Influence of Temperature on the Catalytic Function of Phosphoenolpyruvate Carboxykinase

by

Benjamin Morrow

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Author's Declaration:

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract:

Enzymes are well known for their importance in biological systems. One of these enzymes, PEPCK, has been well studied and characterized over the years. Successful catalysis in PEPCK is very dependent on mobile loop elements within the catalytic cleft folding properly. One loop, the Ω-loop, has been found to regulate access to the catalytic cleft of PEPCK by acting as a lid. Without this loop, PEPCK became inactive. When the Ω-loop was disrupted by an amino acid mutation, the stability of this loop was affected, resulting in a decrease in the loops ability to form the lid complex. This had the consequence of decreasing catalytic completion, because the reactive enolate formed during catalysis was unprotected from solvent, resulting in unintended protonation. Temperature is a well-studied variable that influences the rate of catalyzed and uncatalyzed reactions. Typically, the rate of an enzyme catalyzed reaction will increase in a logarithmic fashion in response to an increase in temperature. Temperature is also observed to affect the stability of enzymes, resulting in potential effects on the observed rate that are independent of the direct effect of temperature on the catalyzed reaction. To date, the influence of temperature on the PEPCK catalyzed reaction has not been assessed. This study investigates the effect of temperature on the stability of the enzyme, the rate of the various catalyzed reactions, and the impact of temperature on the stability of the Ω-loop, and the interpretation of those effects in light of what is known about the importance of loop motion in the catalytic functioning of the enzyme. Data from the kinetic studies of WT rcPEPCK and A467G PEPCK show that as expected, the rate of the catalyzed reaction increases as temperature increases, and that the stability of the Ω-loop in its closed, active state is affected at higher temperatures in A467G PEPCK. Surprisingly, this was not observed in the WT rcPEPCK

enzyme. Surprisingly, the resultant activation energies determined for the A467G variant of PEPCK suggests that the forward direction of catalysis is less favourable than the reverse direction, opposite of what was previously hypothesized. While the Arrhenius equation predicts a linear response in reaction rates relative to temperature change, some of the catalyzed reactions were exhibited to deviate from linearity with a break at approximately 41°C. A rationale for the observed deviation is discussed in terms of the recently proposed equilibrium model of enzyme temperature dependence.

Acknowledgements:

I would like to thank my parents, Paula and George, and my sister Brandy. Without their constant support I would not be where I am right now.

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List of Abbreviations

PEPCK – Phosphoenolpyruvate carboxykinase

WT rcPEPCK – Wild-type rat cytosolic phosphoenolpyruvate carboxykinase

GTP – Guanosine-5’-triphosphate

GDP – Guanosine-5’-diphosphate

ADP – Adenosine-5’-diphosphate

DTT – 1,4-dithiolthreitol

TCEP – Tris-2-carboxyethyl-phosphine

OAA – Oxaloacetic acid

PEP – Phosphoenolpyruvate

IPTG - Isopropyl β-D-1-thiogalactopyranoside

HEPES – 4-(2-hydroxyethyl)-1-poperazineethanesulfonic acid

TIM – triose phosphate isomerase

MDH – Malate dehydrogenase

LDH – Lactate dehydrogenase

PK – Pyruvate kinase

NADH – Nicotinamide adenine dinucleotide

ASU – Asymmetric unit

Introduction:

1.1: Enzymes and Enzyme Catalysis

It is well appreciated that chemical reactions are essential for the functioning of cells and organisms.[36] The majority of these necessary reactions occur at intrinsic rates that are orders of magnitude slower than what is required to maintain biological activity.[36] As such, most chemical reactions occurring in living matter must be catalyzed.[36] The largest category of biological catalysts are the protein-based macromolecules known as enzymes.[36] Enzymes exhibit a great diversity in their primary sequences and ultimately their three-dimensional folds, resulting in many unique categories of enzymes that span the requirements for the various chemical reactions that are necessary in diverse biological systems.[36]

Unlike non-protein based catalysts, enzymes exhibit the property of being highly specific.[36] Typically, enzyme selectivity is such that they will recognize only a single or narrow range of specific substrates to convert into a product.[36] In general, enzyme catalysis follows the cycle where substrates bind in the active site of the enzyme, a location that is typically identified as a cleft or depression in the enzyme surface.[36] It is at this location where through various mechanisms, the enzyme facilitates the lowering of the chemical activation energy barrier, resulting in an increase in the observed rate of conversion of substrates to products.[36]

Historically, several theories have existed that attempted to explain the process of a substrate binding to an enzyme. The first was the lock and key hypothesis that was first postulated by Emil Fischer in 1894.[12] Dr. Fischer had hypothesized that an enzyme and

substrate fit together like a lock (the enzyme) and a key (the substrate).[12] For an enzyme-substrate complex to form, only a specifically sized substrate would fit into the active site of the enzyme.[12] If a substrate did not fit the static shape requirement of the enzymes active site, it would not be able to bind, and the enzyme would not undergo catalysis.[12]

This hypothesis was widely accepted by scientists around the world until 1958 when Daniel Koshland introduced the induced-fit hypothesis.[20] Dr. Koshland hypothesized that not all experimental evidence could be explained by the lock and key hypothesis.[20] Dr. Koshland instead postulated that enzymes do not exist as a single static conformation, but rather the final key-lock state of the enzyme occurs after structural changes to the enzyme that are induced by substrate binding.[20] This change is what allows a transition from a non-active to an active state in the enzyme.[20] In more recent years a different theory was suggested, conformational selection, which hypothesized that rather than a substrate inducing a structural change in an enzyme, an enzyme already exists in multiple conformational states, and a substrate will bind to the key-lock state preferentially compared to all other conformational states.[2,33]

Evidence suggests that enzymes with lid-gated active sites, such as phosphoenolpyruvate carboxykinase (PEPCK), follow the induced-fit model.[33] PEPCK will differ in its structure during an open state, with no substrates bound, fitting a more thermodynamically favourable conformation to aid in the binding of substrates to form the key-lock conformation.[33] Once the key-lock conformation of the enzyme is formed and the enzyme is in the closed state, catalysis occurs and an intermediate enolate is formed, committing the

enzyme to catalysis.[33] After conversion of the enolate to the product, PEPCK will transition back into an open conformation allowing for product release and the next catalytic cycle.[33]

1.2: Steady-State Enzyme Kinetics

Leonor Michaelis and Maud Menten are often viewed as the founders of modern enzyme kinetics.[26] One of the key insights they had was to control and normalize the conditions that enzymes experienced during a reaction so that measured data would be consistent.[26] This included variables such as pH, enzyme concentration, and temperature, as well as having the initial rates of the enzyme measured as a reference point so that abnormalities in collected data could be identified and explained.[26] Their work led to what is now known as the Michaelis-Menten equation, a key equation for enzyme kinetics:

$$\upsilon = \frac{V_{max}[S]}{K_M + [S]}$$

One of the conditions they mentioned was temperature, as it can be an extremely important factor when it comes to measuring the catalytic rate of enzymes.[26] At high enough temperatures, the activity of an enzyme can decrease over time, so high temperatures would want to be avoided when performing steady-state enzyme kinetics.[26]

1.3: Biological Function of Phosphoenolpyruvate Carboxykinase

The functional studies that compose my thesis focus on the characterization of the PEPCK enzyme. Mostly known as a gluconeogenic enzyme, PEPCK is involved in the process of gluconeogenesis and glyceroneogenesis by removal of oxaloacetic acid (OAA) from the citric acid cycle to form phosphoenolpyruvate (PEP) and CO_2.[15,37] Because of PEPCK's role in

gluconeogenesis, it is also key in maintaining blood glucose levels in fasting individuals and fasting hyperglycemia in non-insulin-dependent diabetes.[15] Because of this, diabetes research has been one of the main focuses of PEPCK in the past. More recently PEPCK has been found to have impacts in TB infection, cancer, and aging.[23,25,38] What is generally regarded as the forward physiological direction of catalysis involves the decarboxylation of OAA to form an intermediate enolate.[32] This enolate is then phosphorylated by GTP to form PEP, GDP, and CO_2 (Figure 1.1).[32] This chemical reaction is freely reversible and can occur in the opposite direction through the dephosphorylation and subsequent carboxylation of PEP to form GTP and OAA.[32]

1.4: Catalytic Function of PEPCK

PEPCK is an enzyme that has been highly characterized and is regarded as the first committed step of gluconeogenesis. PEPCK is ubiquitously expressed in organisms from all kingdoms of life.[14] It is classified in two general classes, ATP-dependent and GTP-dependent, based upon the nucleotide used in the chemical conversion.[14] Within the GTP class of enzymes there is also a mitochondrial and cytosolic form which are the products of two different nuclear encoded genes.[14] In my thesis I will be solely focusing on and discussing GTP-dependent PEPCKs, and more specifically the cytosolic form from the rat (WT rcPEPCK). In addition, I will investigate an A467G variant of the WT enzyme.

Structurally, PEPCK is found to be a globular enzyme that can be subdivided into two separate regions; the N-terminal lobe (residues 1-259) and the C-terminal lobe (residues 260-622), a fold unique to PEPCK enzymes (Figure 1.2).[10] PEPCK's fold places the active site in a cleft between the two separate lobes.[10] The reaction catalyzed by PEPCK is freely reversible. In what

is typically considered the forward, physiologically relevant direction, the catalyzed reaction involves the decarboxylation and subsequent phosphorylation of OAA to form PEP and CO_2 (Figure 1.1).[32] *In vivo*, most PEPCKs are thought to preferentially perform the decarboxylation of OAA to form PEP, but some enzymes from some sources have been postulated to have a predominant biological role of fixing CO_2 by carboxylating PEP.[6,8,29] This later reaction is mostly favoured by parasitic eukaryotes.[6,8,29]

In GTP-dependent PEPCKs, catalysis requires the presence of two divalent metal cations, typically Mn^{2+} and Mg^{2+}, to function properly.[32] In this regard, Mn^{2+} operates as a metal cofactor having the ability to bind to the enzyme in the absence of substrates, and Mg^{2+} is required to form a metal-nucleotide complex, which serves as the true substrate of the reaction.[32]

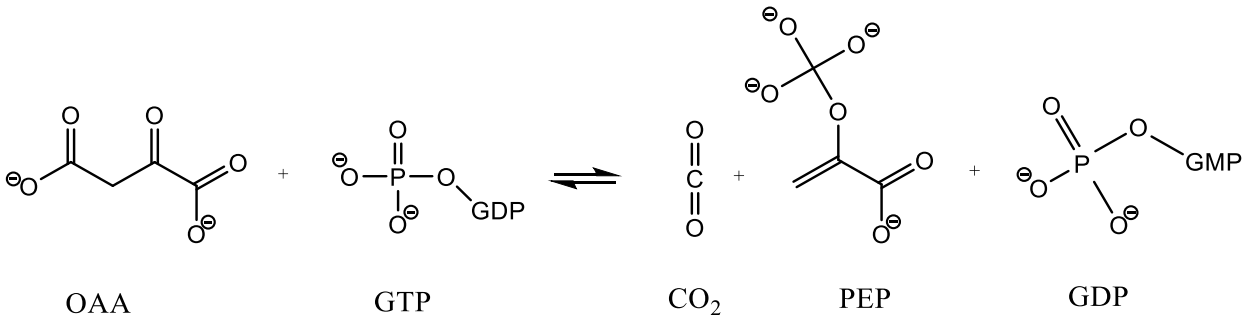

Figure 1.1: Reaction scheme for the freely reversible catalysis of OAA to PEP by PEPCK through decarboxylation of OAA and subsequent phosphorylation by GTP.

