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Mutagenesis of Phytophthora Sojae and Mitochondrial Taurocyamine Kinases to Determine Residues Important in Substrate Specificity

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ABSTRACT: A good model for studying molecular evolution is the Phosphagen Kinase (PK), as it has a diverse family present in both unicellular and multicellular organisms. Through mutation, specialization, and divergence, this diverse PK family may have evolved a diverse set of substrate specializations, similar to what is seen in multicellular animals today. The evolutionary pathway of interest is that of the Glycocyamine Kinase (GK) from the Taurocyamine Kinase (TK). To investigate this pathway, the effect of making a one-residue mutation was observed to see the influence it would have on substrate binding and activity in PsTK and Ab MiTK enzymes. The mutations created to understand the enzymes' activity were V71A, V71T and G26H in PsTK and V71T in Ab MiTK. The mutation in the V71T region of PsTK is suggested to be detrimental for the enzyme as it led to a dead enzyme that was unable to be transformed. The AbMiTK V71T mutant showed a better affinity and higher catalytic efficiency with glycocyamine, suggesting that this mutation was part of the pathway for the evolution of GKs. The binding affinity and the catalytic efficiency of the V71A mutant remained the same for glycocyamine but decreased for taurocyamine. This suggests that this was also part of the pathway for the evolution of GKs as the ability of the TK to use its substrate decreases by 10-fold. The G261H mutation leads to no observable enzyme activity, suggesting that the mutation is deleterious to the enzyme.

I. INTRODUCTION:

Molecular evolution is an area in biology that studies the process of change in DNA, RNA, and proteins across generations. The interest of this paper is in regard to the study of molecular evolution at the level of proteins. Proteins are very vital in an organisms' biochemistry and their evolutionary changes have remained a central subject in evolutionary and molecular biology for a half-century (Zhang et. al., 2015). Understanding protein evolution is critical to reconstructing evolutionary history and mechanisms (Zhang et. al., 2015). Being able to fully understand the evolution of protein would allow us to successfully make changes to an organism's biochemistry, which we can apply in bolstering Earth's survival.

A good model to study the molecular evolution of proteins is the Phosphagen Kinases (PKs). PKs are known to be present in both multicellular and unicellular organisms and bind to different but related substrates. They play a role in energy production and utilization (Uda et. al., 2013) and catalyze the reversible transfer of γ-phosphoryl group from ATP to an acceptor molecule containing a guanidinium group. PKs help maintain energy homeostasis (Uda et. al., 2013; Palmer et al., 2013) and are widespread in cells that have a high demand for ATP, such as myocytes and neurons, and are also present in spermatozoa and protozoan motility apparatus (Uda et. al., 2013). The PK family, which occurs naturally, includes Arginine Kinase (AK), Creatine Kinase (CK), Glycocyamine Kinase (GK), Taurocyamine Kinase (TK), Lombricine Kinase (LK), Hypotaurocyamine Kinase (HTK) Thalassemine Kinase (ThK), and Opheline Kinase (OK), which are named after the various guanidino substrates they bind to. For this paper, we are going to focus on AK, CK, GK, TK, LK, and HTK.

CK, GK, and AK are highly specific for their respective guanidine acceptor while the remaining three PKs (TK, LK, and HTK), are considered promiscuous since they have the ability to bind to two or more guanidino substrates (Suzuki and Ellington; Uda et al, 2005). Their promiscuity suggests a flexibility in their substrate

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binding site, the Guanidino Specificity (GS) loop. The amino acid group around the GS region is highly conserved, and the GS loop was proposed to be involved in distinguishing the size of the guanidino substrate in all PKs except TKs (Uda et al 2013; Tanaka et. al., 2011; Palmer et al., 2013). When comparing the GS loops of the different PKs via a multiple sequence alignment of the amino acid residues (Figure 3), you can see that GKs have no insertion in their GS region, AKs have 5 insertions, CKs have one insertion, and LKs have five insertions in their GS region.

The evolutionary relationship of PKs to each other has yet to be fully elucidated. The most efficient way to analyze and appreciate the distribution of phosphagen kinases is to map these enzymes onto a phylogenetic tree using their sequence data. After analyzing a phylogenetic tree created with the available sequence data, Ellington and his coworkers, 2006, created a possible evolution pathway in which they believed that the common ancestor of the PKs, with the exception of AKs, is CKs.

This, however, is not supported by the work done by Jourden and his coworkers (2007. In their previous work (Jourden et. al., 2007), they tried to determine the sparing number of changes needed to transform a CK into a GK. Part of their interest in this experiment was based on the exquisite level of specificity in reactivity that the CKs and GKs have, although their substrates differ by a single methyl group. They also believed that creating a GK from a CK mutant would not be too difficult, as at the time, it was believed that GKs evolved from CKs. This, however, was not the case. Evidence from their mutagenesis studies suggests that these two PKs radiated from a common ancestor rather than from each other. Similarly, another work done (Lim et. al., 2010) in understanding GKs does not support this. In their work, they discovered the presence of a glutamate residue conserved in GK and AK but missing in CK. This suggests that GK evolved from AK or a common ancestor to AK but not CK. In addition to the work done by Jourden, Lim, and their coworkers (2007; 2010), Ellington's model is also not supported by the work done by Palmer and her coworkers (2013). They discovered that Oomycota TKs group on the outer parts of the CK supercluster (contains GKs, LKs, TKs, CKs, and dimeric AKs) and appear to have diverged after the AK dimers diverged (Figure 1). This suggests that Oomycota TKs shared a common ancestor with the CK, LK, and GK branches before they diverged into separate branches, and it also suggests that TKs evolved from AK lineages (Figure 1; Palmer et. al., 2013).

The lack of support for the model made by Ellington and his coworkers (2006), by these studies, led us as a class to create a putative evolutionary pathway of PKs (Figure 2) applying information and discoveries obtained from previous studies (Jourden et. al., 2007; Lim et. al., 2010; Palmer et. al., 2013).

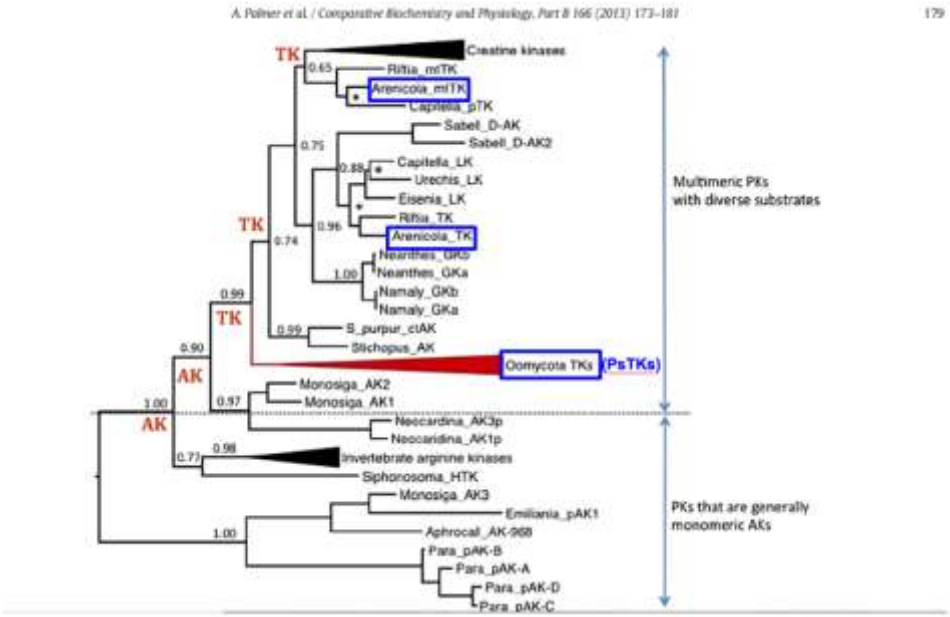

Figure 1: Phylogenetic tree relationship between TK/HTK adapted from Palmer et. al., 2013.

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The figure was modified by the class to show the ancestor we believe to be present at the branching points. The PKs written in red represent the putative ancestral protein candidates. The PKs in blue boxes represent the TKs of interest for this study. The branches of Creatine Kinase, Oomycota TKS, and Invertebrate arginine kinase are collapsed. The branches of organisms below the dotted line are monomeric (except Siphonosome_HTK), while the branches of organisms above the dotted line are dimeric. The numbers represent the approximate likelihood ratio test (aLRT) scores, which indicate the likelihood of the branch to exist on a maximum-likelihood tree.

In this pathway, we suggest that AK is the common ancestral PK and all other PKs, with the exception of HTK, evolved from TK. This putative pathway is very attractive, however, its validity is still being investigated.

It was previously thought that only one kind of TK existed; however, the presence of two types of TKs, a cytoplasmic TK and a mitochondrial TK (MiTK) in Arenicola brailiensis(Ab),was discovered (Uda et al., 2005). PKs were also originally thought to be restricted to the metazoan animal kingdom but were recently found in a protozoan clade, the oomycota (Palmer et al., 2013; Uda et. al., 2013). Origins of several qualities, including substrate binding, enzyme structure, can be illuminated with the help of the successful characterization of protozoan PKs. The oomycota organism of focus in this study would be Phytophthora sojae. Based on previous work (Palmer et al., 2013), the PKs present in P. sojae were characterized to TKs and HTKs (which are going to be referred to as PsTKs).To evaluate the evolutionary relationships of TKs with other phosphagen kinases, a phylogenetic tree was constructed with amino acid sequences (figure 1). The cytoplasmic TKs were shown to cluster outside the region that the MiTKs clustered (figure 1). This suggests that the cytoplasmic and MiTKs evolved independently of each other as they had separate divergences from a common ancestor.

