HUMAN GLUTATHIONE SYNTHETASE REACTION ORDER AND KINETICS EXAMINED USING SPECTROSCOPIC ANDCALORIMETERIC TECHNIQUES

A THESIS

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DEDICATION

To my beloved Jowdar and Dominic who supported me and held down the fort at home whilst I spent long hours of work in the lab. I am forever grateful for you both.

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Thank you first and foremost to Dr. Anderson, an individual that possess the rare ability to both drive students to greater heights and support and encourage them along the way. I am extremely thankful to have someone so full of knowledge to turn to for troubleshooting and support. I am also extremely appreciative of Dr. Anderson and Dr. Tom Cundari's ability to consistently cut through my verbosity and help me to produce a polished thoughtful piece of writing. I would also like to thank my committee members, Dr. Heather Conrad-Webb and Dr. Nasrin Mirsaleh-Kohan for their thoughtful questions and suggestions as well as their overall support and kindness. Thank you to the Texas Woman's University Chemistry and Biology departments as well as the graduate school for their financial support, guidance in completing all research, paperwork and thesis preparation. Also, to my family that helped watch and comfort my son so that I could pursue an education to better all of our lives.

ABSTRACT

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HUMAN GLUTATHIONE SYNTHETASE: NEGATIVE COOPERATIVITY AND BINDING STUDIES

DECEMBER 2018

Glutathione (GSH) is a tripeptide important in preventing cellular oxidative damage. Human glutathione synthetase (hGS) catalyzes the second stage of GSH biosynthesis. Homodimeric hGS is negatively cooperative with respect to its L-γ-Glu-Cys substrate. Although the allosteric effects of substrates binding to hGS have been studied, the order of substrate binding has not. GS in plants and prokaryotes are reported to exhibit opposing random ter and ordered ter ter reaction orders respectively; currently little is known about the reaction order in humans. Knowledge of the mechanism and reaction order of hGS is vital to understand how it contributes to the regulation of the levels of the limiting amino acid cysteine and of glutathione. Using ITC binding studies the mechanism of action and reaction order of hGS has been evaluated and suggests a semi-ordered reaction in human GS.

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ABBREVIATIONS

DSC – Differential scanning calorimeter or differential scanning calorimetry GAB - Glutamyl-α-aminobutyrate

γ-GC - γ-glutamylcysteine synthetase GS – Glutathione synthetase

GSH - Glutathione

HGS – Human glutathione synthetase

ITC – Isothermal titration calorimetry or Isothermal titration calorimeter LB - Luria-Bertani

SUPPLEMENTAL MATERIALS

Figure 1. Sample DSC (differential scanning calorimeter) for wild type hGS, melting point 58.82˚C (Gaussian curve fit) 0.01 mM phosphate buffer temperature 10-90˚C tested.

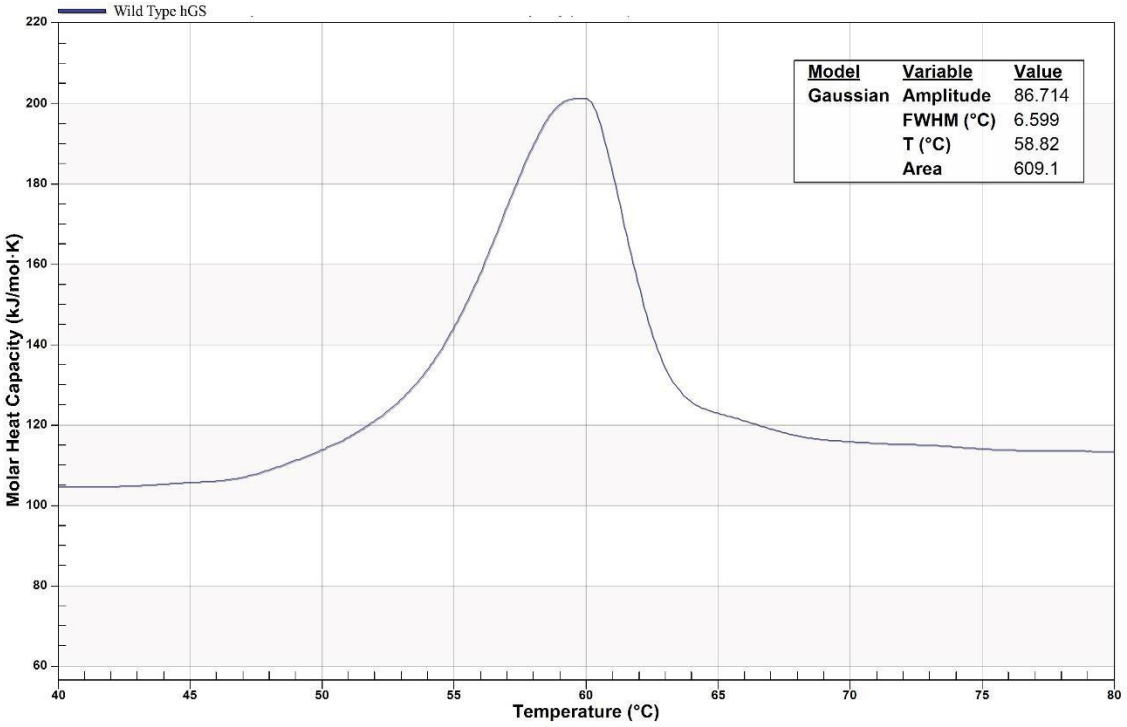


Figure 2. (A) Representative raw data of glycine (50 mM) injected into hGS (0.0126 mM). The final concentration of glycine in hGS was 8.9 mM. (B) Glycine injections with blank control data removed (molar ratio vs enthalpy).

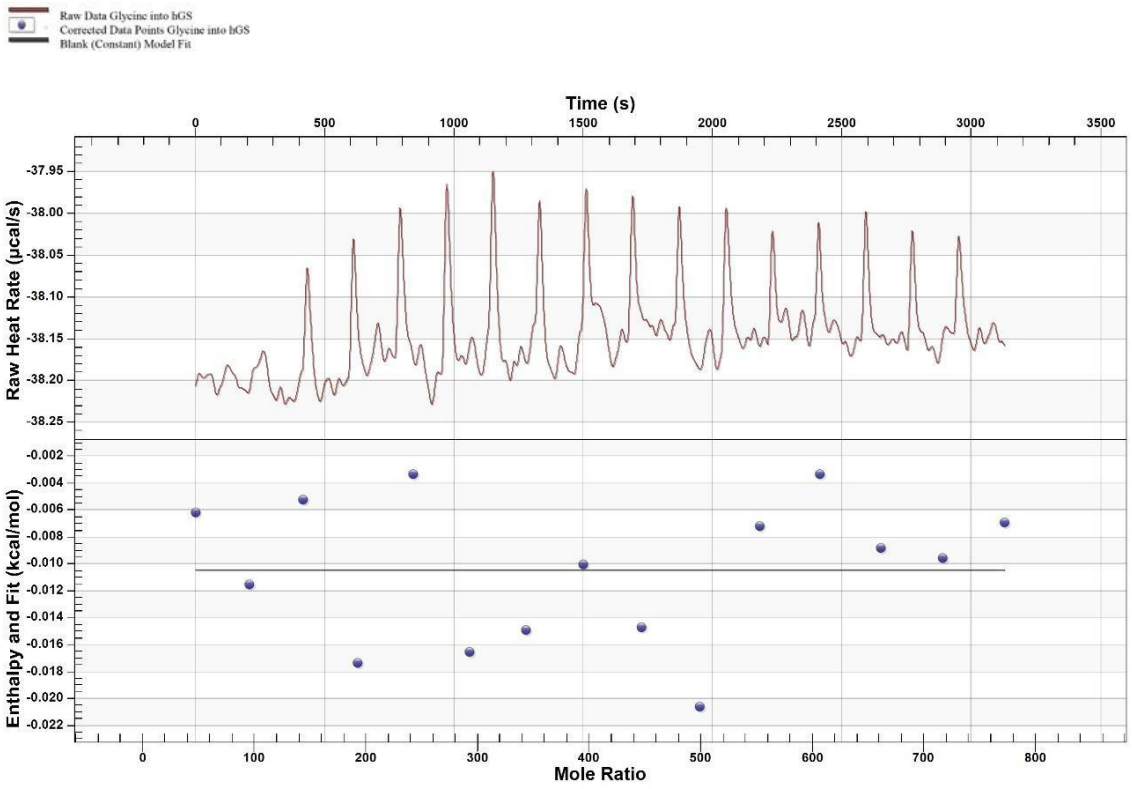


Figure 3. (A) Representative raw data of magnesium ion (0.245 mM) injected into hGS (0.00495 mM) in 0.01 M phosphate buffer. The final concentration of magnesium in hGS was 0.037 mM. (B) Magnesium injections with blank linear control data removed (molar ratio vs enthalpy). -6.611 ± 0.487 µJ, slope 0.0280 ± 0.05, N = 2.