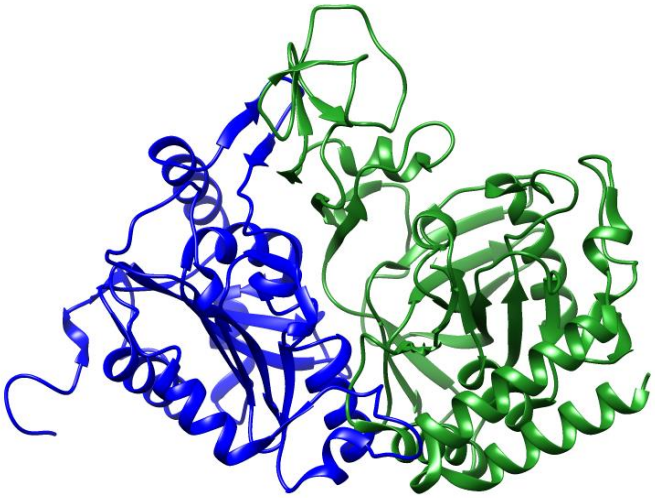

Figure 1.2: The three-dimensional structure of WT rcPEPCK. The N-terminal region is shown in blue (residues 1-259) and the C-terminal region is shown in green (residues 260-622), showing the unique folding pattern of PEPCK. The structural model was generated using PDB ID#2QEW and UCSF Chimera.

1.5: Reaction Mechanism of PEPCK

As previously stated, the PEPCK catalyzed reaction is reversible. In what is commonly known as the reverse direction, the formation of OAA and GTP results from the generation of an enolate intermediate through phosphoryl transfer from PEP to GDP followed by the carboxylation of the enolate by CO_2.[32] The forward direction is simply the opposite of this, where OAA is decarboxylated and the resultant enolate is subsequently phosphorylated by GTP to form PEP and CO_2.[32] Of the two directions of catalysis, the forward direction is typically hypothesized to be favoured over the reverse direction *in vivo*.[16,17,37] The forward direction of catalysis is also postulated to be the predominant direction due to the high apparent K_M for

HCO_3^-/CO_2.[17] In addition, due to the nature of the chemical reaction, specifically the stepwise mechanisms that moves through an enolate intermediate, PEPCK has also been observed *in vitro* to catalyze the decarboxylation of OAA to form pyruvate in the presence of GDP instead of GTP, due to the lack of a phosphoryl donor to the enolate.[16] The physiological relevance of this reaction, if any, is presently unknown.

The complexity of catalysis can sometimes lead to unintended results. In the forward direction of catalysis in PEPCK, an enolate intermediate is created from the decarboxylation of OAA before PEP is formed through its phosphorylation.[33] This intermediate is highly reactive, which can lead to problems completing the reaction. Normally this enolate would be phosphorylated by GTP to form PEP.[33] If solvent enters the catalytic cleft before phosphorylation occurs, protonation of the enolate is favoured over phosphorylation and the unintended side product pyruvate is formed instead.[16] Not surprisingly, through evolution of the PEPCK fold, it has acquired a mechanism to prevent this unintended protonation from occurring. Found within the catalytic cleft of PEPCK, a loop called the Ω-loop has been demonstrated to act as a lid to control access to the cleft, and thereby in normal functioning opening to allow substrate access and product release but closing upon enolate formation to ensure phosphoryl transfer rather than the undesired enolate protonation.[16]

1.6: Catalytic Enzyme Loops

Loops are an often-overlooked element of an enzymes structure as most attention is paid to the regular elements of secondary structure, namely β-sheets and α-helices and how they interact to generate the overall three-dimensional protein fold. A loop is usually defined as

an element of the primary structure that serves as a connector between the α-helices and β-sheets through turns in their structure.[27] Some loops can be found secluded with little direct interaction with an enzyme, whereas other loops can be directly involved in the catalytic function of an enzyme through their involvement in the formation of the active site.[27] In general, due to their non-regular structure, loops can be found in two different states: freely floppy and disordered with no single highly populated conformation, or alternatively they can be more rigid and ordered sampling a single or narrow range of conformational states.[27] In addition, it is generally observed that in enzymes many loops will transition between different conformational states during catalysis, allowing some loops to serve important enzymatic functions.[27]

Based upon a diversity in structure, loops found in enzymes can have many different potential roles. Loops with catalytic functionality are usually found closer to the catalytic cleft, while loops with more general functions can be found further from this area near the surface of the enzyme.[27] While historically loops have been an often-overlooked element of structure in enzymes, more recent studies have demonstrated that loops play key roles in the function of many diverse types of enzymes catalyzing a range of different chemistries.[11,17,22,24] An example of a common class of loops that are essential to a diverse set of enzyme activities are the class of loops known generally as Ω-loops. Typically, it has been observed that Ω-loops can act as lids, which in turn can modulate active site accessibility from the bulk solvent.[11] In general, Ω-loops are identified as a polypeptide segment with an exaggerated loop structure in the shape of the Ω symbol, resulting from close spatial proximity of the exit and entry points of the loop relative to the rest of the globular structure.[11] Ω-loops are also known to lack the presence of repeating

backbone dihedral angles or regular hydrogen bonding patterns typical of regular secondary structural elements.[11] Mutagenesis experiments investigating the function of Ω-loops have shown that these loops are important for diverse processes ranging from substrate binding to catalytic function.[11,16,17]

1.7: The Role of the Active Site Ω-Loop Lid in PEPCK Function

There are three dynamic active site loops that have previously been characterized to have functional roles in PEPCK mediated catalysis.[4,16,17,32] These are the R-loop, the P-loop, and the Ω-loop.[4,16,16,32] The R-loop (residues 85-92), has been shown to interact directly with OAA.[32] This loop is also postulated to work in concert with the Ω-loop lid and facilitate its closure over the active site through a latch mechanism between the residues in the two mobile loops.[17] The P-loop/Kinase 1a motif (residues 284-292) is the canonical motif found in most kinases that is involved in contacts with the beta and gamma phosphates of the nucleotide substrate and facilitates phosphoryl transfer.[4,17] Lastly, as previously mentioned, the Ω-loop (residues 464-474) acts as a lid and controls access to the active site cleft, allowing the substrates and products of the chemical reaction access and exit to/from the active site, respectively.[16] Structural data have shown that the Ω-loop transitions from an open/disordered to a closed/ordered state over the active site of PEPCK during catalysis.[17] The specific importance of this transitioning was recently demonstrated when it was shown that impacting the ability of the Ω-loop to perform this transition resulted in a decrease in the rate of OAA to PEP conversion by 26% compared to the WT rcPEPCK enzyme.[16] This loss in catalytic conversion was postulated to be due to the decreased stability of the closed Ω-loop lid, resulting in water

protonating the reactive enolate intermediate that is formed during the process of catalysis (Figure 1.4; Figure 1.5).[17] This protonation results in the formation of pyruvate, a non-productive molecule for PEPCK catalysis, through this undesired chemical reaction.[17]

As mentioned previously, it is important to note that the Ω-loop lid is not exclusive to the structure of PEPCK and can be found in a diverse range of enzymes, such as triose phosphate isomerase (TIM), tryptophan synthase, and rhizomucor miehei triglyceride lipase.[3,9,19,21] While these enzymes all catalyze different chemical transformations using different protein folds, the Ω-loop in all cases performs the same function as in PEPCK; acting as a lid to protect a reactive intermediate from unwanted chemistries during catalysis.

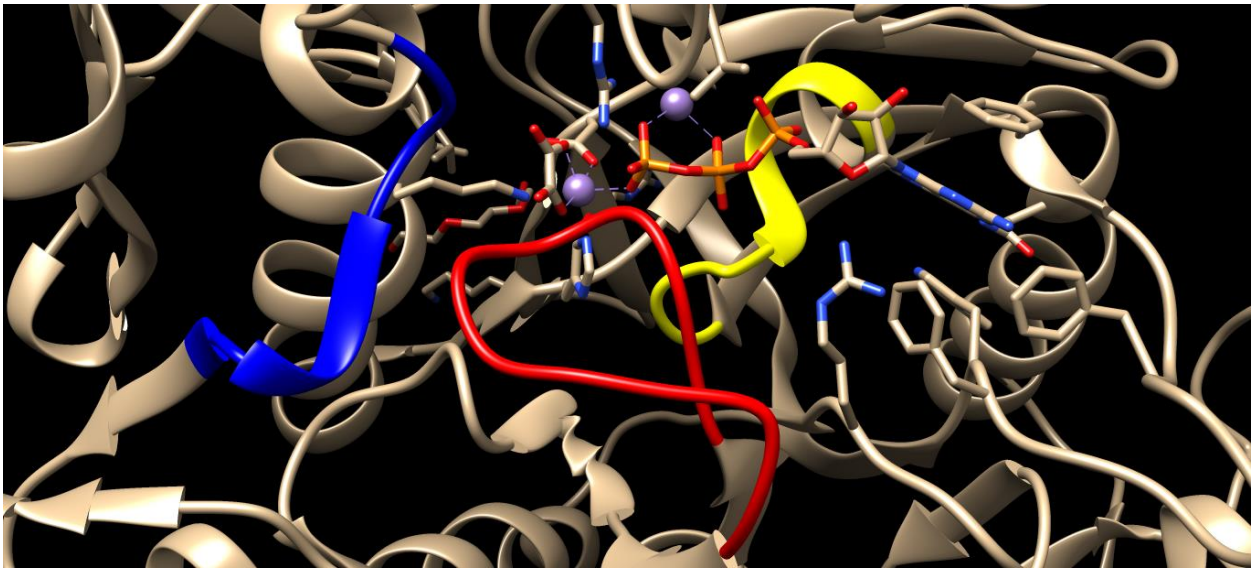

Figure 1.3: Active site of the WT rcPEPCK enzyme in complex with oxalate and GTP showing the important catalytic loops and their position required for PEPCK catalysis. The R-loop, P-loop, and Ω-loop are highlighted in blue, yellow, and red respectively. The image was generated using UCSF Chimera and PDB ID#3DT2.