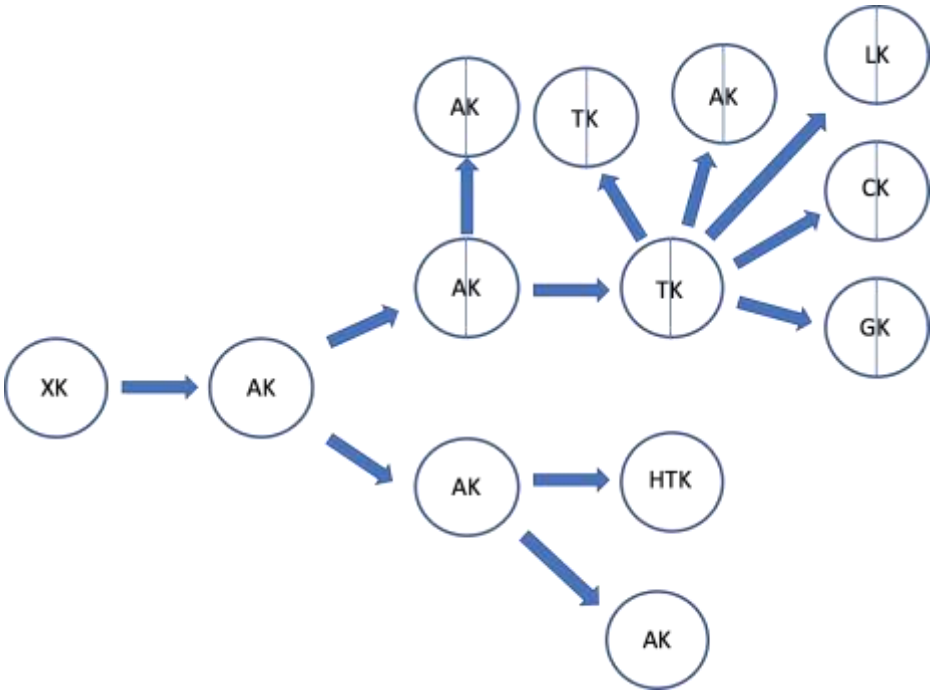

Figure 2: The possible evolution pathway of PKs.

XK is presumed to be a functional monomer of a primordial phosphagen kinase. The circles represent monomers, while the circles with a line drawn in the middle represent dimers. This diagram was put together making reference to Ellington et. al., 2006 and Palmer et. al., 2013.

When analyzing the phylogenetic tree (Figure 1), the putative PKs present at different speciation eventswere determined. Looking at Figure 1, you can see that the AKs are the putative ancestor of all PKs and that a speciation event in which a TK was present led to the divergence of PsTKs.

When comparing the substrate binding of MiTK, PsTK, and cytosolic TKs, both PsTK and cytoplasmic TK were seen to have five insertions

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in their GS loop, and MiTK had no insertions in its GS region. In previous work, when analyzing the enzymatic activities of the three different TKs, they discovered that cytoplasmic TKs showed activity for substrates taurocyamine and lombricine, MiTK showed activity for taurocyamine, lombricine and glycocyamine and PsTKs showed activity for taurocyamine and glycocyamine (Uda et al, 2005; Palmer et al., 2013).

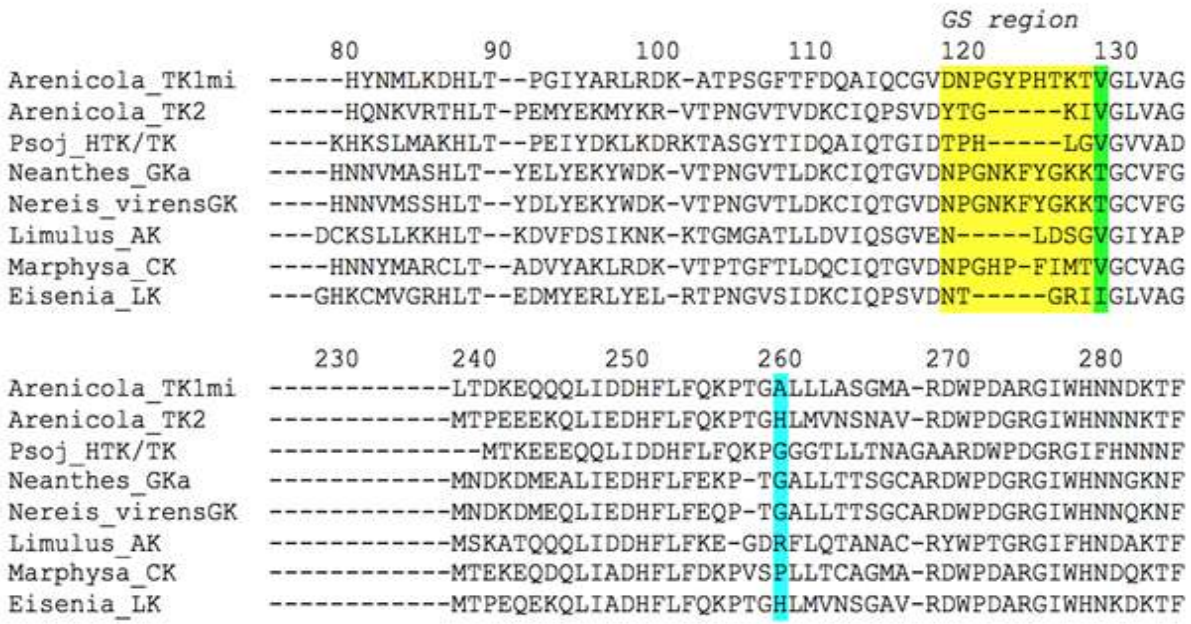

Figure 3: The alignment of various PKs.

These sequences were found on ncbi protein BLAST while the alignment was made with MacVector using the clustalW analyze function and was also manually adjusted. The region highlighted in yellow represents the Guanidino Specificity (GS) loop which is important in substrate binding. Regions outside the GS loop suggested to play a role in substrate binding are highlighted in green and blue. The column highlighted in green represents the 71 region as indicated in Tanaka et. al, 2011, while the column highlighted in blue represents the 261 region as adapted from the Palmer et. al., 2013.

PsTKs, MiTKs and cytoplasmic TKs are all TKs, yet they have different guanidino substrate specificities. This led us as a class to try to understand what residues control substrate specificity. To be able to better understand this, we built tools (mutants) that would allow us to measure and compare taurocyamine and glycocyamine metabolism and activity to that of the wild type (WT) PKs. The mutants created to understand the GK activity are V71A, V71T, and G26H in PsTK and V71T in AbTK. The V71 mutations were of interest to us due to its role in substrate specificity and past work done on it in MiTK (Tanaka et. al., 2011). In past work, the V71A mutation in MiTK led to a change in the enzyme's activity, causing the MiTK to act like a GK (Tanaka et. al., 2011). Repeating this experiment in PsTK was hypothesized to give the same result as that seen in past studies. The V71T mutation in AbTK was proposed due to the presence of T in this GK. It was hypothesized that changing this amino acid residue T would improve glycocyamine binding. The fourth experiment, which was the main focus of my group, was to create a G261H mutant in PsTKs and observe its kinetic activity with taurocyamine and glycocyamine. PsTKs are genetically similar to cytoplasmic TKs but biochemically similar toMiTK. When all these TKS are aligned against each other (Figure 3), you see that MiTK has no deletion in its GS region while PsTKs and cytoplasmic TKs have five deletions in their TKs. These similarities would suggest that both PsTKs and cytoplasmic TKs would then bind to the same guanidino substrate. However, both MiTK and PsTKs showed enzymatic activity for glycocyamine and taurocyamine, while cytoplasmic TKs showed activity for only taurocyamine and not glycocyamine. When looking at the 261 region – identified by previous work to be important in substrate binding – of the

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alignment, you see that cytoplasmic TK has a histidine (H), MiTK has an alanine (A) and PsTKs differ by a glycine (G). G and A are different by one methyl group. This could be the reason for the similarities between MiTK and PsTK substrate specificity. It was thus hypothesized that changing the G in PsTK to an H would lead to a worse affinity of PsTK for glycocyamine, therefore making the 261 region the region of influence in the PsTK and MiTK substrate binding similarities.

II. METHODS

Databases

NCBI database was used for the retrieval of sequences. MacVector was used for alignment and analysis of the sequences.

Site-Directed Mutagenesis

The template of the PsTK used for its mutagenesis was pET45-PsTK. A forward primer with a sequence of 3' CAGAAGCCCGGTGGTCACACCTTGCTGACC AACGCTG 5' and a reverse primer of 5' CAGCGTTGGTCAGCAAGGTGTGACCACCG GGCTTCTG 3'were used for the mutagenesis. The highlighted region in the forward primer was changed from GGC to CAC to ensure that the region codes for a Histidine instead of a Glycine. The sequence was also changed in the reverse primer to ensure it is coded for Histidine. The mutagenesis of the PsTK was done following the instructions of the QuikChange Lightning Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA).