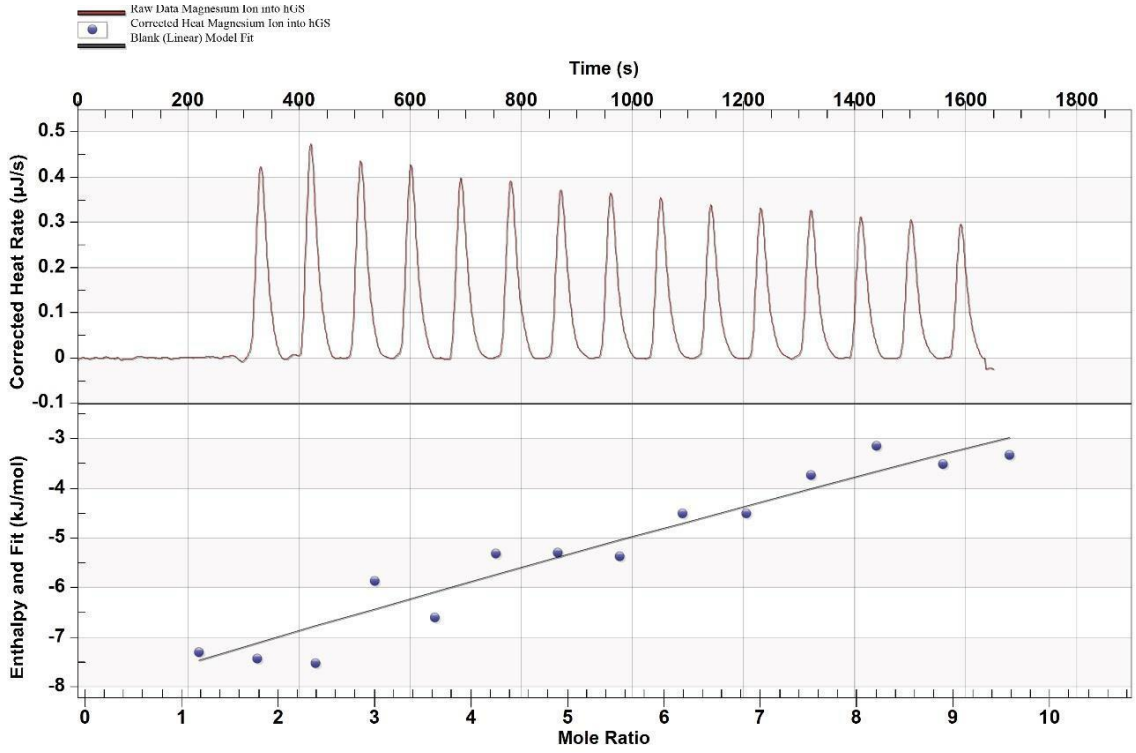
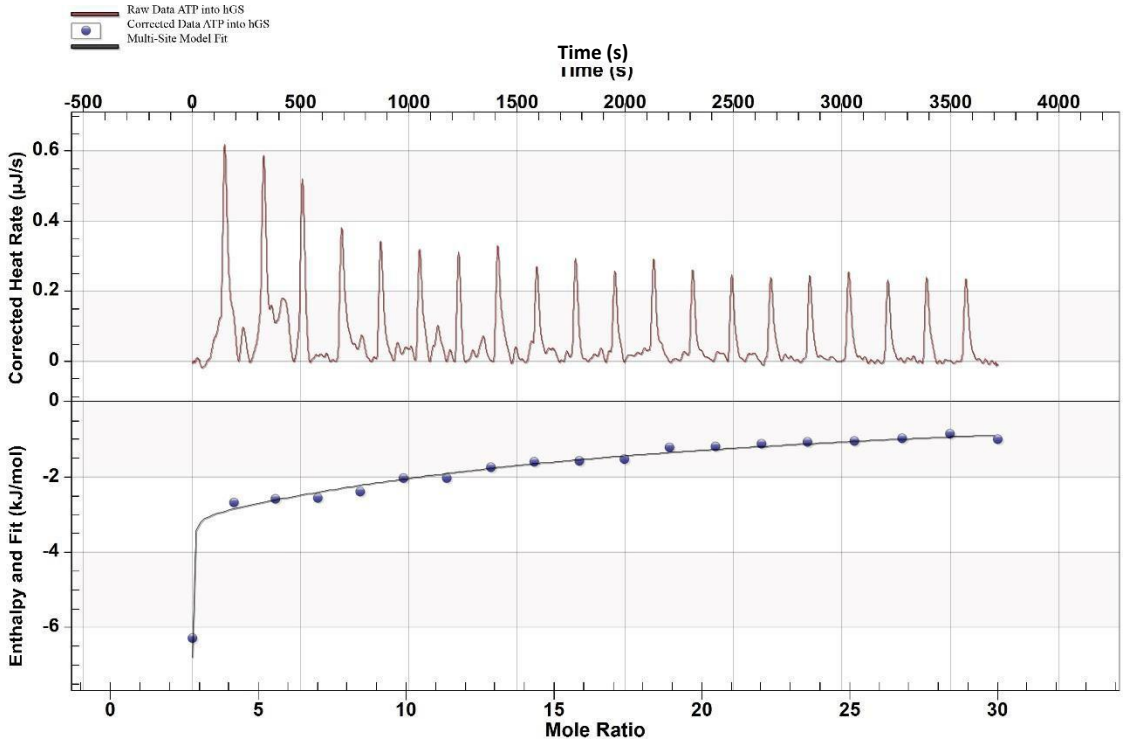


Figure 4. (A) Representative raw data of ATP (1.85 mM) injected into hGS (0.012 mM). The final concentration of ATP in hGS was 0.280 mM. (B) ATP injections with multisite model fit (molar ratio vs enthalpy). $N = 1$.



CHAPTER 1

INTRODUCTION

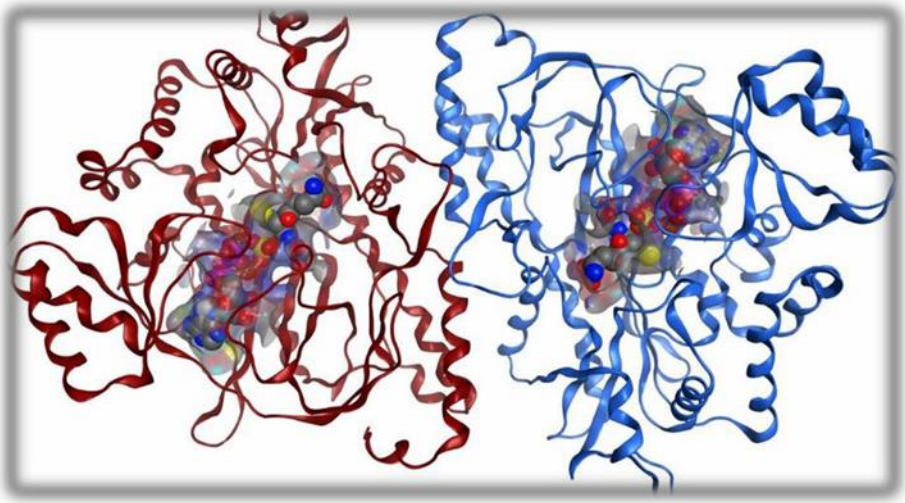
The importance of enzymes in nature is multifold. Their main function is to act as catalysts to lower reaction activation energies thereby making reactions faster. The ability of enzymes to function may be altered in many ways. For example, an enzyme's ability to function may be directly affected through the up or down regulation of its production. Other molecules may also inhibit enzymes reversibly or irreversibly. In addition, an enzyme may use cooperativity in order to self-regulate its function[1].

Regulated enzymes are often found at key regulatory points in metabolism in organisms and provide multiple ways in which to alter their activity. Enzymes have served as targets for research scientists that desire to regulate malfunctioning metabolic systems. Thus studying enzyme activity and its regulation provides key insight into how organisms function and the biochemistry and molecular biology of disease states. Additionally, studies of enzyme regulation have permitted scientists to target drug therapies to treat disorders that result from or are related to enzymatic activity[1].

One enzyme that has been of particular interest is glutathione synthetase (GS). It is found in all organisms (although the structure varies slightly between species [see Figure 1]).[2,3] GS is the second enzyme used in the two-step production of the antioxidant glutathione. Glutathione (γ-glu-cys-gly), GSH, is of interest because it is a tripeptide important in preventing cellular oxidative damage[4]. Both deficient and excessive amounts of glutathione and the enzymes involved in its synthesis are associated with numerous physiological and neurological disorders and diseases. Glutathione imbalance has been

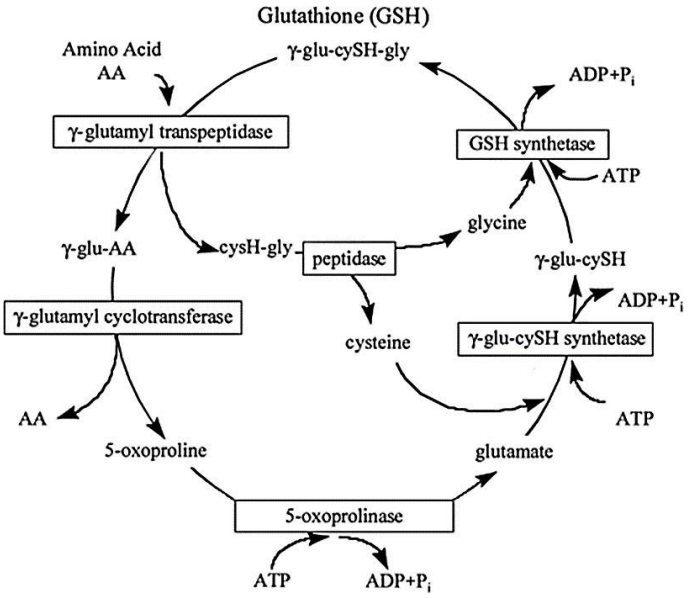
associated with 5-oxoprolinuria, cardiovascular disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), early aging (wrinkling), cataracts, macular degeneration, diabetes and susceptibility to infections and resistance to radiation and chemotherapeutic drugs.[5,6,7,8,9,10] In addition, genetic defects such as decreased neural development are present when the levels of glutathione or its associated enzymes are abnormal during gestation and a complete lack of glutathione is fatal in mammals.[10,11,12]

Figure 1. Human glutathione synthetase.[2]



A proposed reason for the widespread damage caused by glutathione deficiency is the role glutathione plays in the transport and metabolism of crucial biomolecules such as cysteine[13] (see Figure 2) and its possible regulatory function at key metabolic pathways.[5,14] It is theorized that the regulation of glutathione production and the enzymes involved in its production act as a metabolic checkpoint in the human body for glutathione and for the limiting amino acid cysteine.

Figure 2. Mammalian cysteine metabolism.[11]



Human glutathione is synthesized intracellularly in two ATP-dependent steps and utilizes two enzymes: γ-glutamylcysteine synthetase (γ-GCS) and human glutathione synthetase (hGS; see Figure 3).[11,15] The first step (eq 1, P_i = phosphate) of glutathione synthesis is controlled through up or down regulation of the amount of enzyme γ-GCS, and feedback inhibition from glutathione; the enzyme utilized in the second step (eq 2), hGS, appears to only regulate production of glutathione through negative cooperativity, [16] a process by which the binding of one substrate leads to decreased ability of a second substrate to bind in a second site of the enzyme.