1.8: The A467G Mutant of PEPCK

Previous interest in the functional role of the Ω-loop in PEPCK catalysis led to the creation of an A467G variant of PEPCK (Figure 1.5).[16] It was hypothesized that this would increase the entropic penalty against the closing of the Ω-loop lid, resulting in destabilizing the closed, ordered state.[16] This in turn would lead to catalytic defect in the various reactions catalyzed by the enzyme.[16] The structure A467G-Mn^{2+}-oxalate-Mn^{2+}GTP contained two molecules in the asymmetric unit (ASU), where molecule B had an open lid conformation.[16] Molecule A however had a partially closed lid conformation at 70% occupancy (Figure 1.4).[16] In comparison to the WT rcPEPCK enzyme, which had a closed lid conformation occupancy of 100%, this structure only had an occupancy of 35% overall.[16] This showed that the A467G mutation did indeed appear to destabilize the closed state of the Ω-loop lid, as previously hypothesized.[16]

In the forward direction of catalysis, it was shown that this mutation resulted in a 14-fold higher K_M value for OAA than in the WT rcPEPCK enzyme.[16] This mutation also resulted in a reduction in k_{cat} by 26% compared to the WT rcPEPCK enzyme, leading to a reduction in catalytic efficiency (k_{cat}/K_M) to 1.9% of the WT rcPEPCK value.[16] In the reverse direction of catalysis, it was shown that this mutation resulted in a decrease in the K_M value for PEP by 21% compared to the WT rcPEPCK enzyme.[16] This mutation also resulted in a reduction in k_{cat} by 5%, leading to a reduction in catalytic efficiency by a factor of 4 compared to the WT rcPEPCK enzyme.[16] Compared to the WT rcPEPCK enzyme a reduction in the catalytic rate, catalytic efficiency, and higher K_M values for the substrates showed a clear reduction in catalytic output

from the enzyme. Since the Ω-loop was the location of the A→G mutation it was safe to conclude that the Ω-loop became destabilized, preventing proper closure over the active site and formation of the lid complex.[16] These results were consistent with the hypothesis that the Ω-loop lid is important for catalytic function.

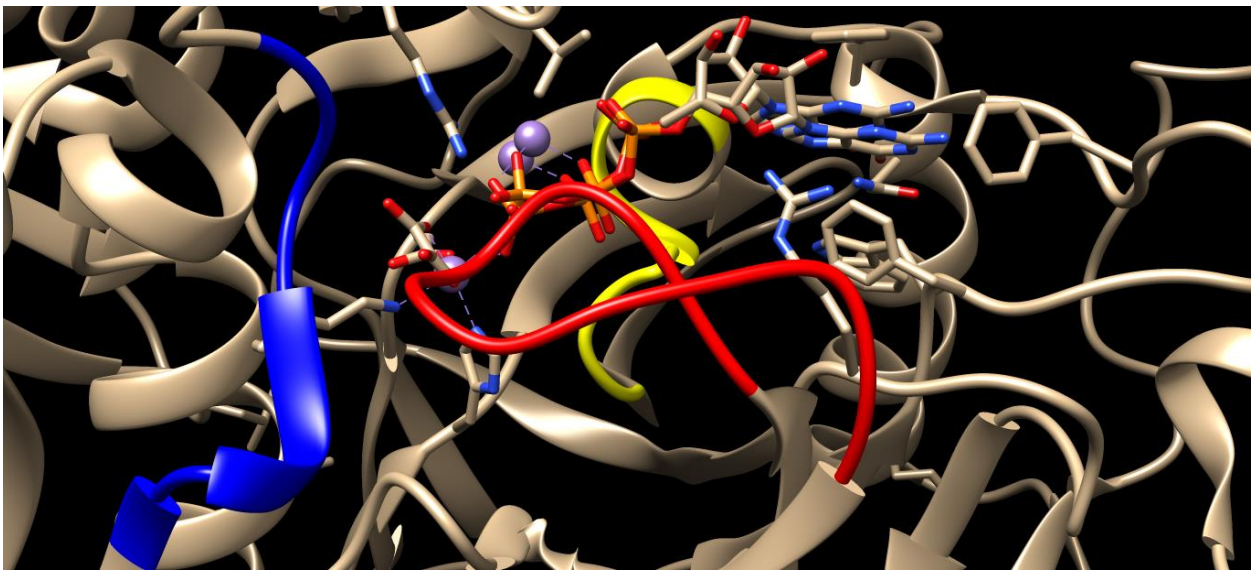

Figure 1.4: The active site region of the A467G PEPCK enzyme in complex with Oxalate and GTP with a partial Ω-loop lid conformation, highlighting the R-loop (blue), P-loop (yellow), Ω-loop (red). This image was generated using UCSF Chimera and PDB ID#3MOF.

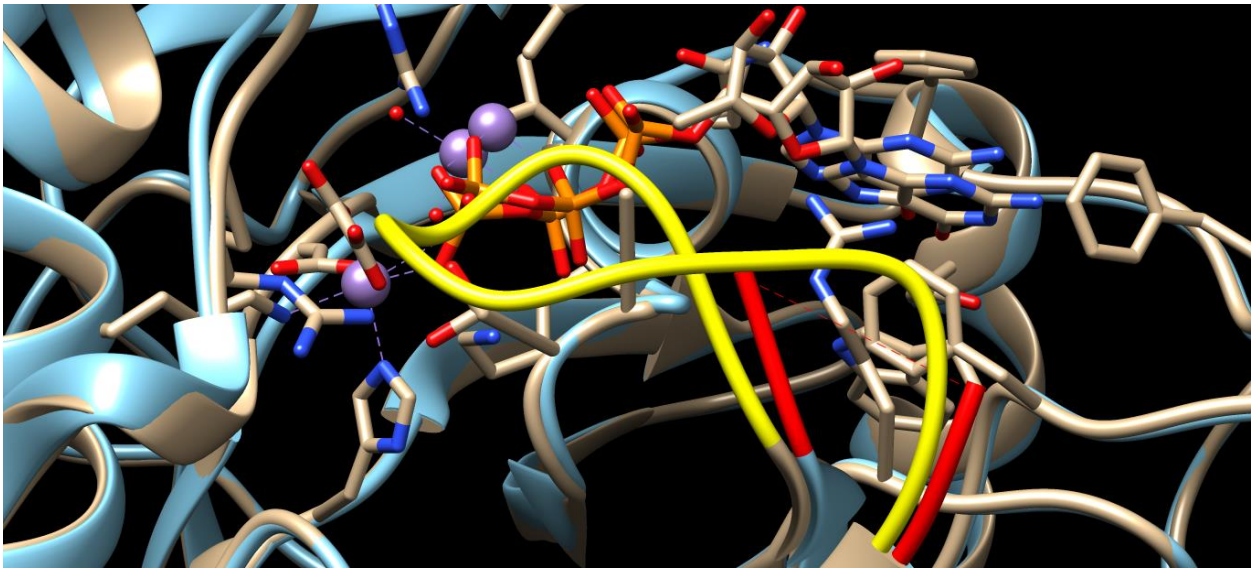

Figure 1.5: The active site region of the A467G PEPCK enzyme highlighting the Ω-loop from molecule A and B in the ASU superimposed over each other. In red the Ω-loop is in the open conformation and in yellow the Ω-loop is in the closed conformation. This shows the Ω-loop lid conformations importance in proper alignment to prevent unwanted chemistries in the active site. The image was generated using UCSF Chimera and PDB ID#3MOF.

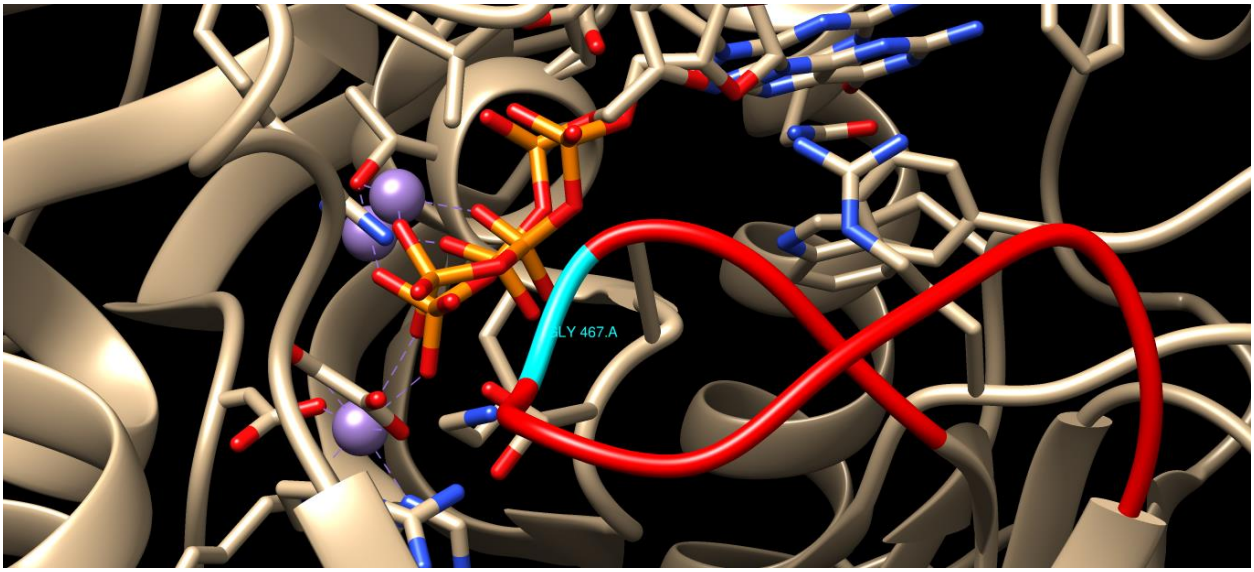

Figure 1.6: A467G PEPCK enzyme highlighting the Ω-loop during closed (red) conformation. Highlighted in cyan is residue 467, showing the alanine to glycine mutation. Structure was generated using PDB ID#3MOF through UCSF Chimera.