Transformation

The PKs used in this experiment are those obtained from rabbit muscle. The mutant PsTK was transformed into ultracompetent cells using the QuikChange Lightning Site-Directed Mutagenesis (Agilent Technologies, Santa Clara, CA) protocol. S.O.C media was used instead of NZY$^+$. The transformed cells were grown in Luria Broth (LB) agar with 1X ampicillin (AMP; 50mg/mL) under sterile conditions and stored at 37°C. Colonies did not grow on my partner and I's plate, so we used four colonies from other members of our group. Overnight cultures of the colonies were made in LB media with 1X AMP. The positive clones of PsTK G261H were transformed into NiCo competent cells (NiCo21 [DE3]). The mutant DNA (50ng/µL) was added to the homemade NiCo cells. These cells were incubated on ice for 30mins and heat shocked at 42°C for 60secs. S.O.C media was then added to the mixture, after which it was incubated at 37°C for 1 hr. The DNA was then plated on an LB-AMP plate and incubated overnight at 37°C. The transformed colonies were grown on LB agar with AMP (50mg/mL) under sterile conditions and stored overnight at 37°C, shaking at 250 – 300rpm

Plasmid Mini Prep

The purification of the mutant DNA from the colonies was done following the protocol obtained from the Qiagen Plasmid Purification Handbook (Qiagen, Hilden, Germany).

Mutagenesis Analysis

The success of the mutagenesis was investigated using gel electrophoresis to view the PCR of the mutagenesis. The PCR of the purified DNA was run on a 1X TAE gel (40mM Tris, 20mM Acetate, 1mM ~8.6 pH) with 1% w/v agarose gel and a log 2 DNA ladder (gotten from NEB) at 95V for 40mins.

Sequence Analysis

After being purified, the PsTK template concentration of 30ng/µL (5.5kb) in plasmid was submitted to the Molecular and Cellular Imaging Center (MCIC) at the Ohio Agricultural Research and Development Center (OARDC) for sequencing. The resulting mutant sequences were aligned with the wild type PsTK using MacVector.

IPTG Induction

The overnight culture was added to Terrific Broth (TB; 450mL) after phosphate buffer (PB; 50mM) and AMP (100µg/µL) were added to it. The flask was incubated at 37°C and shaken at 250 – 300 rpm, with its OD_{600} being monitored every half hour. Once the OD was 0.81, IPTG (1mM) was added to it. The flask was then incubated at 18°C, shaking at 250 – 300rpm.

Protein Purification

The cell pellets were obtained from the IPTG induced cells by centrifuging at 8000xg for 20 mins at 4°C. The collected cell pellets were then stored at -20°C. The proteins from the cell pellets were purified following the protocol of QIAexpress® Ni-NTA Fast Start Handbook (Qiagen, Hilden, Germany). To stabilize the purified protein, it was run through a Bio-Rad DG10 column, after which SB with added DTT was run through the same column. Fractions (0.5mL) were collected in Eppendorf tubes. Bradford protein assay was done by adding aliquots (5µL) of each fraction to aliquots (50µL) of Bradford reagents. The protein fraction corresponding to the Bradford assay that turned

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very blue was concentrated using an AmiconUltra 4 centrifugal unit to centrifuge at 5000xg for 40mins. Its absorbance was measured to be 0.78 at 280nm. Using the ExPasy site and the amino acid sequence of our mutant clone, the extinction coefficient was determined to be 49850$(Mcm)^{-1}$, which was used to determine the protein concentration (16.6µM).

Gel Electrophoresis: SDS-PAGE

The aliquots obtained during the purification of the mutant protein were used to run an SDS-PAGE gel electrophoresis to determine the purity of the purified protein. A pre-cast gel of Tris-Glycine and a protein SDS precision plus dual color standard obtained from Bio-Rad was used. The gel electrophoresis was done following the protocol obtained from the Mini-PROTEAN® Tetra Cell (Bio-Rad, California, United States of America). The gel was given to the professor for Coomassie blue staining.

^{31}P NMR

To measure the proteins' enzymatic capability, ^{31}P NMR was done on the enzyme reacting with substrates and various controls to compare the differences. The reaction mixture for the PsTK G261H and PsTK contained Bicine (pH 8, 100mM), MgATP (20mM), substrate (10mM), MgAc (1mM) and the respective PsTK. Control ^{31}P NMR were done on ADP (pH 8, 100mM Bicine; 1mM MgAc; 20mM ADP), K^+ phosphate (pH 8, 100mM Bicine; 1mM MgAc; 20mM K^+ Phosphate) and PCr (20mM PCr; pH 8, 100mM Bicine; 1mM MgAc). The reaction mixtures were given the professor to be run on the NMR machine.

Linked Assay

NADH linked assay was performed on various amount of substrates to determine the enzymatic properties such as Km, Kcat and catalytic efficiency of the enzyme, PsTK G261H. Each cuvette used to measure the change in absorbance overtime in the spectrometer had MgATP (5mM), substrate (ranging from 0.25 - 5mM), NaOAc (5.7mM), tricine (pH 8, 100mM), PEP (1.6mM), NADH (0.15mM), $Mg(OAc)_2$ (2mM), BSA (0.025mg/mL), LOH (50µL) and H_20 (enough to make the total volume 490µL) added to it. PsTK G261H (10µL; 16.6µM) was added to the cuvette directly before it was put in the spectrophotometer. The absorbance was measured for 2mins at 340nm. Not enough enzyme was left for the linked assay of glycocyamine run.

III. RESULTS

Mutagenesis

The purpose of these experiments was to analyze residues important for substrate specificity in regards to the evolution of GKs from TKs. The tool used in the analysis involved building various PsTK and AbTK mutants to see if these mutations would result in a difference between glycocyamine and taurocyamine activity when compared to the wild type. My group's mutant of focus was PsTK G261H, which was cloned in pET45-PsTK. The investigation of the success of the mutagenesis was done using gel electrophoresis PCR and sequencing (figure 4). The result of the mutagenesis reaction when viewed on the gel was inconclusive. Smearing was only seen in the lane that contained the mutagenesis sample, and no clear bands of DNA was formed at different parts of the lane (Figure 4). This suggests that mutagenesis may have not been successful. No success was seen in our transformation of mutants into ultracompetent cells, so colonies from other members of our group were used.

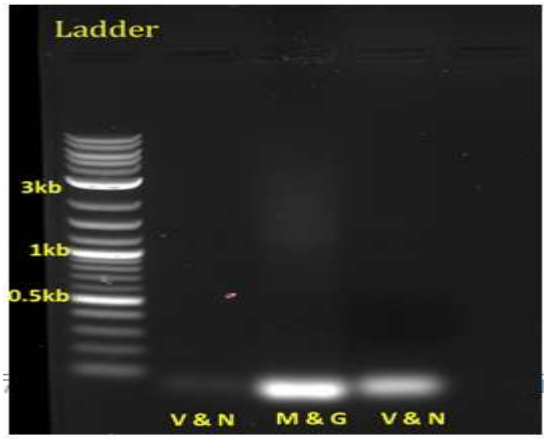

Figure 4: Analyzing the success of Mutagenesis. Gel Electrophoresis of the mutagenesis PCR.

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The sample was run on a 1% w/v agarose gel (1X TAE) with a log 2 DNA ladder. Samples were put in the M&G lane. Lane's V&N contains samples not discussed in this paper.

Plasmid purification was performed on these colonies, and the purified plasmids were given to the MCIC at OARDC for sequencing. The four colonies of PsTK were successfully mutated. The DNA was changed to successfully code for a Histidine (CAC) instead of a Glycine (GGC) at the 261 region (Figure 4). The success rate of the mutagenesis of the class was 59.5% where more than half of the colonies created by the class were successfully mutated.

Table 1: Success rate of Mutagenesis.

Species	Mutation	No of Successful Mutant Created (Out of 8 colonies)
PsTK	V71T	0
	V71A	8
	G26H	6
Ab MiTK	V71T	5

The success rate in mutagenesis, albeit not 100%, enabled us to move to the next step which was assessing substrate specificity and enzymatic kinetics of the mutants.

Purification of PsTK G261H

The mutant was transformed into NiCO bacterial cells, which have a T7 RNA polymerase-IPTG induction system that allows a high level of protein expression. The cell was induced with IPTG and pelleted at an OD_{600} of 0.81. At this OD_{600}, there would be a high level of protein present, which would allow us to isolate the PsTK mutant and measure its activity. The cell pellets were purified to isolate the PsTK mutant. The aliquots obtained from various steps of the protein purification were used to run an SDS-PAGE gel electrophoresis. The size of the proteins of interest was observed at ~40kD in the gel. The amount of protein present at the pre-induction is less than that present at the post-induction (Figure 5). This is expected as during the post-induction, more proteins are expressed.

