological phenomenon ranging from genetic mutations, enzymatic function and protein stability.

The presented hypothesis of the existence of measurable differences due to the single mutation at residue 192 was supported by the data and the prediction was validated through both the Upside MD and atomistic MD simulations. In conclusion, this study offers a comprehensive computational analysis of the impact of a mutation on the structural stability of a highly investigated protein. Future studies can be directed to analyze the specific local adaptations of the protein through other simulations that require more computational power.

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