2.1: Temperature Effects on Enzyme Structure

How temperature influences an enzyme is a key factor in many biological systems. It is well known to the scientific community that increasing temperature will affect enzymes in two ways: the catalytic rate of the enzyme will increase, and at a high enough temperature that is enzyme dependent, denaturation will occur.[34] Denaturation of an enzyme is typically regarded as a two-step process. First, the enzyme will undergo a conformational change leading to a partially folded state, which is reversible in some enzymes.[34] If the temperature continues to increase, a second, irreversibly denatured state of the enzyme will be formed.[34] Since enzymes are only marginally stable in the folded state, most enzymes are highly susceptible to temperature induced denaturation.

2.2: Enzyme Stability

With many different classes and families of enzymes there will be those that are more ordered and those that are more disordered at a modest temperature (e.g. 25°C). Ordered enzymes are hypothesized to be more thermostable as temperature increases, whereas more disordered enzymes are hypothesized to be more stable at lower temperatures.[27,35] This has led to enzymes being classified into different groups based upon their host organisms ability to survive at different optimal temperatures (psychrophilic (<15°C) thermophilic/hyperthermophilic (41-122°C) and mesophilic (20-45°C)).[27]

It is hypothesized that loops are involved in the ability of enzymes to function in different temperature regime and their inherent stability at these temperatures.[28] Freely

mobile and flexible surface loops are postulated to be involved in the stability of an enzyme by offsetting the activation entropy prior to successful catalysis.[1] More specifically, during catalysis a substrate will bind in the active site, changing the active site from a disordered state to an ordered state to allow a reaction to occur.[1] This process is thermodynamically unfavourable for an enzyme and must be offset to bring the enzyme back into a thermodynamically favourable state.[1] One way this thermodynamic compensation could occur is that as the enzyme becomes ordered around the active site during catalysis, flexible, mobile surface loops can become increasingly disordered and in doing so keep the enzyme within a thermodynamically favourable state while still allowing for the ordering of the active site that is required for the catalytic reaction to occur.[1]

2.3: Influence of Temperature on Catalytic Function

As described above, temperature has been a well-known factor affecting the structural integrity of enzymes and proteins in general.[26] Extrapolating from basic chemical kinetics also tells us that increasing the temperature will increase the observed rate of an enzyme catalyzed reaction. However, in contrast to a non-enzyme catalyzed reaction at high enough temperatures, denaturation of the enzyme will occur resulting in a rapid decrease in the observed rate.[34] Based upon this phenomenon, it is known that every enzyme will have its own characteristic response to temperature resulting in a temperature optimum which is the temperature where the observed catalytic rate of the enzyme is at its highest.[34] Beyond this temperature consistent with the preceding discussion, the structural integrity of the enzyme will begin to fail, eventually leading to complete denaturation and the corresponding loss of

enzymatic activity.[34] One of the most established methods for measuring the effect of temperature on the catalytic rate of an enzyme is through application of the Arrhenius equation.

$$k = Ae^{\frac{-E_a}{RT}}$$

Through the analysis of the temperature (T) dependence of the catalyzed rate of the reaction (k), application of the Arrhenius equation can be used to calculate the activation energy (E_a) of the enzymatic reaction.[30] Another factor in measuring the influence of temperature on an enzymes catalytic rate is the intrinsic thermal stability of the enzyme. Thermal stability of an enzyme can be measured through the determination of thermal inactivation rate constant, k_{inact}.[30] These two factors together form a model that has been widely used to evaluate the influence that temperature will have on the enzyme activity.[30]

As is demonstrated in the above equation, the Arrhenius equation predicts a logarithmic increase in rate as a function of temperature. However, typically enzymes will deviate from this behaviour at elevated temperatures, consistent with the ideas presented previously, at which time the catalytic rate will decrease again, in violation of the behaviour predicted by the equation.[30] This anomaly has historically been attributed to temperature induced denaturation (discussed above), but more recently an alternative explanation called the Equilibrium model has been suggested to more fully explain this phenomenon.[30]

2.4: The Equilibrium Model

The Equilibrium model was put forth to account for the decreasing activity at high temperatures observed in enzymes by hypothesizing that an intermediate inactive form of an enzyme that is in equilibrium with the active form of the enzyme is present.[7] With the old model it is hypothesized that the enzyme goes from a folded, active form to a completely inactive and denatured form at higher temperatures resulting in the observed rate decrease with increasing temperature. In this new model it is proposed that an equilibrium exists between the active and intermediate, inactive state of the enzyme and this equilibrium shifts to the intermediate state as temperature increases.[7] If the temperature continues to increase, at some temperature the intermediate inactive enzyme will transition into an irreversible inactive denatured form.[7]

The investigators that put forth this theory think that it provides a better explanation of the observed data. These points of evidence for this model are that if an enzyme transitioned directly from an active form to an inactive denatured form at high temperatures, one could hypothesize that an enzyme's activity from a range of temperatures would increase indefinitely until the temperature at which it completely denatures into this inactive form, at which point the activity would immediately go to zero.[7] In the new equilibrium model, it is hypothesized that as is observed in the primary data, enzyme activity will increase as a function of temperature to a maximum, the temperature optimum, at which time higher temperatures will result in enzymatic activity decreasing at each subsequent temperature increase rather than complete loss of activity as predicted by the old model.[7] This phenomenon is again, suggested

to be the result of more of the active form of the enzyme converting to the intermediate, inactive form as the equilibrium shifts in that direction as a function of temperature.[7] Subsequently the intermediate inactive form can be induced to convert into the fully denatured inactive form if temperature continues to increase, leading to complete loss of catalytic activity.[7]

Hypothesis:

3.1: Project Overview and Hypotheses

The general question that my thesis will address is what influence temperature has on the catalytic function of PEPCK. It is hypothesized that consistent with the Arrhenius equation, increasing temperature will increase the observed catalytic rate of the enzyme. Previously it has been postulated that the reverse direction of PEPCK catalysis, in which PEP is converted to OAA< is rare in living cells. This is because the forward direction of catalysis is hypothesized to be marginally thermodynamically favoured over the reverse direction *in vivo*.[16,17,37] Through the temperature analysis of the reaction rate in both directions this thesis will attempt to determine the effect that temperature has on the activation energy required for PEPCK catalysis in each direction and provide insight into this prior conclusion. The second goal of my research is to investigate the impact of temperature on Ω-loop function due to the essential nature of the lid complex it forms during catalysis. Previous work on the A467G PEPCK enzyme has already established that destabilizing this loop affects the formation of the lid complex and it is of interest in my work to see how temperature may influence the Ω-loop and its role as a lid in PEPCK function. To address this question, I will compare the temperature dependence of the WT rcPEPCK to the A467G PEPCK variant. It is my hypothesis that higher temperatures will lead to a more greatly destabilized Ω-loop in the A467G enzyme as compared to WT which in turn will result in an increasing detrimental effect of the A→G substitution with increasing temperature.

Materials and Methods:

4.1: WT rcPEPCK Construct

The WT rcPEPCK gene encoding the 622 residue PEPCK enzyme containing an N-terminal His6 tag was previously cloned into the pE-SUMOstar vector that contains a His6-SUMOstar fusion tag using the methodology provided by the supplier (Lifesensors).[18] This vector utilizes the T7 RNA polymerase promoter for production of the enzyme, while using kanamycin resistance for plasmid selection.

4.2: IPTG Expression of WT rcPEPCK

WT rcPEPCK was expressed by the *E. coli* cell strain BL21 (DE3) with the pE-SUMOstar vector using isopropyl-β-D-thiogalactopyranoside (IPTG) induction. For expression, cells were grown overnight at 37°C at 180rpm in four 25mL flasks of LB media containing a concentration of 50µg/mL of kanamycin antibiotic. Overnight cultures were diluted 40-fold in fresh LB media containing kanamycin antibiotic at a concentration of 50µg/mL and grown until an OD_{600} of 1.0-1.4 was obtained. Cells were then induced with 1mM of IPTG and grown for another 4 hours at 37°C. The culture was then centrifuged at 7000g for 20 minutes, and the resultant pellets were stored at -80°C.

4.3: Purification of WT rcPEPCK

All steps were carried out at 4°C. The frozen pellets were removed from their tubes and resuspended in Buffer 1 (Table 4.1) to a volume of 50mL. Cells were then lysed twice at 20,000

PSI using a French Pressure Cell that was pre-incubated at 4°C. Lysed cells were centrifuged at 10,000g for 30 minutes to pellet the insoluble fraction. The supernatant was then removed and added to a Qiagen Nickel-NTA resin column pre-equilibrated with 250mL of Buffer 1 (Table 4.1). The column was rocked for 1 hour to allow mixing of the supernatant and nickel resin. The nickel resin was washed with Buffer 1 (Table 4.1) until the flow through A_{280} was less than 0.1. Buffer 2 (Table 4.1) was used to elute the protein off of the nickel resin into fractions. The fractions had their A_{280} absorbance checked to determine which fractions had protein. The fractions with protein were then concentrated with a 50mL Amicon Stirred Cell Nitrogen Spin Concentrator with a 30kDa Amicon HA-filter to a final volume of approximately 8mL. The concentrated protein was then buffer exchanged on a Bio-Rad P6DG polyacrylamide gel column pre-equilibrated in 300mL of Buffer 3 (Table 4.1), which has a 1cm bed of Chelex-100 added to chelate contaminating metal ions. Fractions were taken and had their A_{280} absorbance checked to determine which had protein. The protein was then incubated with SUMO-protease overnight at 4°C to cleave the SUMO-fusion tag. The protein was then added to the Qiagen Nickel-NTA resin column again that was pre-equilibrated with 250mL of Buffer 3 (Table 4.1). This column was then rocked for 1 hour to bind the SUMO-fusion tag and His-tagged SUMO protease. The protein was allowed to flow through the column and collected in fractions. The nickel resin was then washed with Buffer 3 (Table 4.1) into fractions and all fractions containing protein were concentrated to 8mL with the Amicon Stirred Cell Nitrogen Spin Concentrator with a 30kDa Amicon HA-filter. The protein was buffer exchanged into Buffer 4 (Table 4.1) using the Bio-Rad P6DG polyacrylamide gel column pre-equilibrated in 300mL of Buffer 4 (Table 4.1). Fractions of the protein were collected and concentrated down to 10mg/mL using an Amicon

Ultra-15 Ultracel-30K centrifuge filter. Protein concentration was determined using WT rcPEPCK's extinction coefficient of 1.60mL mg^{-1} and a molecular mass of 69560 Da. A molecular mass of 69415 Da was used for the A467G PEPCK enzyme. The protein was then flash frozen in liquid nitrogen in 20µL aliquots. The frozen protein was stored in a -80°C freezer. Purity of the protein was checked using SDS-PAGE.

Table 4.1: Purification Buffers for WT rcPEPCK purification protocol.