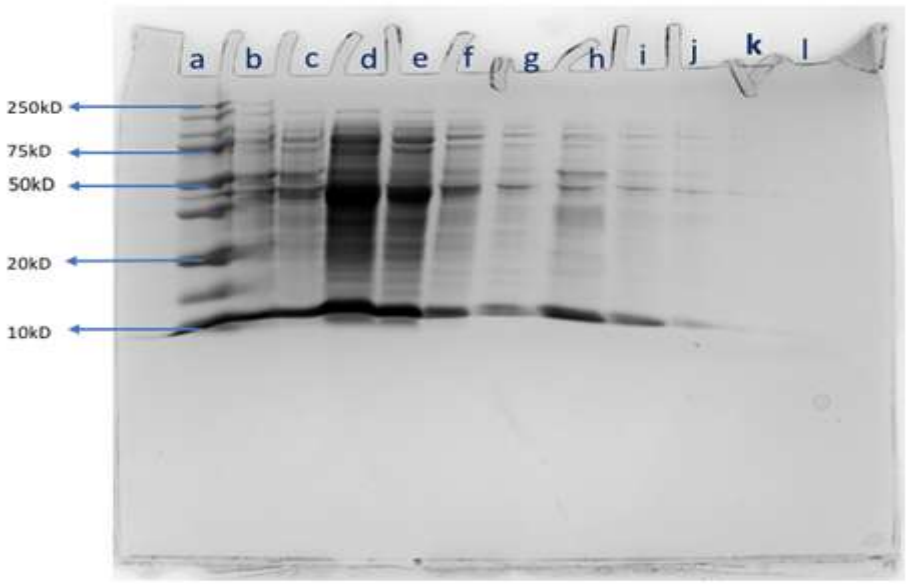

Figure 5: Analysis of PsTK G261H success.

a) represents the standard used in the gel electrophoresis (protein SDS precision plus dual color standard) b) pre- IPTG induction aliquot c) post aliquot induction d) aliquot from supernatant of cell pellet e) cell lysate flow through f) first wash aliquot g) second wash aliquot h) first elution aliquot i) second elution aliquot j) concentrated protein aliquot k) 1:5 dilution of concentrated protein l) 1:10 dilution of concentrated protein.

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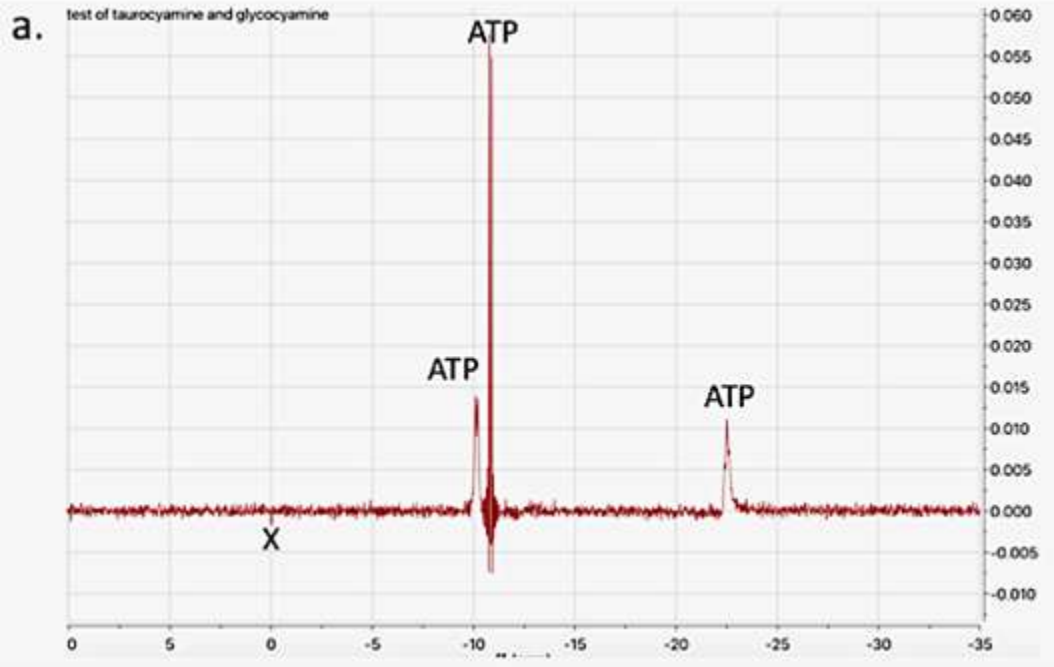
However, the amount of protein seen at ~40kD significantly decreases from the lane representing the cell lysate flow through to the concentrated protein. This could be a result of some of the enzymes not binding tightly to the column, causing a lot of them to flow through to the column during the first wash. In the gel, more protein is observed in the first wash than in the first elution. This suggests that a lot of our protein was lost to the first wash during purification. Some proteins were still able to bind to the column as the amount of protein in the first elution is more than that in the second wash (Figure 5).

Measuring the activity of the mutated enzyme

^{31}P NMR and linked assay were used to measure PsTK G261H activity. The result obtained from the ^{31}P NMR was inconclusive. The phosphorylated products of PKs are not available commercially, so phosphocreatine (PCr) was used as a control to identify the expected peaks of the products of the PsTK mutant. The peaks of PCr are observed at 0 and ~-4ppm while the peaks of ATP are observed at ~-10 and at ~-23ppm (Figure 15c & e; appendix). The signals of peaks of the PCr are, however, observed at 10^2 folds less than that of ATP. When looking at the ^{31}P NMR of the PsTK reaction with taurocyamine and glycocyamine, only ATP peaks can be seen. Thissuggests the peaks of the phosphorylated PsTK products may have been lost in the noise due to the difference of 10^2fold seen in the signal observed in ATP and PCr . This could also be as a result of having too much ATP and a small enzyme concentration in the reaction mixture for the ^{31}P NMR. This could also explain why the ATP peaks are high and strong, and there's barely a peak representing the products of the PsTK mutant. One of the peaks seen in ^{31}P NMR of PCr at 0ppm, can be seen as a tiny peak at 0ppm of the PsTKtaurocyamine control (Figure 15; Appendix), a tiny, inverted peak at 0ppm in the PsTK G261H glycocyamine test, and absent in the PsTK G261H taurocyamine, also making the data inconclusive (Figure 5). Although the data is inconclusive, it does not suggest that the enzyme is dead or inactive. To further analyze the enzyme's activity, a linked assay was performed.



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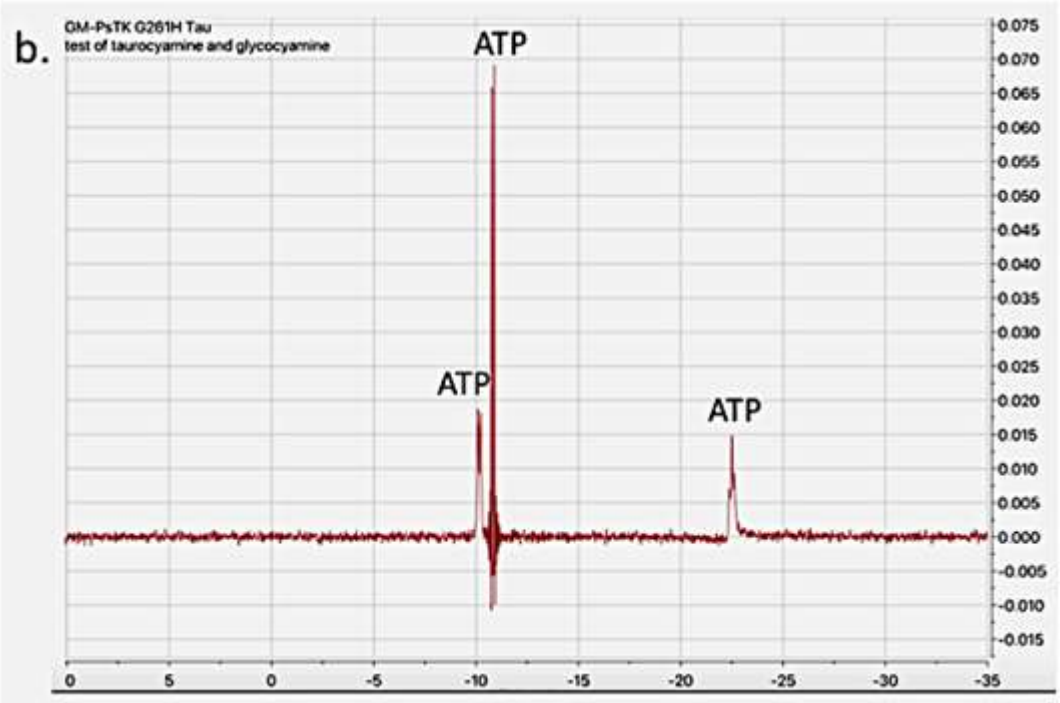
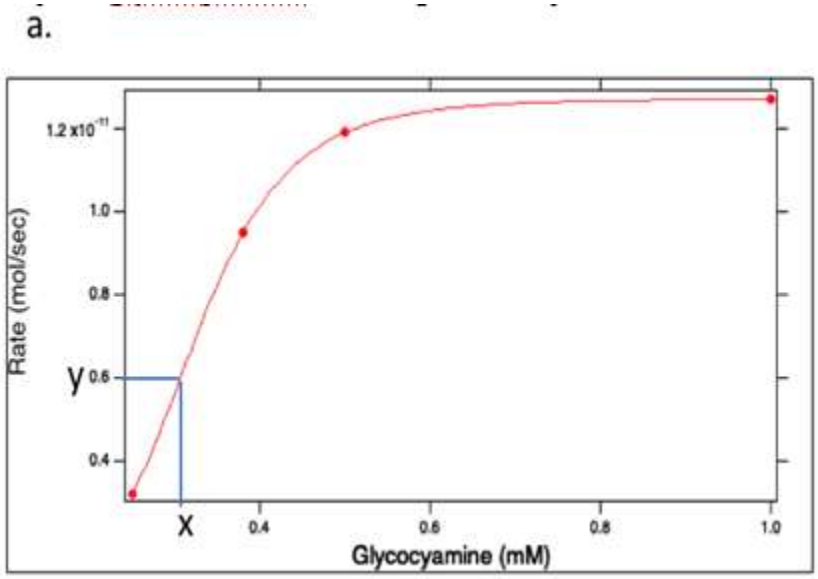


Figure 5: Analysis of PsTK G261H mutant activity using ^{31}P NMR. a) Represents the test of the mutant's activity with glycocyamine. X represents a possible peak for phosphorylated products. b) Represents the test of the mutant's activity with taurocyamine.