Figure 3. Glutathione production steps.[15]

$$\textbf{L-Glu + L-Cys + ATP} \rightarrow \textbf{L-}\gamma\textbf{-Glu-Cys + ADP + P}_i \qquad \textbf{(1)}$$

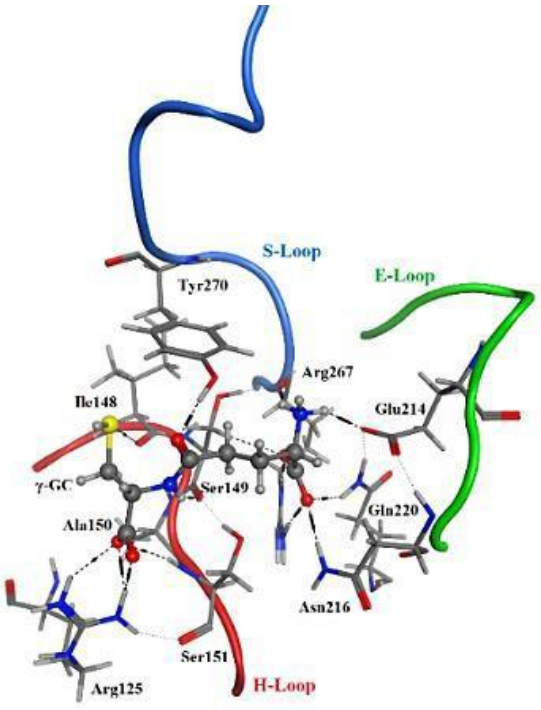
$$\textbf{L-}\gamma\textbf{-Glu-Cys + Gly + ATP} \rightarrow \textbf{GSH + ADP +P}_i \qquad \textbf{(2)}$$

Interestingly, hGS is able to exhibit allostery no matter which binding site the substrate binds to first. This is due the fact that hGS is comprised of two identical subunits and is thus known as a homodimer (see Figure 1).[2,15,17,18] Homodimeric enzymes are

comprised of two structurally identical subunits. Each of hGS's subunits contains an identical active site in which the three substrates ATP, glycine, and γ-L-glutamyl-L-cysteine (L-glutamyl- α-aminobutyrate [GAB] in laboratory studies) bind.

The allosteric movement of secondary structures in hGS and the subsequent binding of substrates to the enzyme have been studied using computational analysis[19] These studies have shown that the three loops in hGS's active site, which have been named the A, G, and S loops (see Figure 4) move fluidly to allow for the binding of its three substrates with great specificity to the active site. Analysis has shown that these substrates likely bind through specific potentially ordered molecular interactions at these sites.[19] Once substrates are bound to the active site it is believed that an ATP-Grasp site closes and allows the production of glutathione to happen more readily.[19,20]

Figure 4. hGS active site.[19]



Enzymes such as hGS comprise the ATP-grasp superfamily of enzymes and consist of only 21 known proteins that exhibit an ATP-grasp fold. ATP-grasp enzymes all contain two domains consisting of an α-helix and β-pleated sheets that allow ATP to bind under a protective lid region within the active site. [20] These folds allow for tighter binding of ATP and additional protection of the catalytic site from hydrolysis. Enzymes that have this region are known to be important to key metabolic functions in the human body such as pyruvate carboxylase, which helps regulate gluconeogenesis, as well as propionyl-CoA carboxylase that is necessary to breakdown fatty acids.[20]

We propose that the unique ATP-binding lid is one indicator that the binding of substrates to hGS is a semi-ordered or completely ordered process. We posit that ATP must bind to the ATP-grasp fold region of the active site in an ordered fashion to allow for the production of glutathione. This binding allows for the release of energy from the hydrolysis of ATP to ADP and inorganic phosphate that simultaneously allows the ATP-grasp lid to close and provides energy for the reaction to synthesize glutathione. The release of energy and the protected lid structure likely provides a favorable environment for glycine and γ-glutamylcysteine to interact and convert into glutathione.

While the reaction order of mammalian GS was previously unstudied, its negative cooperativity related to its active sites has been investigated at length.[21] Homodimeric hGS is negatively cooperative with respect to its L-γ-Glu-Cys substrate.[21] Therefore, as L-γ-Glu-Cys increases, hGS's affinity for it decreases. This further suggests that L-γ-Glu-Cys participates in a regulatory mechanism for the synthesis of glutathione. As stated by Bush, negative cooperativity is theorized to be a poorly understood method of regulation of important metabolic pathways in the human body.[22] In recent years increasing numbers of

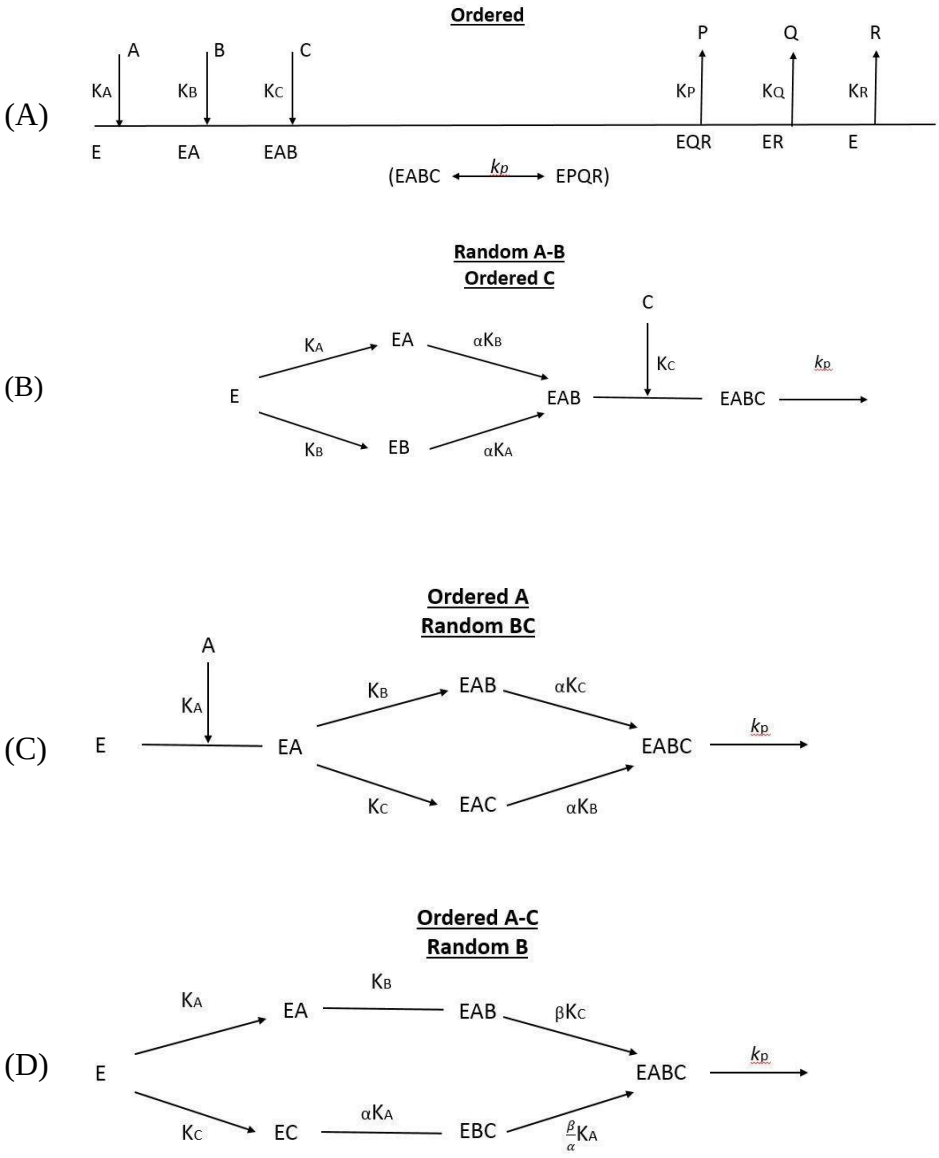
enzymes have been found to exhibit negative cooperativity thereby bolstering Bush's claim that negative cooperativity plays a larger role in metabolism than previously thought.

The active site regions of hGS are known to be highly conserved across species.[19] Mutations in the active site region of hGS have been tied to specific disease and disorders in humans[19]. These types of mutations often lead to a build-up or a deficiency of glutathione, which often causes severe medical complications or even death.[5,6,7,8,9,10, 11,12]

The importance of the specific structure of hGS may be due to the need of hGS's substrates to bind in a specific order. If structural changes occur at hGS's active site this could affect the order and the ability for substrates to bind. To better understand the effects of structural changes either from mutations and or negative cooperativity it is important to know if the three substrates involved in hGS's reaction must bind in a specific order or not.

Previous experiments indicate that GS in plants exhibits a random ter reaction order whereas GS in prokaryotes had an ordered ter ter reaction order; no potential reaction order has been reported in humans.[23,24] Possible mechanisms for GS in humans include random ter (no order) in which all substrates bind in any order or simultaneously, semi-ordered in which at least one substrate must bind first, and completely ordered in which each substrate must bind in a specific order (see Figure 5).[25]

Figure 5. Reaction orders for enzyme binding.[25]



(A) A completely ordered reaction in which each of the three substrates must bind one after the other in a specific order. (B) A semi-ordered reaction in which one of the three substrates must always bind last and the other two bind randomly. (C) A semi-ordered reaction in which one substrate must always bind first and the other two randomly. (D) A reaction in which one substrate must always bind second and the other two bind randomly.

A random ter reaction in this case would mean that ATP, glycine, or γ-L-glutamyl- L-cysteine could bind in any order to hGS and the reaction would proceed in the same manner regardless of which substrate was bound first or if they bound concurrently. A semi-ordered reaction for hGS would require that one specific substrate binds first, second or third and the remaining two substrates bind randomly to the active site (see Figure 5).[25] Finally, a completely ordered reaction would mean that each of the three substrates are required to bind to hGS's active site in a specific order for the reaction to proceed to completion.

Reaction order may be studied using coupled kinetic assays or using newer methods involving isothermal titration calorimetry (ITC) to measure the heat of binding. An isothermal titration calorimeter (ITC), which reveals the enthalpy of binding, may be used to study the heats of reaction of binding of hGS with its substrates in order to determine cooperativity and reaction order.[22,26] Thus, if little or no reaction occurs when one substrate is in the presence of hGS then other substrates likely need to bind first or simultaneously for the production of glutathione.

Studies of the reaction order of hGS as well as its corresponding kinetic data should reveal more about the role that this enzyme's negative cooperativity plays in the regulation of amino acids and glutathione metabolism in humans. If this enzyme is shown to act as an essential regulator of glutathione levels, it could uncover novel ways in which humans maintain levels of vital constituents of metabolic functions.