Buffer	HEPES (mM) pH 7.5	NaCl (mM)	Imidazole (mM)	Glycerol (%)	Reducing Agent
1	25	300	10	10	2mM TCEP
2	25	0	300	0	2mM TCEP
3	25	0	0	0	2mM TCEP
4	25	0	0	0	10mM DTT

5.1: WT rcPEPCK and A467G PEPCK Reverse Direction Catalysis

Substrate and nucleotide saturation conditions were determined at the lowest (14°C) and highest (60°C) temperatures for the WT rcPEPCK enzyme, and lowest (8°C) and highest (52°C) temperatures for the A467G PEPCK enzyme, by increasing the amount of substrate or nucleotide in each assay mixture until the enzymatic rate did not increase further. Each reaction

was measured at 340nm in a Cary 100 UV spectrophotometer by the oxidation of NADH at temperatures ranging from 8°C up to 60°C for the WT rcPEPCK enzyme, and 8°C up to 52°C for the A467G PEPCK enzyme. Temperatures were recorded using both the spectrophotometer temperature setting as well as a thermocouple inserted into the cuvette for accuracy. Each assay mixture contained 50mM HEPES pH 7.5, 10mM DTT, 100µM $MnCl_2$, 1mM GDP, 5mM PEP, 300µM NADH, 50mM $KHCO_3^-$, 10 units of malate dehydrogenase (MDH), and 2.5µg of PEPCK. The addition of PEPCK and MDH occurred simultaneously, and each reaction mixture was incubated at the determined temperature for a minimum of 5 minutes to ensure each mixture was fully equilibrated and each temperature was stable.

5.2: WT rcPEPCK and A467G PEPCK Spontaneous Pyruvate Reaction

Each reaction was measured at 340nm in a Cary 100 UV spectrophotometer by the oxidation of NADH at temperatures ranging from 8°C up to 61°C for the WT rcPEPCK enzyme, and lowest (8°C) and highest (48°C) temperatures for the A467G PEPCK enzyme. Temperatures were recorded using both the spectrophotometer temperature setting as well as a thermocouple for accuracy. Each assay mixture contained 50mM HEPES pH 7.5, 10mM DTT, 200µM $MnCl_2$, 4mM $MgCl_2$, 500µM GTP, 1mM ADP, 300µM NADH, 30 units of LDH, and 500µM OAA. Each assay mixture was incubated at the determined temperature for a minimum of 5 minutes to ensure each assay mixture was fully equilibrated and each temperature was stable. OAA was added to the assay last to start the reaction so that an accurate reading of the spontaneous formation of pyruvate could be measured.

5.3: A467G PEPCK Enolate Protonation Reaction

Substrate and nucleotide saturation conditions were determined at the lowest (8°C) and highest (48°C) temperatures by increasing the amount of substrate or nucleotide in each assay mixture until the enzymatic rate did not increase further. Each reaction was measured at 340nm in a Cary 100 UV spectrophotometer by the oxidation of NADH at temperatures ranging from 8°C up to 48°C. Temperatures were recorded using both the spectrophotometer temperature setting as well as a thermocouple for accuracy. Each reaction mixture contained 50mM HEPES pH 7.5, 10mM DTT, 200µM $MnCl_2$, 4mM $MgCl_2$, 500µM GTP, 1mM ADP, 300µM NADH, 30 units of LDH, 5mM OAA, and 2.5µg of PEPCK. Each assay mixture was incubated at the determined temperature for a minimum of 5 minutes to ensure each assay mixture was fully equilibrated and each temperature was stable. PEPCK was added to the assay, with OAA then being added last to start the reaction so that an accurate reading of the rate of enolate protonation forming pyruvate could be measured.

5.4: WT rcPEPCK and A467G PEPCK Forward Direction Catalysis

Substrate and nucleotide saturation conditions were determined at the lowest (8°C) and highest (52°C) temperatures for the WT rcPEPCK enzyme, and lowest (8°C) and highest (48°C) temperatures for the A467G PEPCK enzyme, by increasing the amount of substrate or nucleotide in each assay mixture until the enzymatic rate did not increase further. Each reaction was measured at 340nm in a Cary 100 UV spectrophotometer by the oxidation of NADH at temperatures ranging from 8°C up to 52°C. Temperatures were recorded using both the spectrophotometer temperature setting as well as a thermocouple for accuracy. Each assay

mixture contained 50mM HEPES pH 7.5, 10mM DTT, 200µM $MnCl_2$, 4mM $MgCl_2$, 500µM GTP, 1mM ADP, 300µM NADH, 30 units of LDH, 50µg of pyruvate kinase (PK), 5mM OAA, and 2.5µg of PEPCK. Each assay mixture was incubated at the determined temperature for a minimum of 5 minutes to ensure each mixture was fully equilibrated and each temperature was stable. PEPCK was added to the assay, with OAA then being added last to start the reaction so that an accurate reading of the forward reaction of PEP production could be measured.

Results:

The kinetic experiments for the WT rcPEPCK enzyme and the A467G PEPCK enzyme were performed to demonstrate how temperature may influence the catalytic function of each enzyme for the forward and reverse direction of catalysis, as well as the enolate protonation reaction. These experiments were also performed to show any differences in catalysis between the WT rcPEPCK enzyme and the A467G PEPCK enzyme.

6.1: Catalytic Rate Analysis of PEPCK

The WT rcPEPCK enzyme in the forward direction of catalysis, in which OAA is converted to PEP, shows an increase in the catalytic rate as temperature is increased (Figure 6.1). The A467G PEPCK enzyme in the forward direction of catalysis has an increased rate as well, with little deviation in the catalytic rate compared to the WT rcPEPCK enzyme (Figure 6.2). The non-enzymatic decarboxylation of OAA by free Mn^{2+} shows in increase in the rate of decarboxylation as temperature is increased (Figure 6.5).

As expected, The WT rcPEPCK enzyme in the reverse direction of catalysis, in which PEP is converted to OAA, also shows an increase in the catalytic rate as temperature is increased (Figure 6.3). At 41°C an inflection point can be seen, where the catalytic rate at higher temperatures has slowed and the linearity of the Arrhenius plot has shifted (Figure 6.3). The A467G PEPCK enzyme in the reverse direction of catalysis has an increased catalytic rate as well (Figure 6.4). The A467G PEPCK enzyme does not have an inflection point like the WT rcPEPCK

enzyme (Figure 6.4). The WT rcPEPCK enzyme has a two-fold increase in its overall enzymatic rate compared to the A467G PEPCK enzyme (Figure 6.3; Figure 6.4).

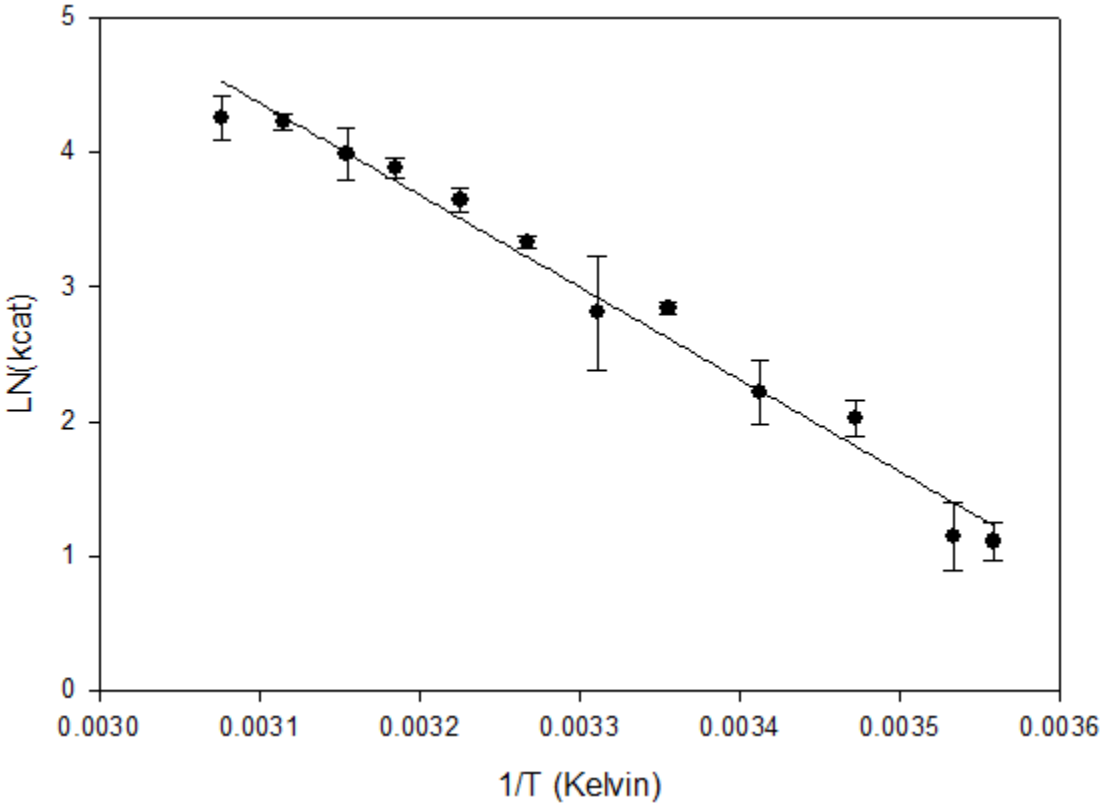

Figure 6.1: WT rcPEPCK forward direction of catalysis with a linear trend showing the relationship between temperature and catalytic rate. Temperatures range from 8°C to 52°C.