NADH-linked assay was performed with varying amounts of substrate to determine the enzymatic properties of the enzyme, such as Km, $K_{cat,}$ and catalytic efficiency of the enzyme. Based on the low concentration of the enzyme used, we were not able toget conclusive data (Table 2) on our mutant. Based on the work done by the other group with the PsTKWT, PsTK was shown to have a better binding affinity for glycocyamine but a higher catalytic efficiency for taurocyamine (Table 2; Figure 6). This data contrasts that observed in previous data (Palmer et. al., 2013). In previous work, the enzyme has a better binding affinity for taurocyamine and its affinity for glycocyamine is 10 folds worse than that of glycocyamine (Palmer et. al., 2013).



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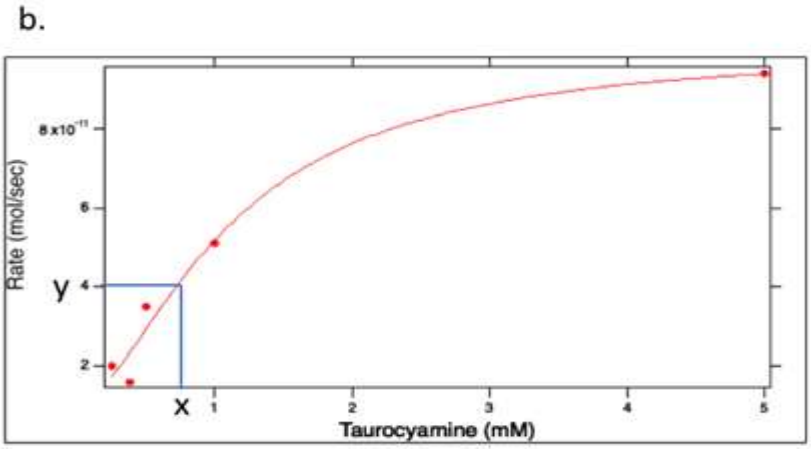

Figure 6: The Michaelis Menten Graph of PsTK WT a) represents the result gotten from the glycocyamine test of the NADH linked assay b) represents the result obtained from the taurocyamine test of the NADH-linked assay. In both graphs, y represents ½ Vmax, and x represents the Km.

IV. DISCUSSION

A fundamental aspect of evolution is that mutations generate novel alleles that are either favored or disfavored by selection (Haiwei et. al., 2004). The key role that mutagenesis of protein-coding sequences plays in evolution led us to use it as a tool to determine the amino acid residues important for substrate specificity; thereby giving an insight into the evolution of TKs to GKs. The relative ease with which substrate specificity can be converted was demonstrated by Suzuki and co-workers, who found that at least one variant of TK can be readily converted in substrate specificity from taurocyamine to glycocyamine (2011). The objective of this experiment, which was to determine the amino acid residues important for substrate specificity, was done by creating different PsTK and Ab TK mutants and seeing the effect on their catalytic activity with their substrates. This study was of interest as it can help illuminate possible evolutionary pathways of the PK family. Taking class and literature data together, our results show that the V71 region, which was previously reported to play a role in substrate specificity in P. infestans, also plays the same important role in PsTK and Ab Mito TK (Tanaka et. al., 2011). Our result for G261H mutant is, however, inconclusive, and there is no past documentation on this specific mutant to use for comparison.

Table 2: Enzymatic Characteristics of WT and Mutant PKs.

Enzyme	Substrate	Km (nM)	Kcat (1/sec)	Kcat/Km	Ratio of Taur/Glyco (Kcat/Km)
Ab MiTK WT	Taurocyamine	0.9143	17.38	19.0091	30.8857
	*Glycocyamine	10.58	6.5116	0.6155	
Ab MiTK V71T**	Taurocyamine	1.4135	12.6055	8.9254	0.904
	Glycocyamine	0.377	3.4765	9.87245	-
PsTK WT	Taurocyamine	1.68647	21.5262	12.7641	-
	Glycocyamine	-	-	-	-
PsTK G261H	Taurocyamine	-	-	-	-
	Glycocyamine	-	-	-	-
PsTK V71A	Taurocyamine	1	0.5	0.5	0.8989
	Glycocyamine	0.8	0.445	0.55625	
PsTK WT	Taurocyamine	1.5	23.4	15.6	1.6049
	Glycocyamine	0.67	6.52	9.72	
PsTK WT	***Taurocyamine	0.52	-	-	-
	***Glycocyamine	6.1	-	-	-

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*Tanaka et. al, 2011
**Due to closeness between the two sets of data for AbmitoTK, their averages represented on the table.
***Palmer et. al., 2013

Regarding the substrate specificity in the V71T mutant, glycocyamine catalytic efficiency increased by 10-fold when compared to that of the Ab MiTK WT. When looking at both mutant and WT, you see that the ratio of taur/glyco catalytic efficiency decreased and the Km decreased by 10-fold (Table 2) in the mutant. This means that the mutant had a faster reaction rate and a better affinity for glycocyamine than taurocyamine when compared to the WT, as the mutant required a lesser amount of substrate for its reaction rate to be maximum. The Km and Kcat of the mutant's taurocyamine activity remain about the same when compared to the WT, but its catalytic efficiency decreases by 10-fold. Our Ab MiTK WT data very closely matched that of published data (difference of ±3 and ±0.01 in $K_{cat}$and Km of taurocyamine respectively), allowing us to confidently make these comparisons. This data supports our hypothesis, as we were successfully able to increase glycocyamine activity by our mutation. This suggests that the V71A mutation could have been a part of the mutation that led to the evolution of GK from TK, as glycocyamine activity in TK increases when compared to that of the WT.

Considering the differences between our PsTK WT data and that of published data, our comparison between the WT and the mutant was made based on the WT we created, as it closely resembles the conditions created for the mutant in the laboratory. According to our PsTK WT data, glycocyamine binding is better than that of taurocyamine, which contrasts published data (Palmer et. al., 2013). Regarding the substrate specificity in the V71A mutant, glycocyamine catalytic efficiency decreased by 10-fold when compared to that of the WT. When analyzing the data of the mutant and the WT, it is observed that the ratio of taur/glyco catalytic efficiency and the Km in the mutant is similar to that of the WT (Table 2). When looking at the taurocyamine, its Km is similar to that of the WT; however, its Kcat and catalytic efficiency with the mutant decreased by more than 10-fold. This implies that the mutation made the PsTK worse at its catalytic reaction with taurocyamine. Although the data does not support our hypothesis, it suggests that this mutation could be part of the path taken by GK to evolve from TK. GK's only substrate is glycocyamine. This mutation makes TK's ability to utilize taurocyamine worse, thereby making its activity closer to that of GK and reducing its promiscuity.

****Data shown in boxes highlighted blue
*****Making the cat in Kcat (K_{cat}) made the cat barely visible, so it was left as it is in the table

In regard to the G261H mutant, the data for its enzymatic characteristics gave unusable values K_{cat}, V_{max}, or Km. This suggests that this mutation could have made a modification that permanently reduced the catalytic activity of PsTK. This also suggests that the enzyme is easily saturated, which could explain the presence of negative V_{max}, K_{cat}, and Km. The enzyme could have also been destroyed by this mutation because residue-specific amino acid mutagenesis can create a risk of the protein misfolding (Goltermann et. al., 2010). Our data suggests that the 261 region is more important in PsTK general activity than it is for substrate specificity.

In previous work, the V71 region was identified to play a common role in binding guanidino substrates in AK, CK and MiTK (Tanaka et. al., 2011). Our results suggest that the V71 region also plays the same important role in PsTK and Ab MiTK and could also be a possible pathway in which the GKs evolved from the TKs. The data also supports the putative pathway, which shows that GKs evolved from TKs (Figure 2) as all the mutations in the V71 region --with the exception of the V71T mutation in PsTK-- led the TKs to act more like a GK. Although inconclusive data wereobtained from the G261H, for future work, it would be interesting to see if a nonconserved mutation would decrease the PsTK's affinity for glycocyamine.

REFERENCE

[1]. Zhang, J., Yang, J. R., 2015. Determinants of the rate of protein sequence evolution. Nature Reviews. Genetics, 16(7), 409–420.
[2]. Palmer, Begres B. N., Van Houten J. M., Snider M. J., Fraga D., 2013. Characterization of a putative oomycete taurocyamine kinase: Implications for the evolution of the phosphagen kinase family. Comp. Biochem. Physiol. B Biochem. Mol. Biol. Chem. Biol. 166, 173-181.
[3]. Ellington, W.R., Suzuki, T., 2006. Evolution and divergence of creatine kinase. In: Vial, C.
[4]. (Ed.), Creatine Kinase. Nova Science Publishers, Inc., New York, pp. 1–26.