CHAPTER 2

METHODS AND MATERIALS

Enzyme Preparation – Human hGS protein was prepared by expression of the HGS-pET-15b plasmid in BL21(DE3) *E.coli*. The *E.coli* was grown at 37˚C on LB (Luria-Bertani) agar plates (5×10^{-3} g/mL NaCl, 5×10^{-3} g/mL yeast extract, 0.01 g/mLpeptone, 0.015 g/mL agar, 2.4×10^{-3} M NaOH, 150 mL reverse osmosis [R.O.] water and containing 50 µg/mL ampicillin) for 12-14 hours until distinct colonies formed. Two isolated colonies were then selected and streaked onto two different LB agar plates containing 50 µg/mL ampicillin and grown for another 12-14 hours. One isolated colony from each plate was then placed into mini cultures (10mL of LB broth containing 50 µg/mL of ampicillin) and then incubated on a shaker set to 37˚C and 275 rpm for 12-16 hours. Each of the 10 mL mini cultures were then placed into a separate 2L flask containing 1L each of LB broth containing 50 µg/mL of ampicillin each. These flasks were incubated on a shaker at 37˚C and 275 rpm. *E.coli* growth was checked on a Shimadzu UV-2600 spectrophotometer at 600 nm approximately every hour until the absorbance reached approximately 1. The cultures were then chilled on ice for 30 minutes to suspend growth. A 1 mL sample was saved as a before induction control to be tested on a 12% SDS page gel.

Enzyme Induction – The GS enzyme was induced by placing 1.91×10^{-4} g/mL of isopropyl β-D-1-thiogalactopyranoside **(**IPTG) into each of the two flasks and incubating cultures on a shaker at 19˚C and 275 rpm for four hours. An after induction control (1 mL) was collected for later testing. The induced cells were then harvested by centrifugation the cultures (10 mins at 4 ºC, 5000 g) in centrifuge tubes. The supernatant was discarded and

the cell pellets were washed with 15 mL of 0.85% NaCl and resuspended by shaking in the incubator at 4˚C at 275 rpm for approximately 10 minutes. The cells were then consolidated into two centrifuge tubes and centrifuged for 10 mins at 4 ºC, 5000 g and the saline solution discarded. The cells were stored overnight on ice in a 4 ºC refrigerator. The following morning the cells were resuspended in centrifuge tubes with 7 mL of MCAC-0 (metal-chelate affinity chromatography solution (20 mM Tris-Cl, 10 % sterile glycerol and 0.5 M NaCl) and resuspended by shaking in the incubator at 4˚C at 275 rpm for approximately 10 minutes. The resuspended pellets were consolidated into one 50 mL falcon tube. The cell solution was pipetted into a Constant Systems model O0/40/AA constant cell disruptor 5 mL at a time and ruptured under 15 kpsi of pressure. The mixture was subsequently sonified using a Branson digital sonifier on ice at pulse intervals of 0.5 seconds on and 0.5 seconds off for two minutes total time at an amplitude of 35%. A lysis control before centrifugation sample of 100 µL was then collected to be tested later. The lysed cells were centrifuged for 20 minutes at 4˚C at 10,000 g and a lysis control after centrifugation sample of 100 µL was collected. The supernatant that contained the hGS was placed into a sterile 50 mL falcon tube and the pellet saved for further testing.

Purification – The supernatant containing the cell lysate was purified using Ni-NTA chromatography beads (Novagen 70666). The nickel column was cleaned with 50 mL of MCAC-1000 (0.5 M NaCl, 20 mM Tris, 1mM imidazole, 10% sterile glycerol), 50 mL of reverse osmosis water and charged with 50 mL 0.1 M Ni-SO_4 the day prior to use. MCAC-0 was then washed through the column overnight.

The lysed cells were added to a glass Biorad econo-column (1 × 20 cm) filled approximately 5 cm deep with nickel chromatography beads and the Biorad econo pump

was set to 0.5 mL/min and cells were monitored using a Biorad econo chart recorder (model 1325) until nearly all of the sample passed through the column. The column was then developed with MCAC-0 buffer (1.0 mL/min) and fractions were taken every 14 minutes. The OD was checked at 280 nm by the econo chart recorder to monitor protein elution. Once the chart recorder showed that the sample had reached baseline, MCAC-55 (5.5% MCAC-1000 and 94.5% MCAC-0) was then added and the column and fractions were collected every 14 minutes until the chart recorder showed that the sample had once again reached baseline. Lastly, MCAC-100 (10% MCAC-1000 and 90% MCAC-0) was added and samples were taken every 2 minutes until the recorder exhibited a return to baseline. The fractions of MCAC-100 were collected until the chart recorder showed that the sample had reached baseline. The 2 mL fractions collected contained the hGS and were subsequently dialyzed at 4˚C to prepare the samples for storage and further experimentation. One hundred microliters of every fraction were saved and run on a 12% polyacrylamide gel with the previously saved samples before and after induction, before and after centrifugation as well as the pellet to confirm hGS production.

Purified samples from the MCAC-100 fractions were then placed in dialysis tubing (VWR Scientific MWCO 12-14,000) and then into dialysis buffer (0.02 M Tris-Cl, 0.001 methylene diamine triacetic acid [EDTA]) and stored at 4˚C with stirring.

Assays – Concentrations of the dialyzed samples were determined using a Folin-Lowry assay with standards between 0-40µg of bovine serum albumin (BSA).

Activity and kinetics were determined using a pyruvate kinase (PK) coupled assay (see Figure 6). This is assay indirectly measures the conversion of GAB into ophthalmicacid. The ADP produced along with ophthalmic acid reacts with

phosphoenolpyruvate (PEP) and creates pyruvate and ATP. Pyruvate and NADH then bond and form lactate and NAD^+. NAD^+ production is monitored as a reduction of absorbance at OD 340 nm on a spectrophotometer. These results are then refined by subtracting controls causing OD changes due to the reactivity of ATP, GAB, and glycine.

Figure 6. Pyruvate Kinase coupled assay.

(1) γ-glutamylcysteine + ATP + glycine $\xrightarrow{hGS}$ glutathione + ADP + P_i

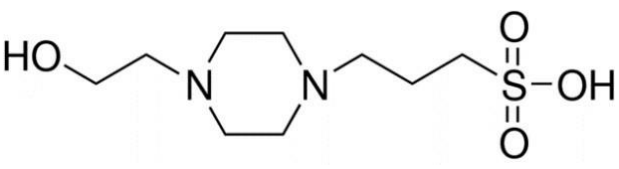
(2) phosphoenolpyruvate + ADP $\xrightarrow{PK}$ pyruvate + ATP

(3) pyruvate + NADH $\xrightarrow{LDH}$ lactate + NAD^+

Assay data was analyzed using Sigma Plot 12.5 to confirm the activity and kinetics of the purified enzyme was within the known parameters for wild type hGS (K_{cat} = 18 ± 2/sec, K_m = 1.42 ± 0.1 mM, V_{max} = 12.4 ± 1.0 µmol/min, Hill = 0.69 ± 0.03, T_m = 60˚C [by DSC, *Supplement 1*]).

Preparation of hGS for ITC - Samples with higher concentrations of wild type (WT) hGS were dialyzed in an ITC compatible buffer, 0.01M EPPS **(**4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid, 4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid, *N*-(2-Hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid) pH 7.4)[28] for 6-8 hours at 4˚C with stirring (see Figure 7).

Figure 7. EPPS Buffer.[27]



Protein Estimation of ITC Samples - Because EPPS buffer interacts with the compounds in the Folin-Lowry method of protein estimation, ITC protein samples were run on 12% acrylamide gels then stained using coomassie blue and analyzed against samples with known concentrations on a Li-Cor Odyssey CLX imager. The gels were scanned using the Li-Cor imager and the intensity of the signal of a known protein sample was compared to the signals of the unknown samples to determine the final concentration of the unknown samples containing EPPS buffer.

Preparing Samples for ITC – All substrate samples were prepared by adding powdered substrates into 0.01 M EPPS buffer excluding the preparation of the cofactor for ATP, magnesium ions which were diluted from a 1 M $MgCl_2$ solution using 0.01 M EPPS buffer and the pH was tested using Jovitech (B079K71HJM) and Whatman brand pH paper to confirm the samples were pH 7.4 so as to avoid buffer mismatch. Aliquots of hGS were taken from the stocks for each experiment to avoid exposure to air and damage of the stock solutions of hGS due to temperature and pressure changes. All samples, controls, and reverse osmosis water (used for the reference cell) were degassed on a TA Instruments degassing station for 10 minutes at 25˚C and 380 mmHg (0.5 atm) to remove bubbles and achieve similar temperature prior to experimentation.

Determination of Substrate Concentrations – Substrate concentrations need to be within a range that is large enough to exhibit a reaction between the titrant and titrand (if one exists) but small enough to prevent large heats of reaction simply due to dilution effect. In determining conditions of an ITC experiment, the titrant and titrand are usually referenced by the ratio of each sample to one another rather than exact concentrations. Determination of appropriate ratios requires some trial and error. Ratios that are too low

result in flat isotherms whereas excessively high ratios exhibit sharp vertical isotherms both of which are not as accurate when fitting ITC binding models. Typical starting ratios are 10-100 times more titrant than titrand.

Two methods were used to determine the appropriate concentrations of substrates. One, the traditional method of finding a ratio as listed above between titrant and titrand. Using this method, the concentrations of substrates were calculated by multiplying the concentration of the sample of hGS in the sample cell by the desired ratio of hGS to substrate(s). For example, if the sample cell contained 0.5 mM hGS and the test was to be conducted with the titrant glycine that was one hundred times (100X) the concentration of the sample of hGS then the concentration of the glycine titrant would be:

0.5 mM hGS × 100 x glycine = 50 mM glycine

Substrates were tested in ranges from 1X-2000X the concentration of the sample in the cell to the sample in the syringe. The atypically high ratios were used to confirm that in certain circumstances a lack of binding of substrate to hGS was due to an inability to bind and not due to insufficient substrate.

The second method of determining substrate concentrations was based on known K_m values of the substrates for wild type hGS. For these experiments hGS samples were diluted to a concentration of 0.012 mM hGS and the substrate concentrations were set based on known K_m values of: ATP (0.012 mM-0.248 mM), γ-GC (1.26 mM) and glycine (0.529 mM).[21,29] For experiments with 20 injections the K_m value of the substrate was reached between the 17th and 18th injection and for reactions with 30 injections the K_m value was reached at the 26th injection.

ITC Settings, Loading – A TA Instruments NanoITC low volume (model 601000) was utilized for all experiments. The machine was set to 25˚C, between 150-300 rpm stirring rates, 180-second intervals between injections, expected equilibrium for baselines were set as either small or medium, with a timeout of the equilibrium between 600-2100 seconds. A volume of degassed reverse osmosis water was placed into the reference cell for each experiment that was equal to the initial volume placed into the sample cell these volumes ranged from 280-380µL.

The Hamilton loading syringe as well as the 50µL injection syringe were washed with degassed R.O. water and subsequently with 0.01M EPPS buffer prior to loading samples into the ITC to clean the syringes and to prevent any dilution from residual water.