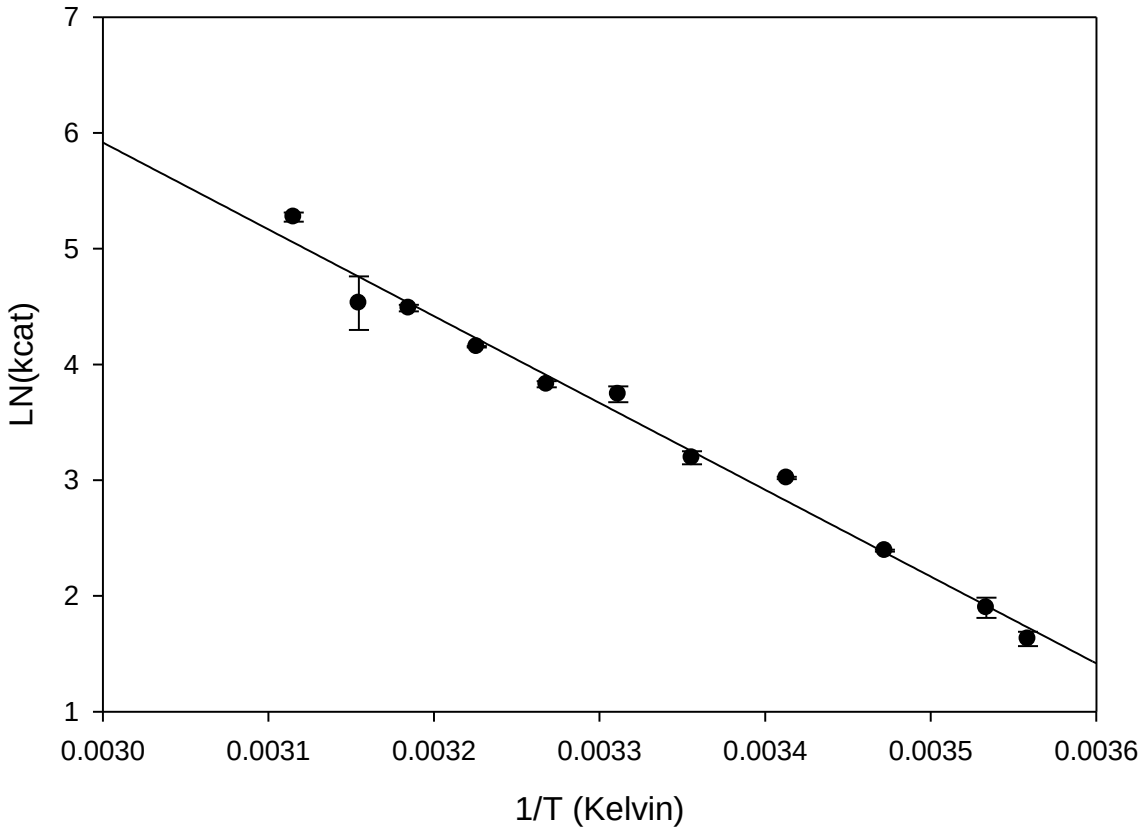

Figure 6.2: A467G PEPCK forward direction of catalysis with a linear trend showing the relationship between temperature and catalytic rate. Temperatures range from 8°C to 48°C.

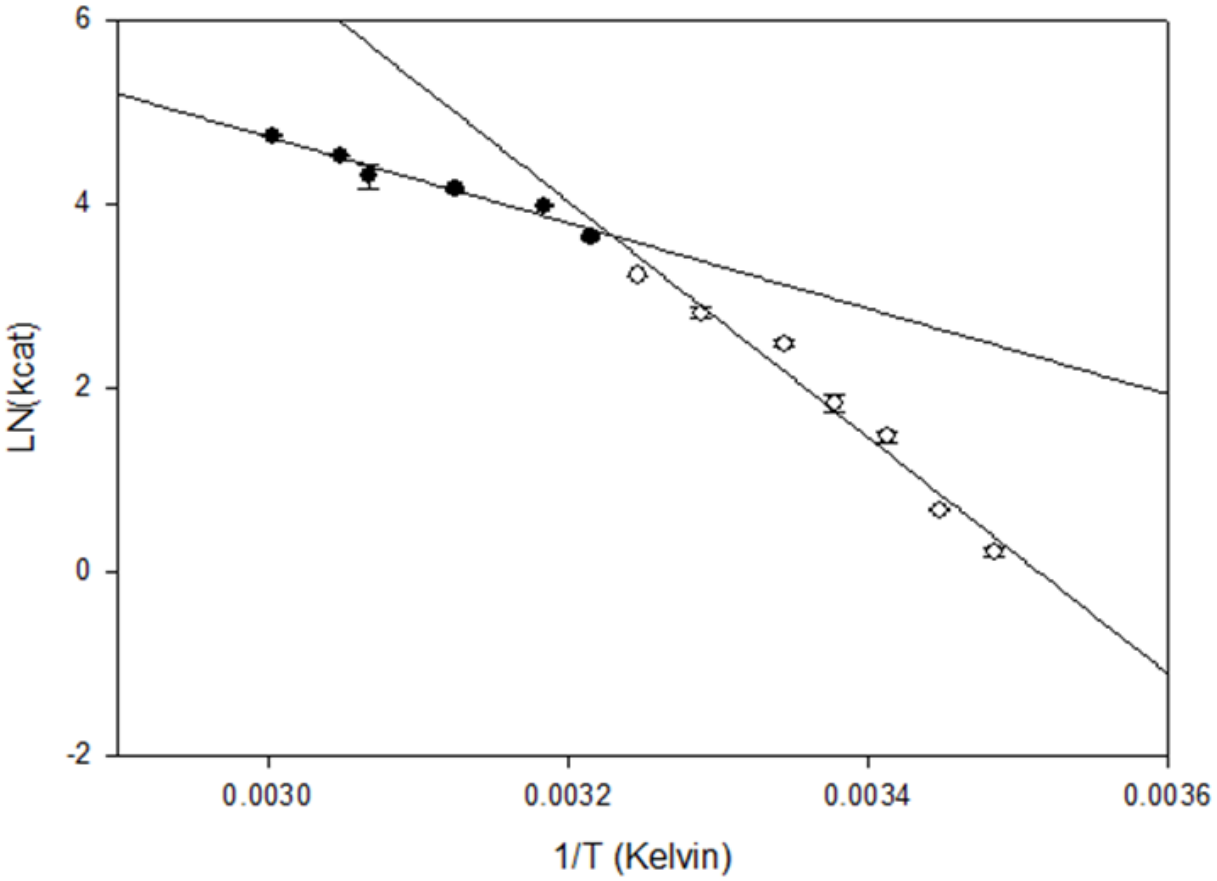

Figure 6.3: WT rcPEPCK reverse direction of catalysis with a linear trend showing the relationship between temperature and catalytic rate. A change in linearity occurs at 41°C. Temperatures range from 14°C to 60°C.

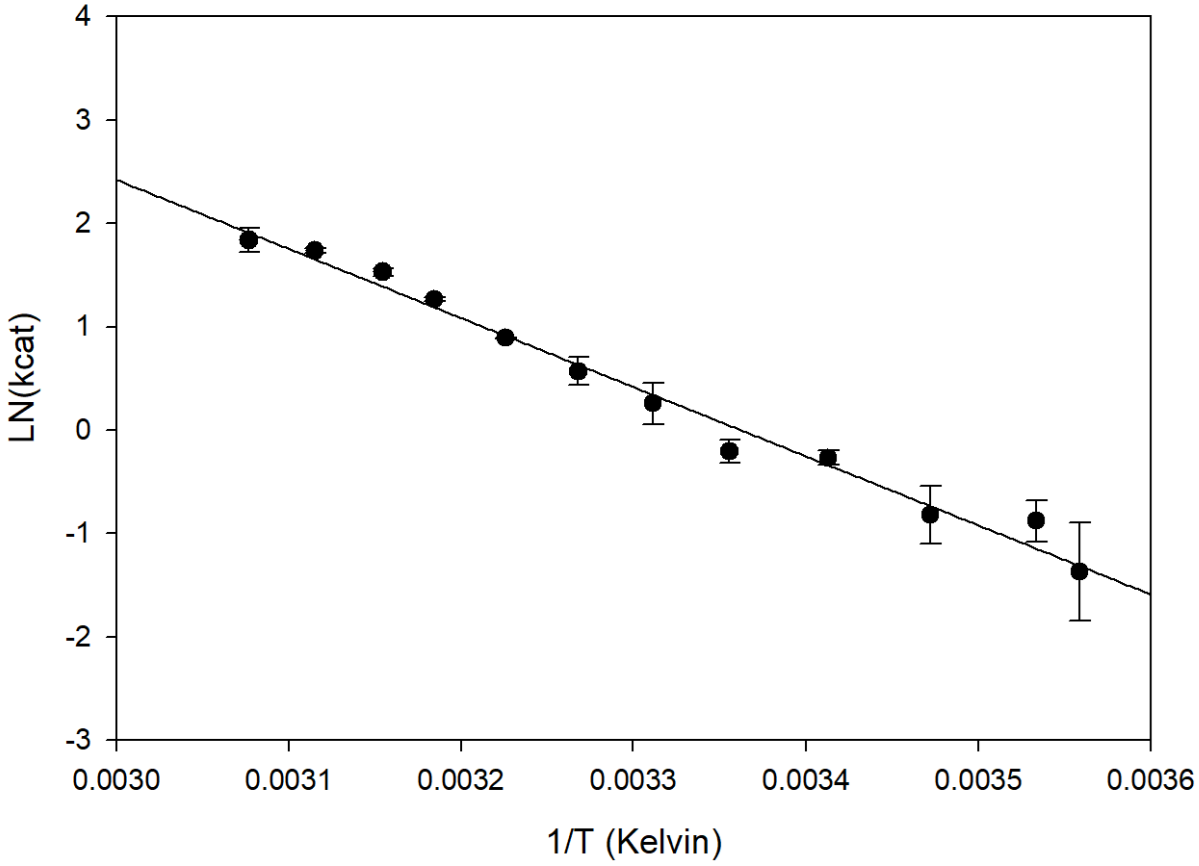

Figure 6.4: A467G PEPCK reverse direction of catalysis with a linear trend showing the relationship between temperature and catalytic rate. Temperatures range from 8°C to 52°C.