International Journal of Advances in Engineering and Management (IJAEM)
Volume 7, Issue 08 Aug. 2025, pp: 13-30 www.ijaem.net ISSN: 2395-5252

[5]. Jourden M. J., Clarke C. N., Palmer A. K., Barth E. J., Prada R. C., Hale R. N., Fraga D., Snider M. J., Edmiston P. L., 2007. Changing the substrate specificity of creatine kinase from creatine to glycocyamine: Evidence for a highly evolved active site. Biochim. Biophys. Acta, 1774, 1519–1527.

[6]. Lim K., S. Pullalarevu, K.T. Surabian, A. Howard, T. Suzuki, J. Moult, O. Herzberg, Structural basis for the mechanism and substrate specificity of glycocyamine kinase, a phosphagen kinase family member, Biochemistry 49 (2010) 2031–2041.

[7]. Uda, K., Saishoji, N., Ichinari, S., Ellington, W. R., Suzuki, T., 2005b. Origin and properties of cytoplasmic and mitochondrial isoforms of taurocyamine kinase. FEBS J. 272, 3521–3530.

[8]. Uda, K., Hoshijima, M., Suzuki, T., 2013. A novel taurocyamine kinase found in the protist Phytophthora infestans. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 165, 42–48.

[9]. Tanaka, K., Matsumoto, T., Suzuki, T., 2011. Identification of amino acid residues responsible for taurocyamine binding in mitochondrial taurocyamine kinase from Arenicola brasiliensis. Biochim. Biophys. Acta 1814, 1219–1225.

[10]. Haiwei H. G., Juno C., Lawrence A. L., 2004. Protein tolerance to random amino acid change. PNAS. 101 (25), 9205-9210.

[11]. Goltermann, L., Larsen, M. S. Y., Banerjee, R., Joerger, A. C., Ibba, M., Bentin, T., 2010. Protein evolution via amino acid and codon elimination. PLOS ONE 5(4): e10104

APPENDIX

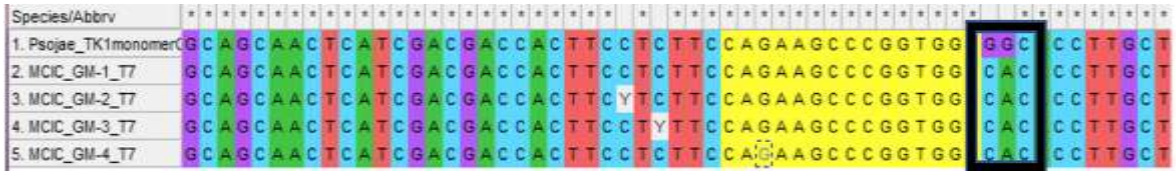

Figure 7: Sequencing of Purified Plasmids. The WT sequence is in the first row, while the sequence of the mutated colonies is in the remaining rows. The region of interest is highlighted in a black box.

Table 3: Linked Assay result of Taurocyamine with PsTK G261H

Sample (Taurocyamine) (µL)	Slope (Abs/min)	Abs/sec	Concentration of NADH (M)
0	0.0039	0.000065	1.04502E-08
6.3	0.0057	0.000095	1.52733E-08
9.4	0.0106	0.000176667	2.8403E-08
12.5	0.001	1.66667E-05	2.67953E-09
25	0.0136	0.000226667	3.64416E-08
125	0.0041	6.83333E-05	1.09861E-08

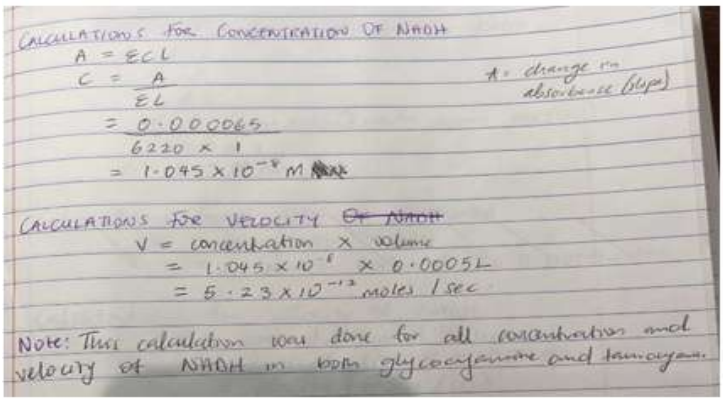

Figure 8: Sample calculations of data present in the tables.

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The data gotten from the linked assay was used to calculate the data points used in determining the enzymatic characteristics of the PsTK G261H. A = change in absorbance over time, concentration = concentration of NADH calculated from A, velocity = variation of absorbance per unit time, volume = total volume of reacting mixture presents in each cuvette used in the linked assay.

Table 4: Data used to obtain the Michaelis Menten Graph for Taurocyamine

Concentration of Taurocyamine (μM)	Velocity (mol/sec)
0	5.22508E-12
0.252	7.63666E-12
0.376	1.42015E-11
0.5	1.33976E-12
1	1.82208E-11
5	5.49303E-12

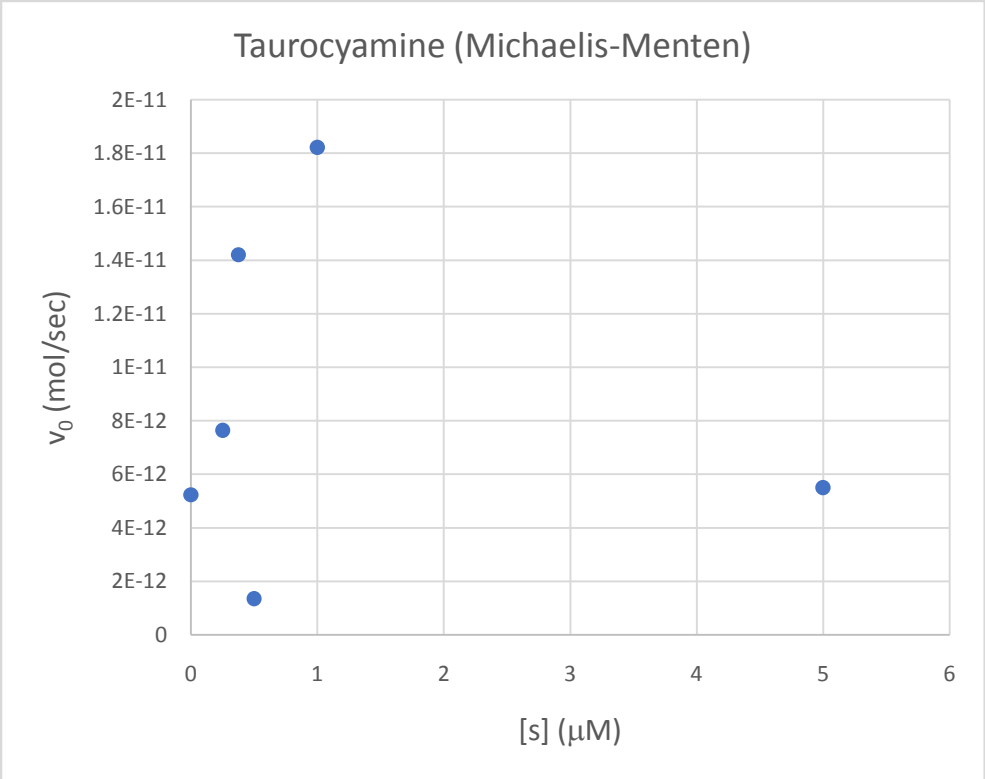

Figure 9: Michaelis-Menten Graph of Taurocyamine Reaction with PsTK G261H. The data obtained from the linked assay was used to create the graph.

Table 4: Data used to obtain the Lineweaver Burk for Taurocyamine

1/[s] (1/μM)	$1/v_0$ (mol/sec)
3.968253968	1.30947E+11
2.659574468	70415094340
2	7.464E+11
1	54882352941
0.2	1.82049E+11