ITC Software – Data was collected using TA Instrument ITC Run software and a best-fit line was found (when applicable) by fitting TA's companion program NanoAnalyze's models for known thermodynamic reactions to the data. These models either were added individually or were added as a global fit where two or more models were fit together. Data was also converted for AFFINImeter software by using "Time(s)" for the primary x-axis, "Injection Time(s)" for the secondary x-axis, "Raw Heat Rate (µcal/s)" as the primary y-axis and "Enthalpy fit kJ/mol" as the secondary y-axis. This graph was then exported as a tab-delimited text (.ta) file for use in AFFINImeter. Once this file was uploaded into the software then the titrant and titrand concentrations, temperature, cell volume, number of injections and volume per injection were entered manually into the program.

The experiments were analyzed by subtracting a corresponding control experiment and determining if the resulting isotherm showed a reaction between the titrant and titrand. Multiple models were then fit to the data to determine the type of binding that was occurring (if any).

CHAPTER 3

RESULTS

The experiments were categorized into four groups: All three substrates injected at once into hGS, one substrate (glycine, ATP or GAB) injected into hGS by itself, glycine injected into a solution of hGS with ATP or GAB, and lastly glycine injected into a solution of hGS with ATP as well as GAB.

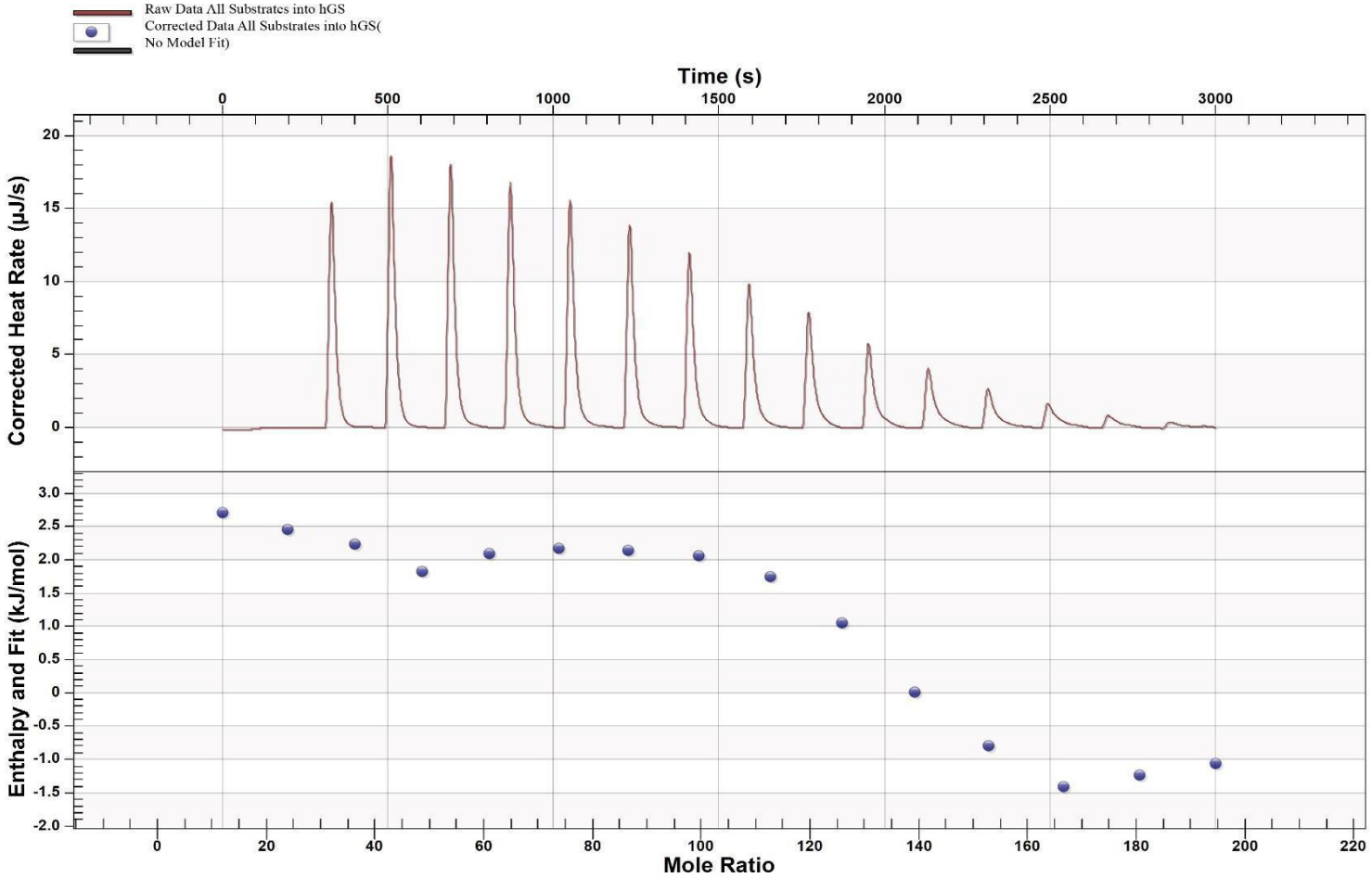
All Substrates into hGS

These experiments served as a method to confirm that when all substrates were present in the cell a reaction could be observed on the ITC.

The first experiments in which all three substrates (glycine, ATP, and GAB) were injected into the enzyme simultaneously resulted in a multiphase isotherm of alternating exothermic and endothermic phases (see Figure 8).

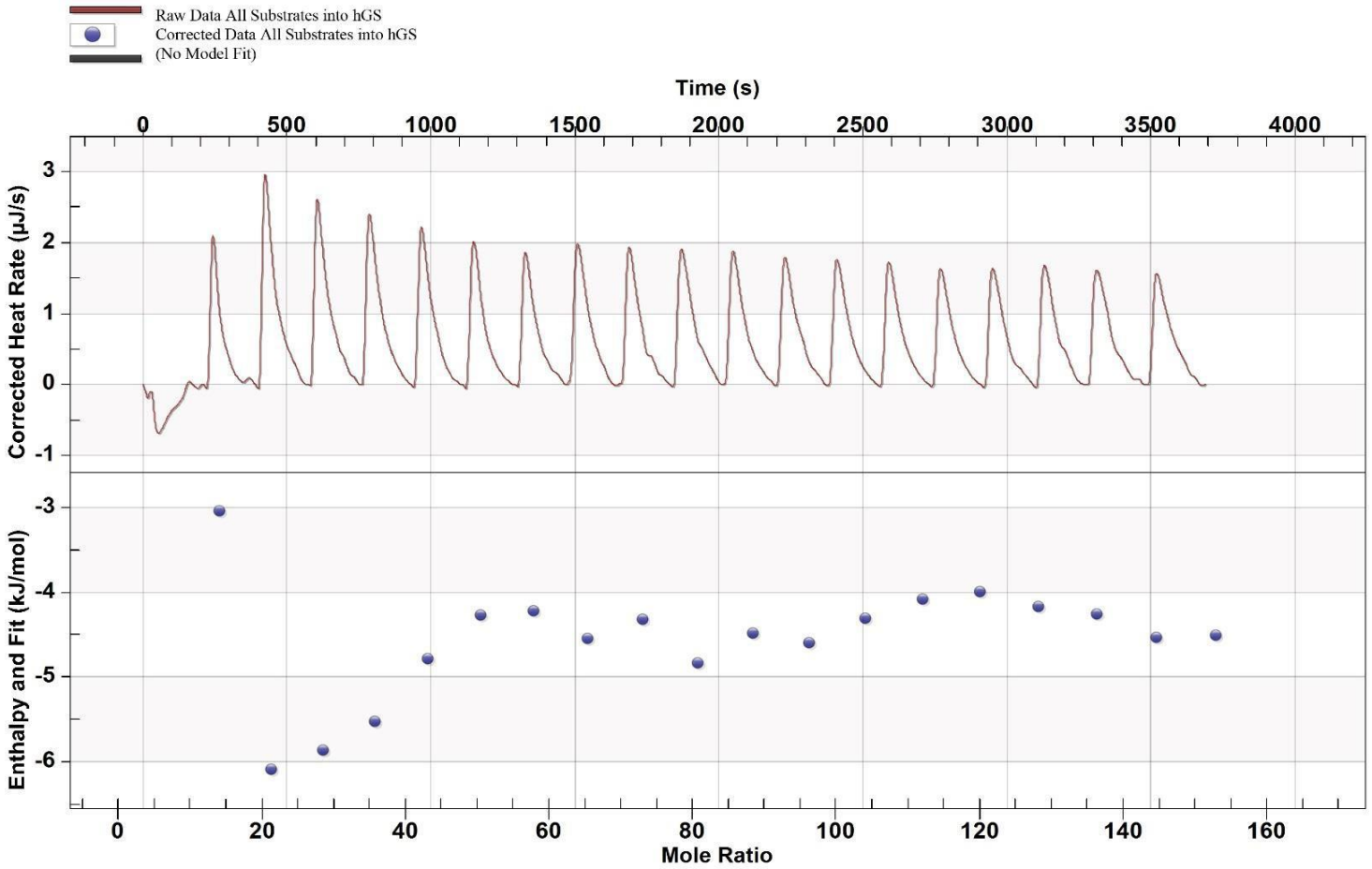
Experiments in which substrates were injected so that they reached their known K_m values exhibited similar subtle changes in binding and included an early distinctive exothermic shift indicating an immediate interaction, which leveled out slightly with increased concentrations of substrates (see Figure 9).

Figure 8. Representative raw data of all of the substrates (13.9 mM) injected hGS (approximately 0.0139 mM).



The final concentration of all of the substrates in hGS was 2.48 mM (Top). Molar ratio vs enthalpy of glycine, GAB, ATP with Mg2+ into hGS (Bottom). *N* = 2.

Figure 9. Representative raw data of all of the substrates (GAB 9.421 mM, ATP 1.85 mM, Mg^{2+} 3.70 mM and glycine 3.95 mM) injected hGS (0.012 mM).