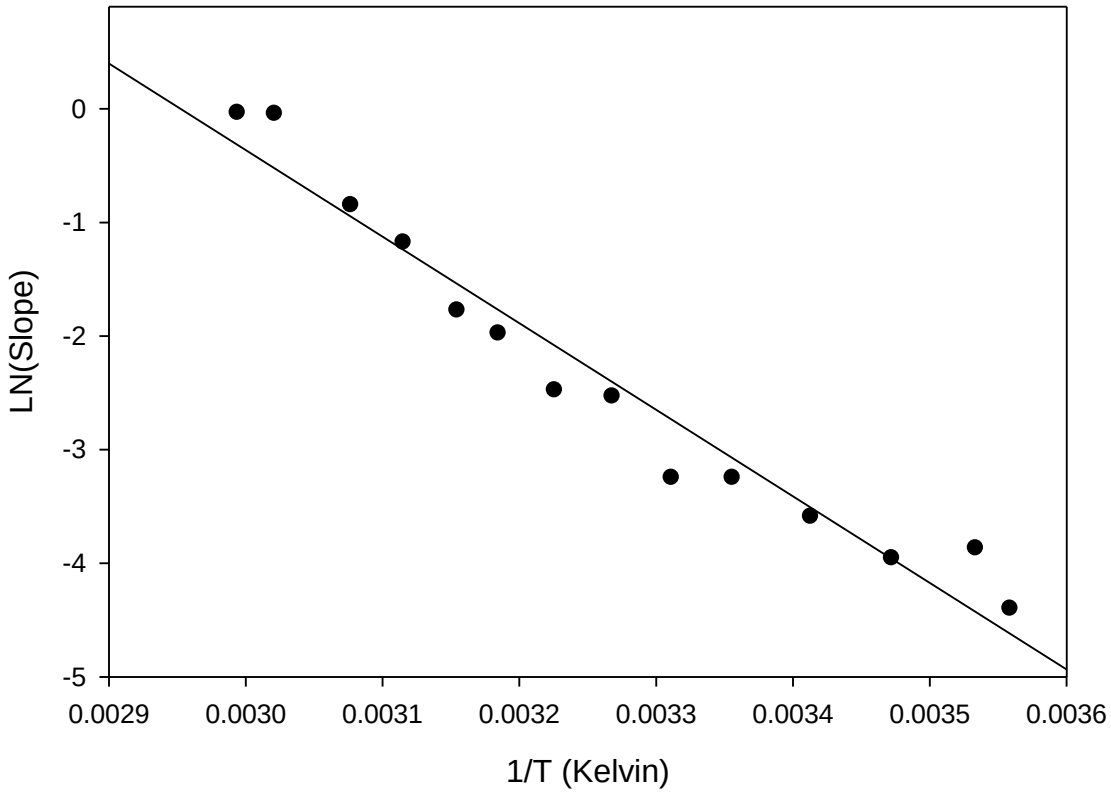

Figure 6.5: Non-enzymatic decarboxylation of OAA by free Mn^{2+} with a linear trend showing the relationship between temperature and a spontaneous chemical reaction. Temperatures range from 8°C to 61°C.

6.2: Enolate Protonation Reaction

The A467G PEPCK enzyme enolate protonation reaction, in which the enolate is converted to pyruvate, shows an increase in the catalytic rate as temperature is increased (Figure 6.6). The enolate protonation reaction rate has an almost two-fold increase in its overall catalytic rate compared to the A467G PEPCK forward direction of catalysis (Figure 6.4). The WT rcPEPCK enzyme did not produce a measurable amount of pyruvate for the enolate protonation reaction at temperatures ranging from 8°C to 61°C.

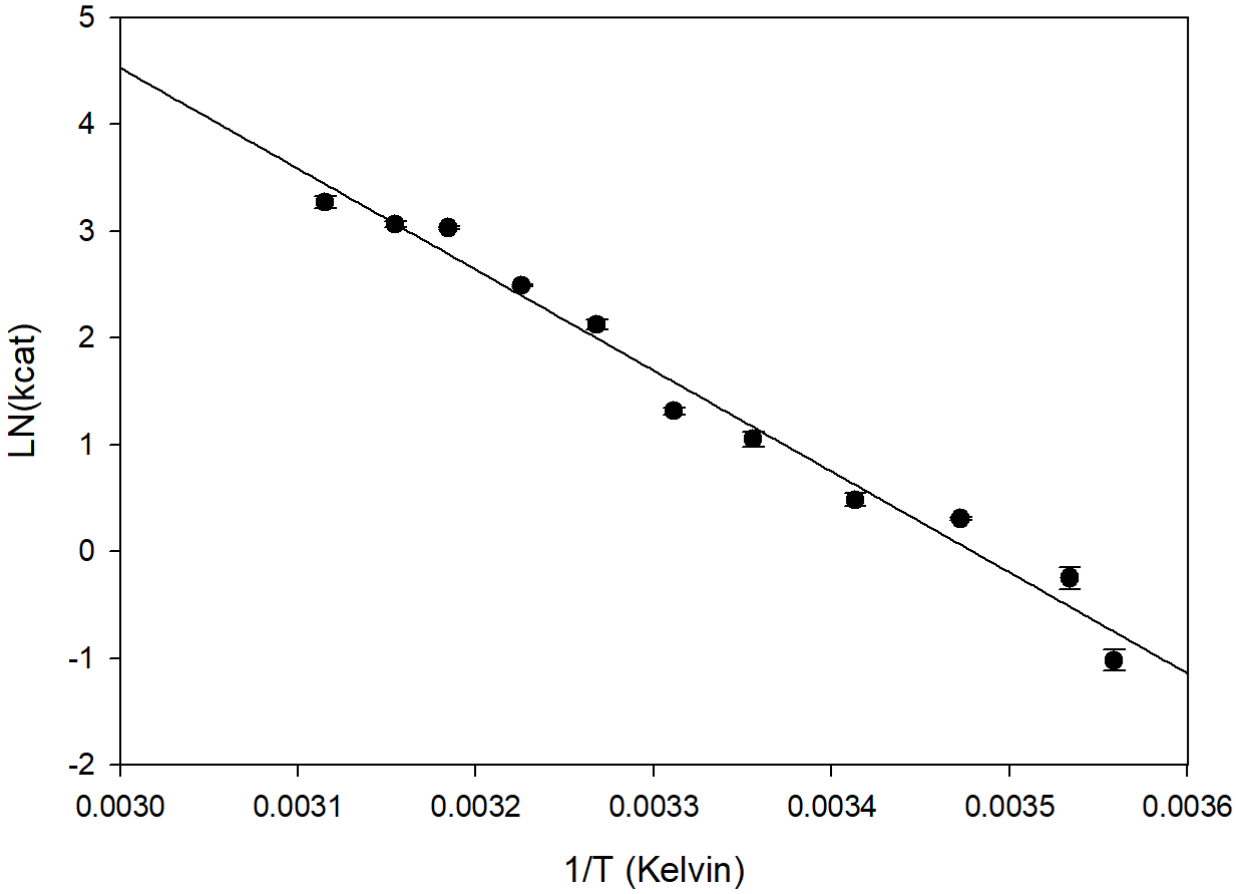

Figure 6.6: A467G PEPCK enolate protonation reaction with a linear trend showing the relationship between temperature and catalytic rate. Temperatures range from 8°C to 48°C.

6.3: Activation Energies of WT rcPEPCK and A467G PEPCK

Activation energies were calculated from the Arrhenius plot slopes from the reaction assays outlined in sections 5.1, 5.2, 5.3, and 5.4. The results are found in Table 6.1 below. The activation energies were determined to be 56.9 $\pm$ 1.1kJ/mol and 76.0 $\pm$ 1.6kJ/mol for the forward and reverse directions of catalysis for the WT rcPEPCK enzyme respectively. The activation energy of the non-enzymatic decarboxylation of OAA by free Mn^{2+} was found to be 63.4 $\pm$ 1.4 kJ/mol. For the A467G mutant PEPCK, the activation energies were determined to be 62.4 $\pm$ 1.1kJ/mol, 55.6 $\pm$ 1.2kJ/mol, and 78.6 $\pm$ 1.4kJ/mol for the forward and reverse directions of catalysis, and the enolate protonation reaction respectively.

Table 6.1: Activation Energies

Enzyme	Reaction	Activation Energy (kJ/mol)
WT rcPEPCK	OAA→PEP	57.0 $\pm$ 2.8
WT rcPEPCK	PEP→OAA (<41°C)	106.8 $\pm$ 5.8
WT rcPEPCK	PEP→OAA (>41°C)	38.8 $\pm$ 3.2
Non-enzymatic	OAA + Mn^{2+}→Pyr	63.4 $\pm$ 4.0
A467G PEPCK	OAA→PEP	62.4 $\pm$ 2.0
A467G PEPCK	PEP→OAA	55.6 $\pm$ 2.6
A467G PEPCK	OAA→Pyr	78.6 $\pm$ 2.6

Discussion:

7.1: The Role of Temperature Influence on Enzymes in Research

Temperature's influence on enzymes and the catalytic rate observed became clear in 1913 when Leonor Michaelis and Maud Menten hypothesized the importance of a controlled environment for steady-state enzyme kinetics.[26] This knowledge influenced the standard practice of controlling variables during experimental procedures that researchers still follow to this day. A better understanding of the relationship between temperature and enzymes became an important topic of discussion and has led to many contributions to the scientific community. In recent years, many researchers have focused on how the stability of enzymes during catalysis can be affected by temperature and how this can lead to complications during catalysis.

7.2: Activation Energy Requirement During PEPCK Catalysis

Activation energy is one of the most important factors in determining whether enzyme catalysis will occur. The reverse direction of PEPCK catalysis, in which PEP is converted to OAA, is postulated to be rare in living cells.[16,17,37] Whether the activation energy required is affected differently in each direction of catalysis is something that was unknown, however.

The forward direction of catalysis for the WT rcPEPCK enzyme showed a slightly lower activation energy in comparison to the non-enzymatic decarboxylation of OAA by free Mn^{2+} (Table 6.1). In the A467G PEPCK enzyme however the activation energy of the non-enzymatic decarboxylation of OAA by free Mn^{2+} was found to be very similar to that of the forward

direction of catalysis for the A467G PEPCK enzyme (Table 6.1). This result is consistent with previous kinetic data on the A467G enzyme and suggests that k_{cat} is measured by the decarboxylation of OAA to form the enolate, making this the rate-limiting step, since this is seen in both reactions.[16]

For the WT rcPEPCK reverse direction of catalysis an anomaly was found at 41°C for the Arrhenius enzymatic assay data. This anomaly shows a transition point in the slope of the reaction at this specified temperature (Figure 6.3). At temperatures higher than 41°C, the activation energy of the reverse direction of catalysis was significantly lower than the activation energy of the reaction for the temperatures below 41°C (Table 6.1). The activation energy of the reverse direction of catalysis above 41°C was also significantly lower than the activation energy of the forward direction of catalysis (Table 6.1). Above 41°C, the hypothesis that the reverse direction of catalysis is rarely seen in living cells appears to be false based on the reaction's activation energy. At temperatures lower than 41°C, the WT rcPEPCK enzyme was shown to have a significantly higher activation energy than the forward direction (Table 6.1). This result appears to support the hypothesis that the reverse direction of catalysis is rarely seen in living cells.[16,17,37] These results together appear to be contradicting based on this transition point in the slope. What could be determined from this result is that in a controlled kinetics environment (~25°C for PEPCK), this hypothesis is true. At temperatures above 41°C it appears that temperature is having a significant effect on lowering the activation energy barrier for PEPCK and is the cause for the change in this result. True Arrhenius plots should follow a linear slope through all the data points. Since this is not the case with the reverse direction of catalysis, an alternative explanation is required to explain the abnormality.