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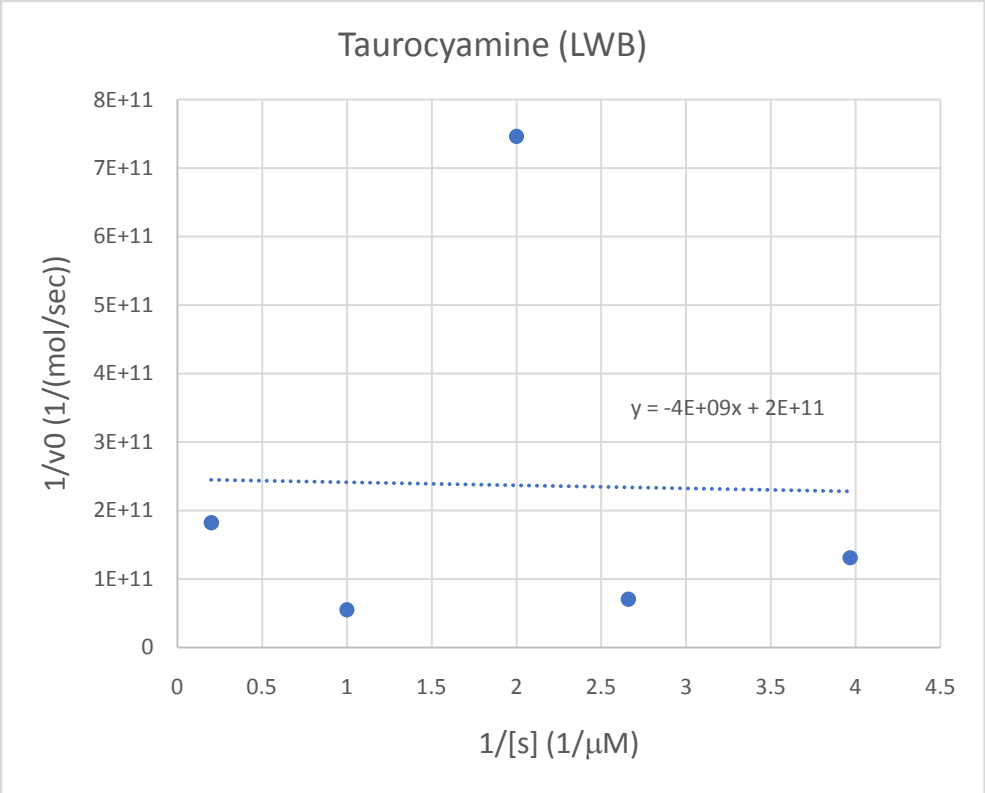
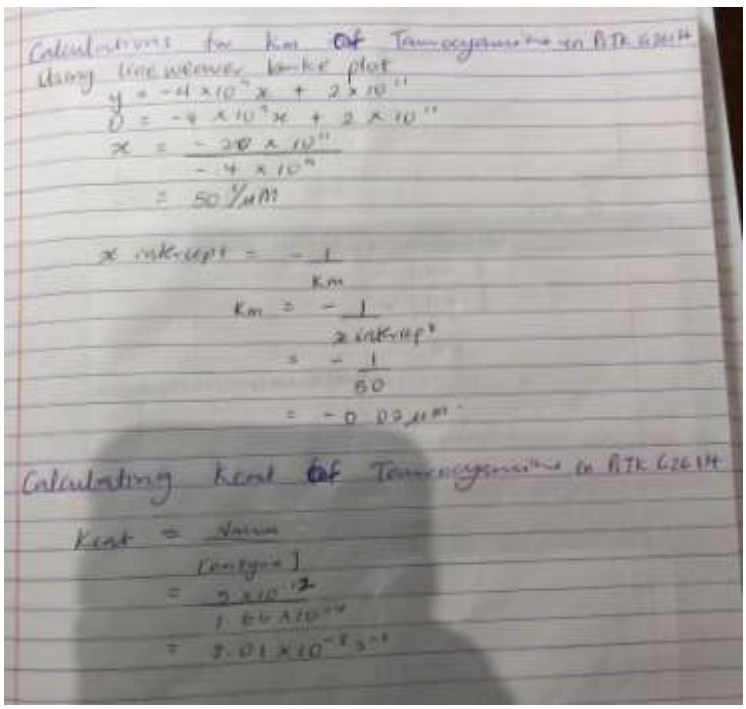


Figure 10: Lineweaver-Burk Graph of Taurocyamine Reaction with PsTK G261H. This data was used to calculate the Km, Vmax, Kcat, and catalytic efficiency of the enzyme's reaction with Taurocyamine.

a.



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b.

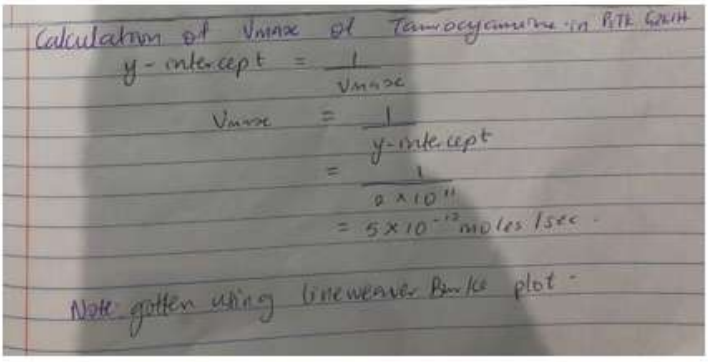

Figure 11: Calculations on the enzymatic characteristics of PsTK G261H with Taurocyamine. The data obtained from the Lineweaver-Burk was used to calculate the Vmax, Km, and Kcat.

Table 5: Linked Assay result of Glycocyamine with PsTK G261H

Sample (Glycocyamine) (µL)	Slope (Abs/min)	Abs/sec	Concentration of NADH (M)
0	0.0006	0.00001	1.60772E-09
6.3	0.0052	8.66667E-05	1.39335E-08
9.4	0.0017	2.83333E-05	4.5552E-09
12.5	0.0009	0.000015	2.41158E-09
25	0.0005	8.33333E-06	1.33976E-09

Table 6: Data used to obtain the Michaelis Menten Graph for Glycocyamine

Concentration of Glycocyamine (µM)	Velocity of NADH (mol/sec)
0	8.03859E-13
0.252	6.96677E-12
0.376	2.2776E-12
0.5	1.20579E-12
1	6.69882E-13

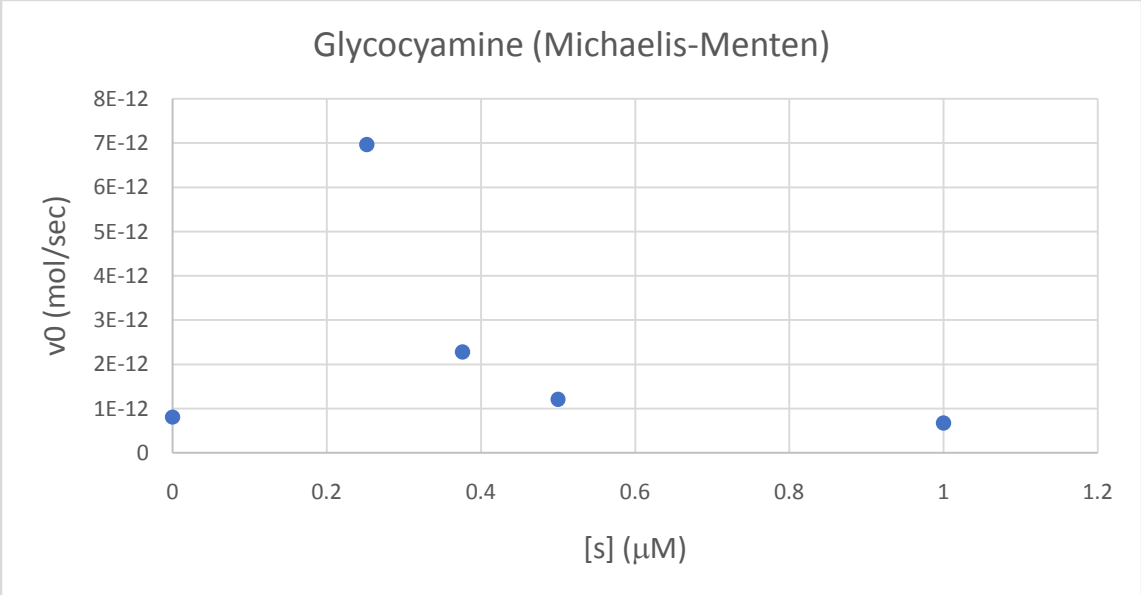

Figure 12: Michaelis-Menten Graph of Glycocyamine Reaction with PsTK G261H. The data obtained from the linked assay was used to create the graph.

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Table 4: Data used to obtain the Lineweaver Burk for Taurocyamine

1/[s] (1/μM)	1/v0 (mol/sec)
3.968253968	1.30947E+11
2.659574468	70415094340
2	7.464E+11
1	54882352941
0.2	1.82049E+11