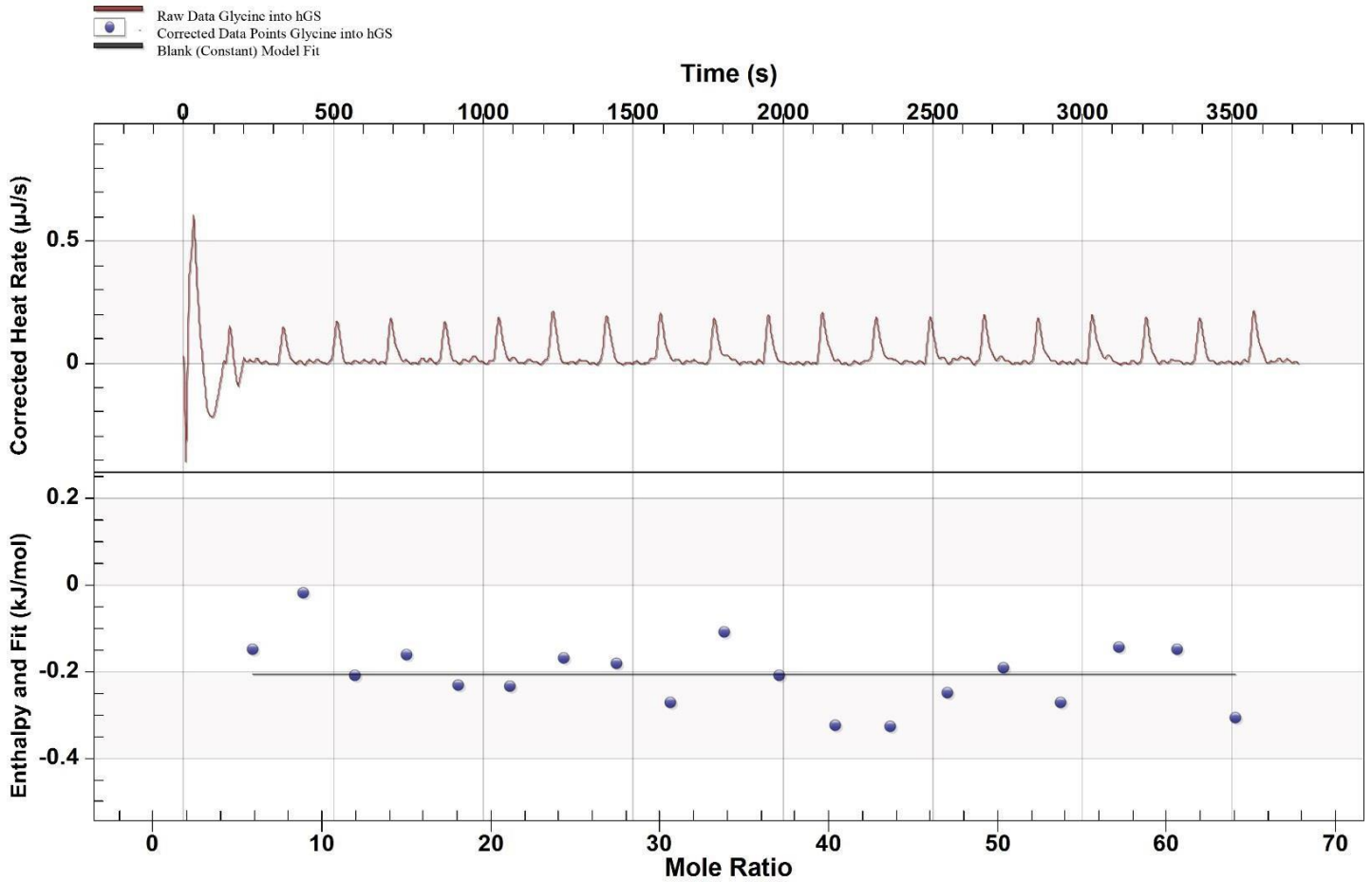


The final concentration of all of the substrates in hGS was GAB 1.68 mM, ATP 0.330 mM, Mg^{2+} 0.660 mM and glycine 0.71 mM (Top). Molar ratio of GAB:hGS vs enthalpy of glycine, GAB, ATP with Mg^{2+} into hGS (Bottom). $N = 2$.

Individual Substrate Binding (ATP, GAB or Glycine) with hGS Glycine into hGS

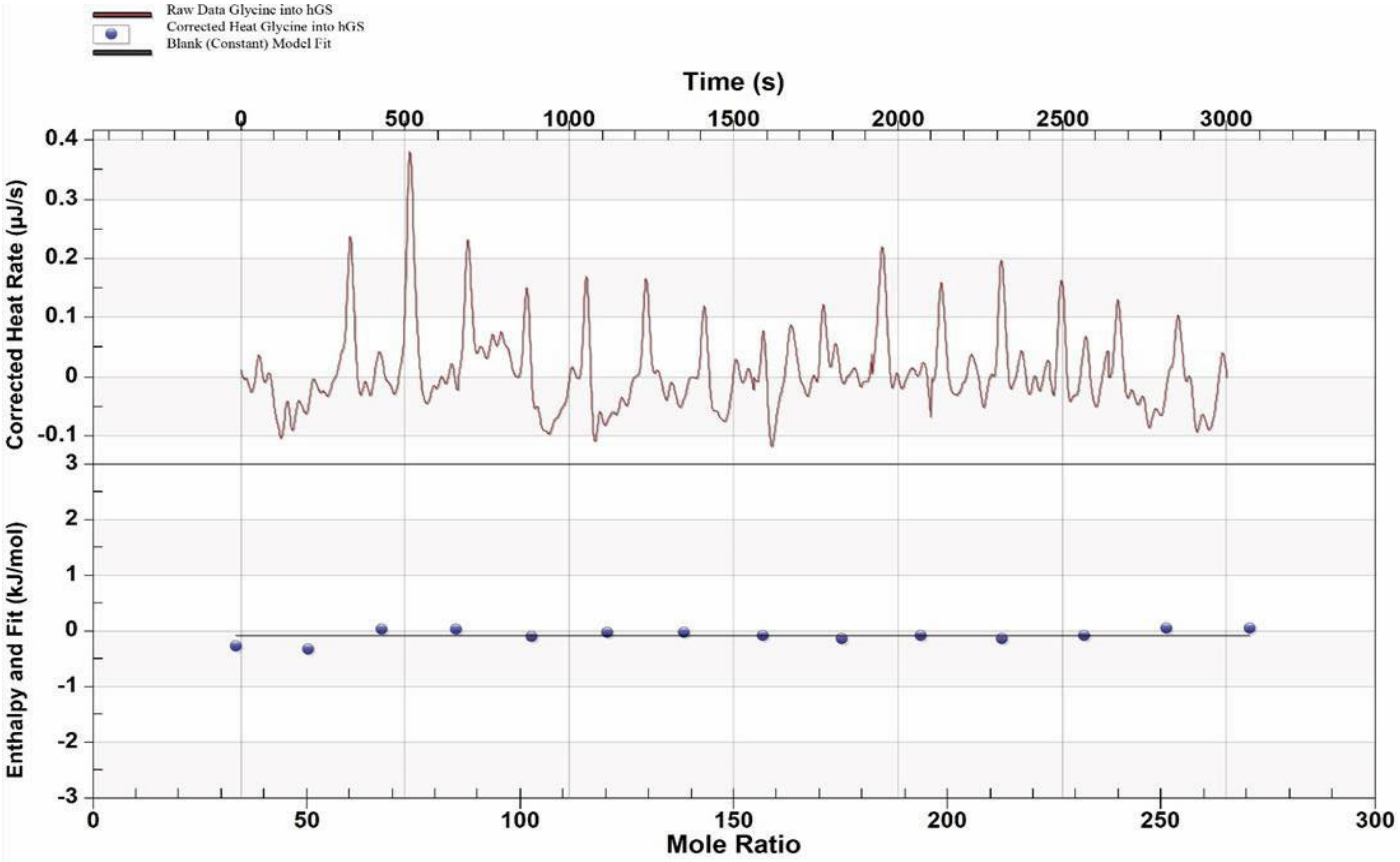
ITC experiments in which glycine was injected into hGS by itself exhibited erratic low heats of reaction and an apparent lack of binding (see Figures 10 and 11). No distinctive isotherms were observed. Due to the lack of a distinctive isotherm, a blank constant model was fitted to the data. The constant blank model yielded a heat of reaction of -3.9431 ± 1.26 µJ for large amounts of glycine and -2.0049 ± 0.488 µJ when concentrations in the cell were near K_m values for glycine of 0.529 mM. These heat values are similar to that found in control experiments injecting water into water of -3.0 ± 1.0 µJ, indicating that any heat present was due to the injection process and not due to binding of glycine to the enzyme.

Figure 10. Representative raw data of glycine (3.95 mM) injected into hGS (approximately 0.012 mM).



The final concentration of glycine in hGS was 0.598 mM (Top). Molar ratio vs enthalpy of glycine into hGS (Bottom). $N = 2$.

Figure 11. Representative raw data of glycine (13.9 mM) injected into hGS (0.01 mM).