With this result my hypothesis is that the rate-limiting step of the reverse direction of catalysis has changed at temperatures above 41°C. At high temperatures chemistry becomes easier and the rate-limiting step requires less energy to complete. In terms of the chemistry of the reaction, this could mean that the speed of product release (OAA) or GTP has increased, altering the speed of the reaction since the slowest step determines the overall reaction rate. At temperatures below 41°C it is logical to conclude that chemistry is occurring slower which is supported by the high activation energy seen for the WT rcPEPCK enzyme reverse direction of catalysis at low temperatures. More kinetic data would aid some of the conclusions made, such as a more complete analysis of the kinetic parameters of these enzymes. An important parameter would be k_{cat}/K_M at each temperature measured which would give more insight into the change in slope seen in some of these reactions.

The activation energies for the A467G PEPCK reaction showed a higher activation energy in the forward direction of catalysis compared to the reverse direction of catalysis, a change in the thermodynamic favourability of PEPCK catalysis (Table 6.1). The activation energy needed for the reverse direction of catalysis for the A467G PEPCK enzyme is significantly lower than that for the WT rcPEPCK enzyme, while the forward direction activation energy is relatively similar for both enzymes (Table 6.1). This result suggests that a change in the normal catalysis of the enzyme has changed in the reverse direction of catalysis rather than the forward direction. It can be hypothesized that the rate of chemistry over low and high temperatures is the cause of such a result. Lower temperatures will have slower chemistry, as seen in the activation energy for the WT rcPEPCK enzyme reverse direction of catalysis. Chemistry will become less rate-limiting as temperature increases, also seen in the activation energy for the

WT rcPEPCK enzyme reverse direction of catalysis. This means that as temperature increases the rate-limiting step in the reaction has become more energetically favourable and the conformational change that is necessary to get the enzyme back to the proper alignment for catalysis to occur again has become easier. For the A467G PEPCK enzyme the activation energy of the reverse direction of catalysis did not shift at a higher temperature as seen in the WT rcPEPCK enzyme, but rather stayed consistent from low temperatures to high temperatures in the middle of what the WT rcPEPCK activation energies were. It could be hypothesized that the mutation of the Ω-Loop has caused the conformational change of the enzyme during catalysis to become partially rate-limiting over the entire range of temperatures. Previous data on this enzyme had hypothesized that the formation of the lid complex had become disrupted and this data appears to suggest the same conclusion.

A continuation on this work with the same experimental procedures utilizing other isozymes of PEPCK would add to the information gathered and give a more encompassing conclusion to the influence of temperature on PEPCK. In the future a crystallographic experiment could be performed to determine any structural changes to PEPCK caused by high temperature exposure to validate some of the hypotheses made based upon this data.

The activation energy for the protonation of the enolate for the A467G PEPCK enzyme was significantly higher than that for the reverse and forward directions of catalysis (Table 6.1). This result shows that the thermodynamic favourability of the protonation of the enolate is less than the favourability of the phosphorylation of the enolate in the forward direction of catalysis. This is shown by the activation energies that both reactions exhibit under varying temperature conditions (Table 6.1).

7.3: Stability of the Ω-Loop in PEPCK

The Ω-loop in PEPCK has become a structure of interest to researchers because of its large conformational shift during catalysis and importance in catalysis. It was previously found that the Ω-loop functioned as a lid to limit accessibility of the active site of PEPCK to substrates, metals and nucleotides.[17] This was found to be a necessary mechanism to protect the active site from unwanted chemistries, because a highly reactive enolate formed during PEPCK catalysis.[17]

To follow up on these previous experiments, I carried out enzymatic assays for both the forward and reverse direction of catalysis at temperatures ranging from 8°C to 61°C. The catalytic rates collected at these temperatures were linearized along with temperature so that Arrhenius plots could be utilized to help determine any trends as temperature increases. For the WT rcPEPCK enzyme no catalytic rate of pyruvate production could be measured, leading to inconclusive results on whether protonation of the enolate occurs in the WT rcPEPCK enzyme. This kinetic assay was attempted previously with the same inconclusive result for the WT rcPEPCK enzyme, but a small rate of pyruvate production in the A467G PEPCK enzyme at 25°C.[16]

Previous experiments on the WT rcPEPCK enzyme had determined a small rate of pyruvate production at 25°C for the GDP-dependent decarboxylation of OAA to form pyruvate.[5,16] Although this PEPCK reaction is different in that decarboxylation is occurring instead of phosphorylation, it indicates that with the presence of GTP in the assay mixture, which is required for phosphorylation to occur, protonation of the enolate simply isn't possible

even at higher temperatures. Phosphorylation appears to be the thermodynamically favourable outcome for WT rcPEPCK.

I determined the effect of temperature from 8°C to 48°C in the A467G PEPCK enzyme, showing a linear increase in the catalytic rate of the enzyme as temperature increased (Figure 6.6). The non-enzymatic decarboxylation of OAA by free Mn^{2+} was corrected for in the rates observed to give a true representation of pyruvate formed specifically by protonation of the reactive enolate. Since there is no pyruvate production for the protonation of the enolate in the WT rcPEPCK enzyme, no comparison can be made between the WT rcPEPCK enzyme and A467G PEPCK enzyme. Temperature stability of the Ω-loop could be determined by a crystallographic experiment to determine any structural changes to PEPCK caused by high temperature exposure to validate some of the hypotheses made based upon this data.

7.4: Temperature Influence on PEPCK Catalysis

At high temperatures, molecules will be moving faster and colliding much more frequently, leading to chemistry occurring at a much faster rate. Since enzymes are the catalysts for biochemical reactions, this results in an increased speed in the chemical changes of substrates, increasing the catalytic rate observed. Determining the influence that temperature will have on an enzymes catalytic rate is usually done using the Arrhenius equation, in which a visual representation in the form of an Arrhenius plot can be utilized. These Arrhenius plots are linearized, using the natural log of k_{cat} and 1/T, so that the relationship between temperature and the catalytic rate can be observed. There are cases where a non-linear trend can be observed instead. It has been suggested that a break in the linearity of an Arrhenius plot could

be caused by two factors: the enzymes normal catalytic conformation has changed, or the rate-limiting step of the catalytic reaction has changed.[31] The difficulty with pinpointing any one cause to anomalous behaviour is the fact that enzymes are very susceptible to temperature, they are complex, and multiple steps are involved during catalysis.[13]

In enzymatic catalysis, increasing temperature will result in an increase in the catalytic rate of the enzyme. Previously it was believed that past the enzyme's optimal temperature, complete denaturation will occur. New theories suggest a different model: The equilibrium model. The equilibrium model follows the principle that an enzymes catalytic rate will increase with increasing temperature to an optimal temperature, at which point temperatures higher than the optimal temperature will result in an equilibrium being formed.[7] The active form of an enzyme in solution will be in equilibrium with an inactive form of the enzyme. With increasing temperature past the temperature optimum, the equilibrium will shift more and more towards the inactive form of the enzyme.[7] As this equilibrium shifts more towards the inactive form of the enzyme, a decrease in the catalytic rate would be observed.[7] Eventually at a high enough temperature, complete denaturation of the enzyme will occur, resulting in complete loss of catalytic activity. This model was introduced to explain the decrease in enzymatic activity that can be observed before complete denaturation occurs.

My experiments determined the temperature influence on the forward and reverse direction of catalysis for both the WT rcPEPCK and A467G PEPCK enzymes using Arrhenius plots. My results were consistent with typical Arrhenius plots, where the catalytic rate increased in a linear fashion from low temperatures to higher temperatures (Figure 6.1, 6.2, 6.4, 6.5, 6.6). Data was not collected at higher temperatures than these, however, due to decreased catalytic

rates that were inconsistent, therefore not usable in the plots. There was an exception, where an abnormality was spotted with one of the Arrhenius plots. The WT rcPEPCK enzyme in the reverse direction of catalysis shows a decrease in the catalytic rate starting at 41°C (Figure 6.3). This catalytic rate was still increasing in a linear fashion past 41°C, but the catalytic rate was slower than before, resulting in this linear trend shifting. The equilibrium model can explain this shift in the linear trend. It is believed in this model that there are three states to an enzyme: a fully intact and active enzyme, an intermediate inactive enzyme, and a fully inactive and denatured enzyme.[7] If this model is true, 41°C appears to be the temperature optimum before the catalytic rate observed decreases (Figure 6.3).[7] Between 41°C and 60°C the catalytic rate is slower, which could be because of an increase in the intermediate inactive enzyme in the equilibrium formed. Unfortunately, one of the coupling enzymes of the reverse direction of catalysis, MDH, cannot survive past 61°C, so further data points could not be measured to determine if there would be more of a drop in the catalytic rate, and eventually complete loss of a catalytic rate, indicating a fully denatured form of the enzyme. In the future, a thermophilic form of the MDH enzyme could be obtained and utilized for temperatures beyond the normal MDH's temperature limits to allow for more data collection.

Conclusions and Future Directions:

In closing, this kinetic data shows some key conclusions about the questions and hypotheses presented in this thesis. At temperatures above 41°C, the reverse direction of catalysis was found to be more thermodynamically favourable in the WT rcPEPCK enzyme than the forward direction of catalysis. Below 41°C the opposite result was found true. For the A467G PEPCK enzyme the reverse direction of catalysis was found more thermodynamically favourable than the forward direction of catalysis. This result has shown that high temperature can have a significant impact on the activation energy requirement for PEPCK catalysis. The stability of the Ω-loop was found to be affected by higher temperatures in the A467G PEPCK enzyme, since pyruvate production through enolate protonation was occurring more frequently. The WT rcPEPCK enzyme saw no pyruvate production through enolate protonation, leading to inconclusive results in the stability of the Ω-loop for this enzyme. Finally, as expected, the catalytic rate of both enzymes increased as temperature increased.

The results of this thesis suggest temperatures influence on enzymes is still not fully understood. It appears enzymes can be disrupted very easily, resulting in abnormal behaviour one would not expect to see. This thesis has determined that temperature plays a significant role in PEPCK's catalytic functionality, although more work is required to give a complete understanding of the influence of temperature on PEPCK. In the future other isozymes of PEPCK can be utilized to determine any differences between them and to determine if a specific trend is seen in multiple PEPCK enzymes. A crystallographic study would also aid in allowing a visual representation of the structural changes temperature causes in PEPCK enzymes.

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Appendix:

Equations:

$$\upsilon = \frac{V_{max}[S]}{K_M + [S]}$$

Equation 8.1 – Michaelis-Menten equation. υ is the reaction rate, V_{max} is the maximum reaction rate at saturating substrate concentration, [S] is the substrate concentration, and K_M is the substrate concentration at which the reaction rate is half of the V_{max}.

$$k = Ae^{\frac{-E_a}{RT}}$$

Equation 8.2 – Arrhenius equation. k is the rate constant, T is temperature in kelvin, A is the pre-exponential factor, E_a is the activation energy of the reaction, and R is the universal gas constant.