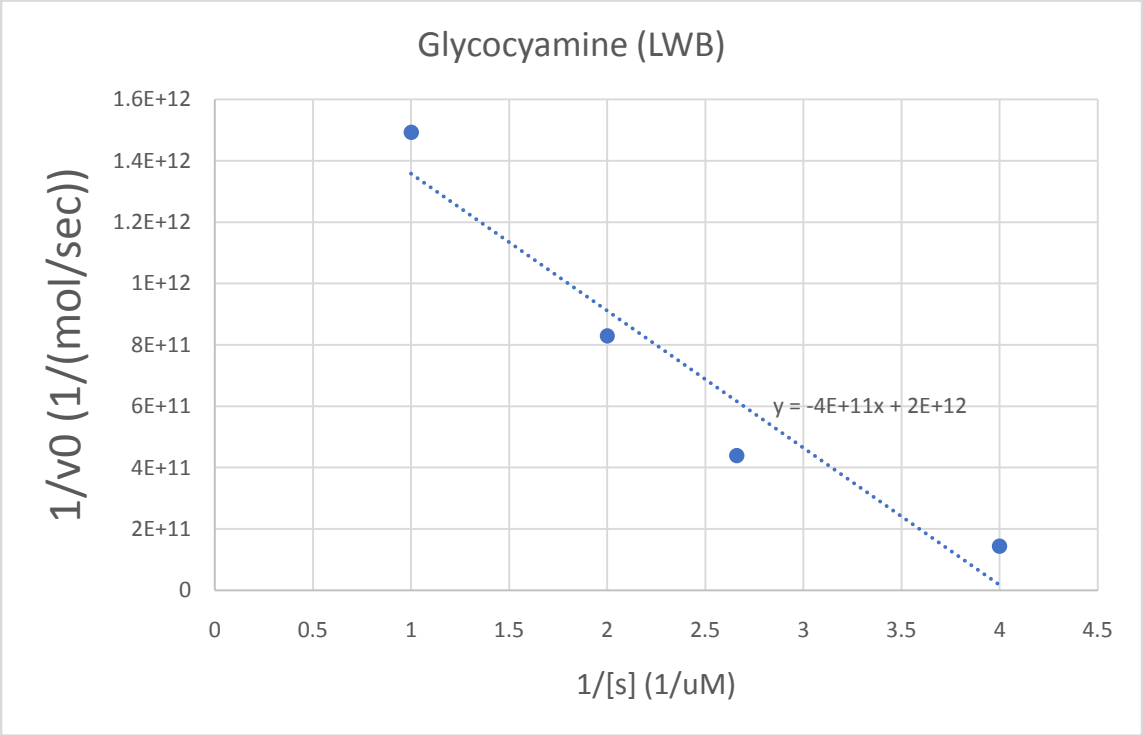
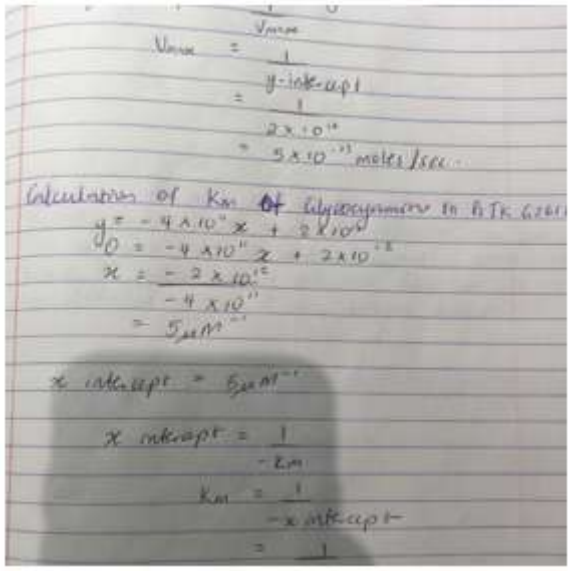


Figure 13: Lineweaver-Burk Graph of Glycocyamine Reaction with PsTK G261H. This data was used to calculate the Km, Vmax, Kcat, and catalytic efficiency of the enzyme's reaction with Taurocyamine.

a.



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b.

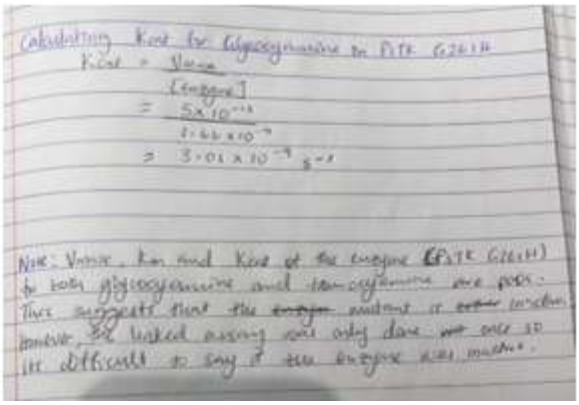
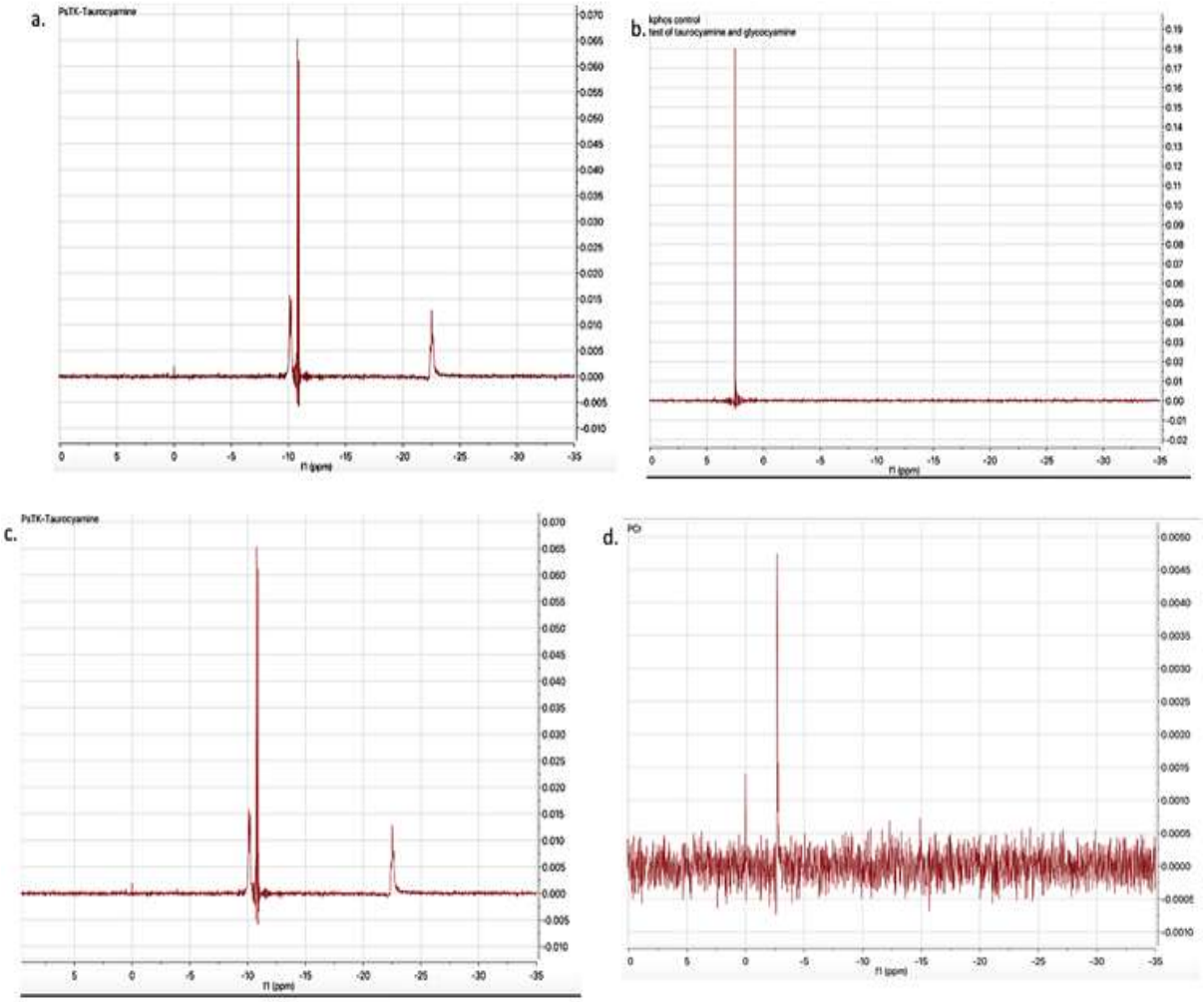


Figure 14: Calculations on the enzymatic characteristics of PsTK G261H with Taurocyamine. The data obtained from the Lineweaver-Burk was used to calculate the Vmax, Km, and Kcat.



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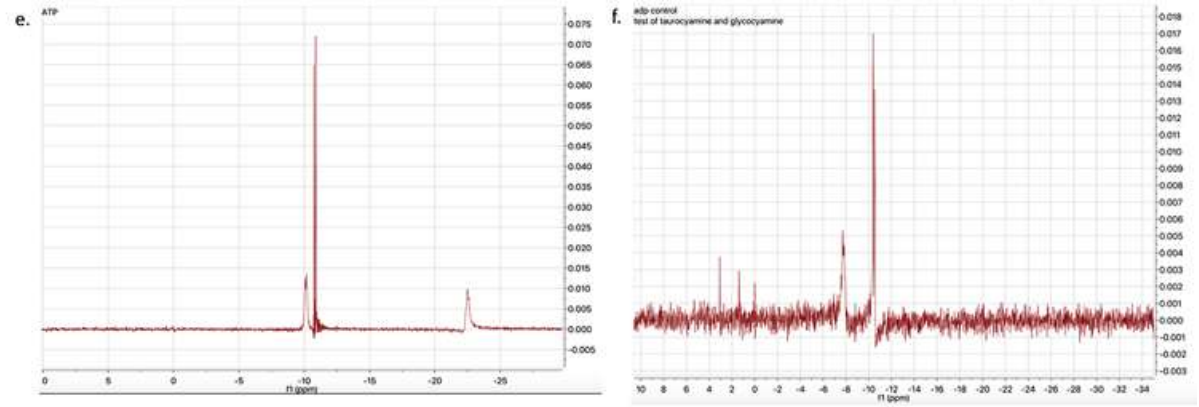

Figure 15: Control data for ^{31}P NMR .a) ^{31}P NMR data for PsTK WT with Taurocyamine b) ^{31}P NMR data for Potassium Phosphate c) ^{31}P NMR data for PsTK WT with Taurocyamine d) ^{31}P NMR data for PCr e) ^{31}P NMR data for ATP f) ^{31}P NMR data for ADP