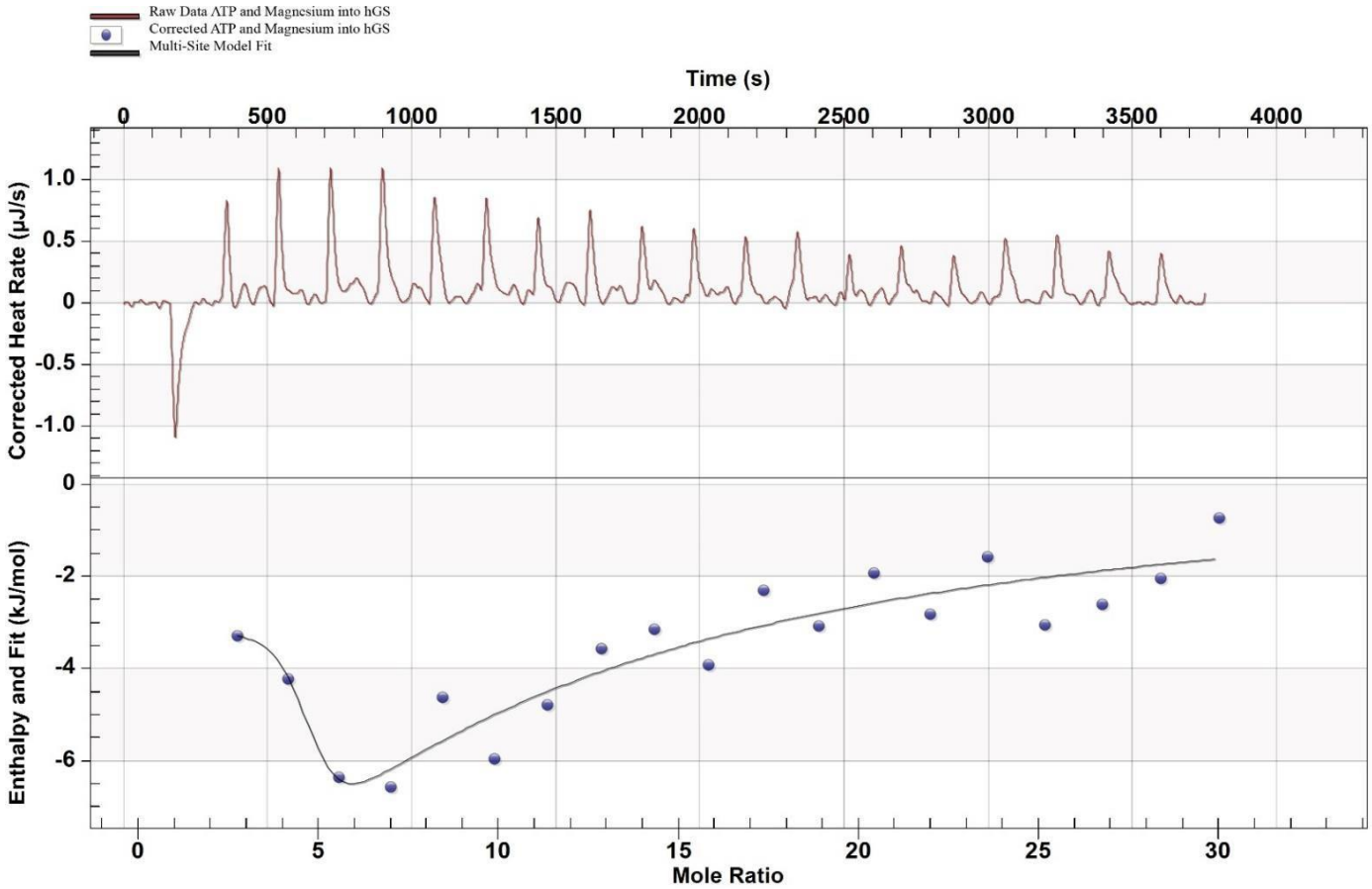
The final concentration of glycine in hGS was 2.1 mM (Top). Molar ratio vs enthalpy of glycine into hGS (Bottom). $N = 3$.

It is possible that the lack of binding of glycine to hGS at these levels could be due to the concentration of glycine being too low; therefore, increasing concentrations of glycine were added to the enzyme in subsequent experiments (*Supplement Figure 2*). However, increasing the concentration enhanced the noise due to the heat of dilution. Because of this, the reaction of the blank experiments exceeded the experimental data itself and was not valid.

ATP (with the Cofactor Mg^{2+}) into hGS

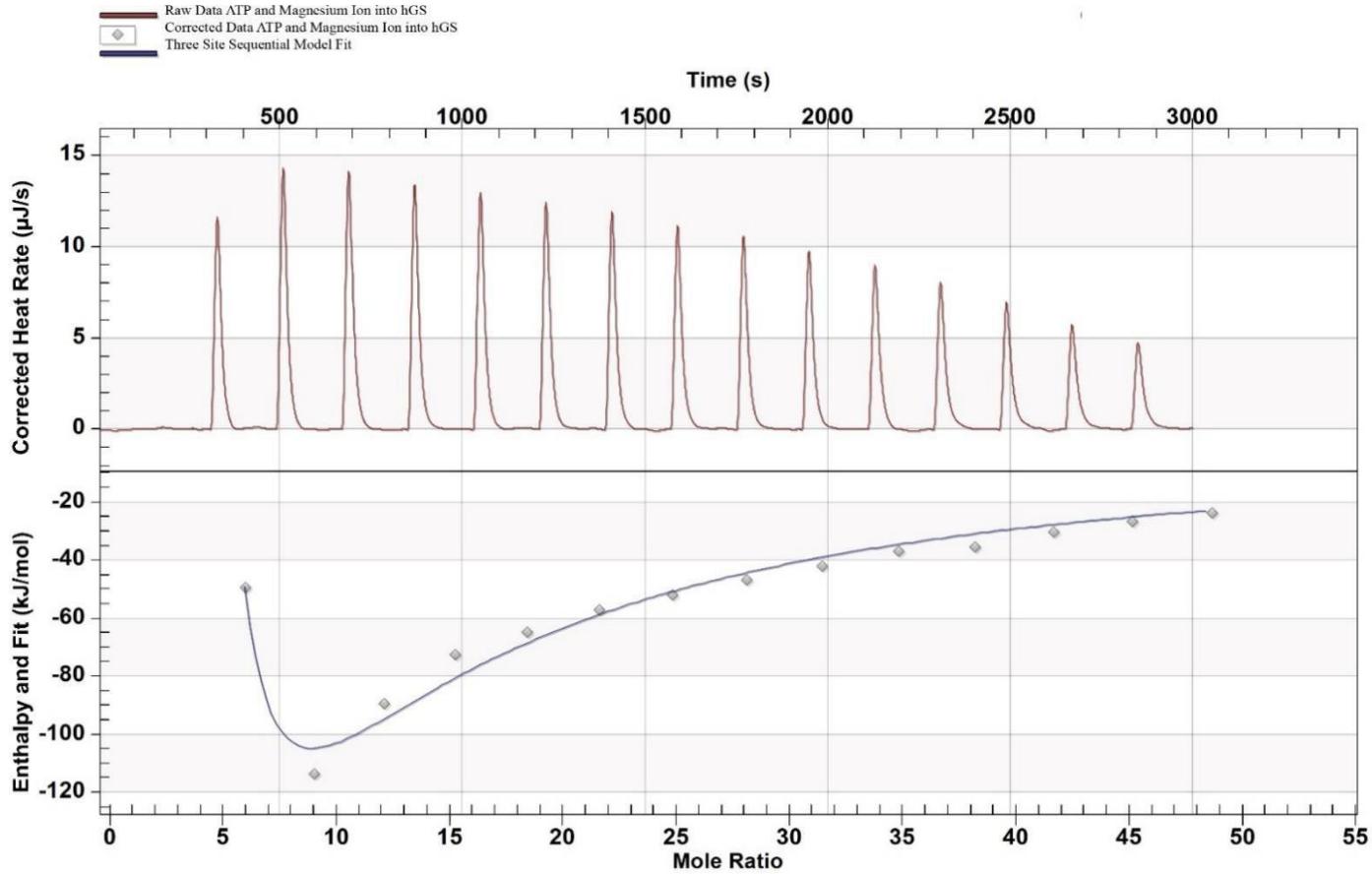
Injections of ATP into hGS exhibited binding. The isotherms that resulted were fit with a multisite model (see Figure 12) and a three site sequential model (see Figure 13) for the experiments with increasing concentrations of ATP as well as those testing K_m values respectively. These isotherms indicate that ATP interacts with the hGS without any other substrate present. The presence of a slightly biphasic isotherm is likely due to the presence of the cofactor Mg^{2+} in the ATP solution having a slight constant binding reaction with the hGS and or with the buffer as was observed in preliminary experiments (see Supplement Figure 3) and by injecting ATP by itself into hGS (see Supplemental Figure 4).

Figure 12. Representative raw data of ATP and Mg^{2+} (1.85 mM, 3.70 mM) injected into hGS (0.012 mM).



The final concentration of ATP and Mg^{2+} in hGS was 0.280 mM and 0.560 mM (Top). Molar ratio vs enthalpy of ATP and Mg^{2+} into hGS (Bottom). *N* = 3.

Figure 13. Representative raw data of ATP and Mg^{2+} (3.475 mM both substrates) injected into hGS (0.0139 mM).

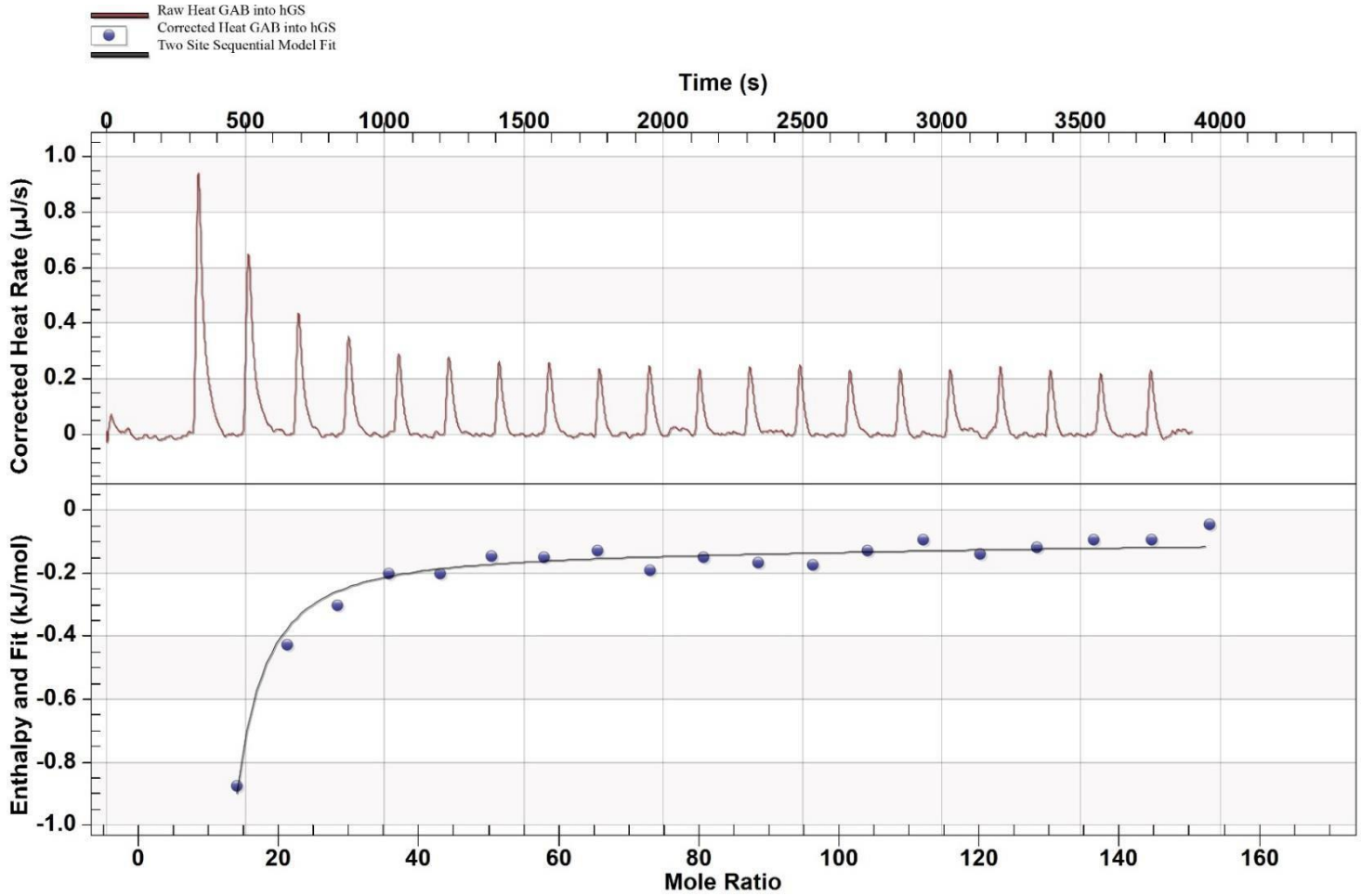


The final concentration of ATP and Mg^{2+} in hGS was 0.53 mM (Top). Molar ratio vs enthalpy of ATP and Mg^{2+} into hGS (Bottom). $N = 3$.

GAB into hGS

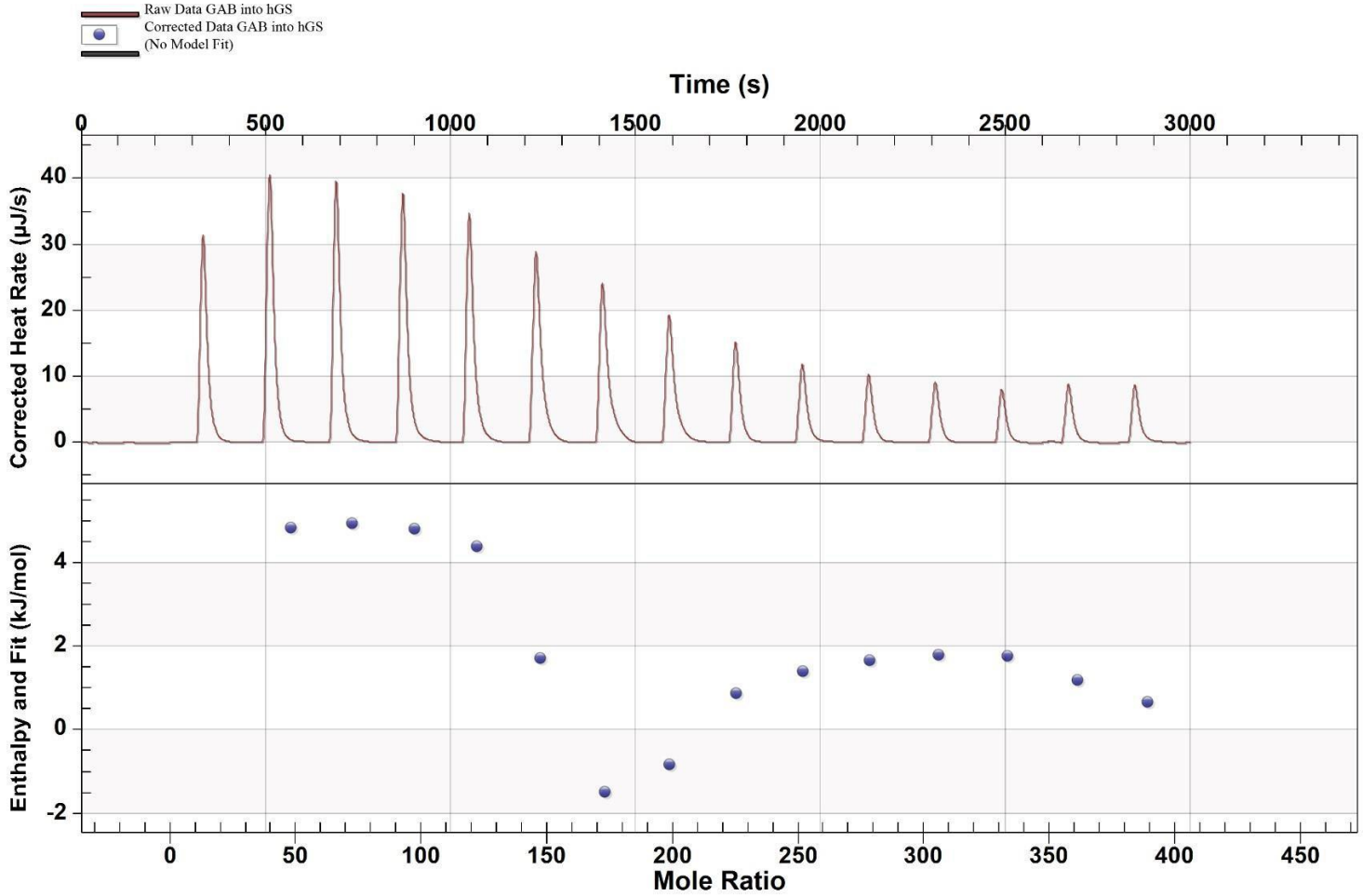
GAB when injected into hGS showed binding by itself to the enzyme. The resulting isotherms differed dramatically based upon the molar ratios injected. Experiments where only the lower K_m values were present in the cell, which only reached 150:1, exhibited a single-phase isotherm that could be fitted with a two site sequential model (see Figure 14). At higher concentrations the isotherms were notably biphasic possibly due to the enzyme being negatively cooperative (see Figure 15). Notably, the isotherm shifted when the ratio of substrate to enzyme reached higher than 170:1. It has been stated that an isotherm with two unique phases may be attributed to cooperativity.[28] This is because as one active site becomes fully bound an allosteric shift occurs on a larger number of the hGS enzymes and in this case causes the second active site on the homodimer to allow less binding to occur.

Figure 14. Representative raw data of GAB (9.421 mM) injected into hGS (0.012 mM). The final concentration of GAB in hGS was 1.43 mM (Top).



Molar ratio vs enthalpy of GAB into hGS with a two site sequential model fit (Bottom). $N = 3$.

Figure 15. Representative raw data of GAB (20.8 mM) injected into hGS (approximately 0.0104 mM).



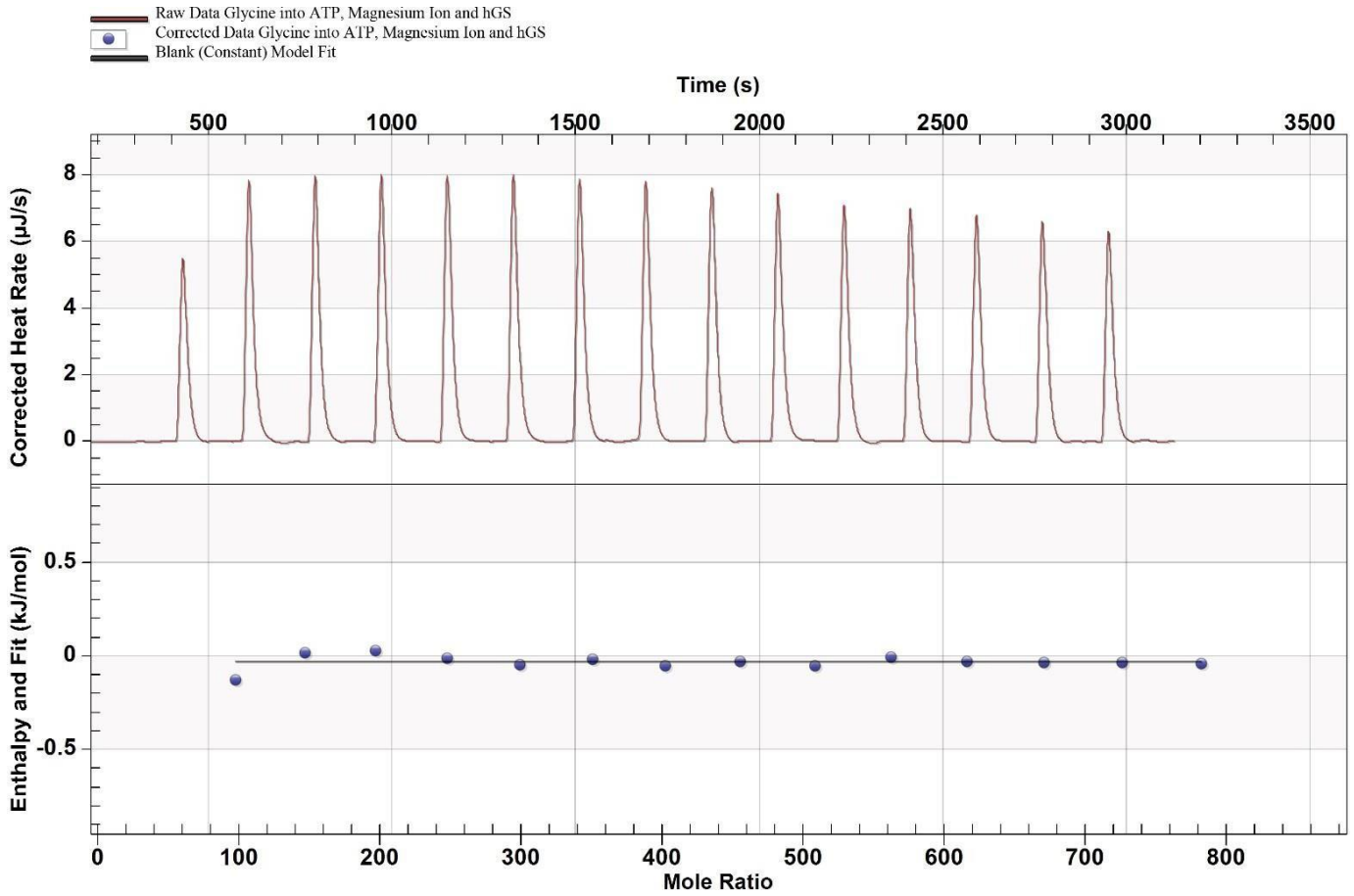
The final concentration of GAB in hGS was 3.15 mM (Top). Molar ratio vs enthalpy of GAB into hGS (Bottom). *N* = 2.

Glycine Added to Enzyme Substrate Solutions

Glycine into ATP, Mg^{2+} and hGS

The data obtained from injecting glycine into hGS in the presence of ATP and Mg^{2+} showed little to no binding even at extremely high concentrations. Excluding the extremely small exothermic second injection, the observed the data showed a lack of binding of glycine to hGS when ATP and Mg^{2+} were present in the cell (see Figure 16).

Figure 16. Representative raw data of glycine (50 mM) injected into ATP with Mg^{2+} (7.57 mM) and hGS (approximately 0.01 mM).



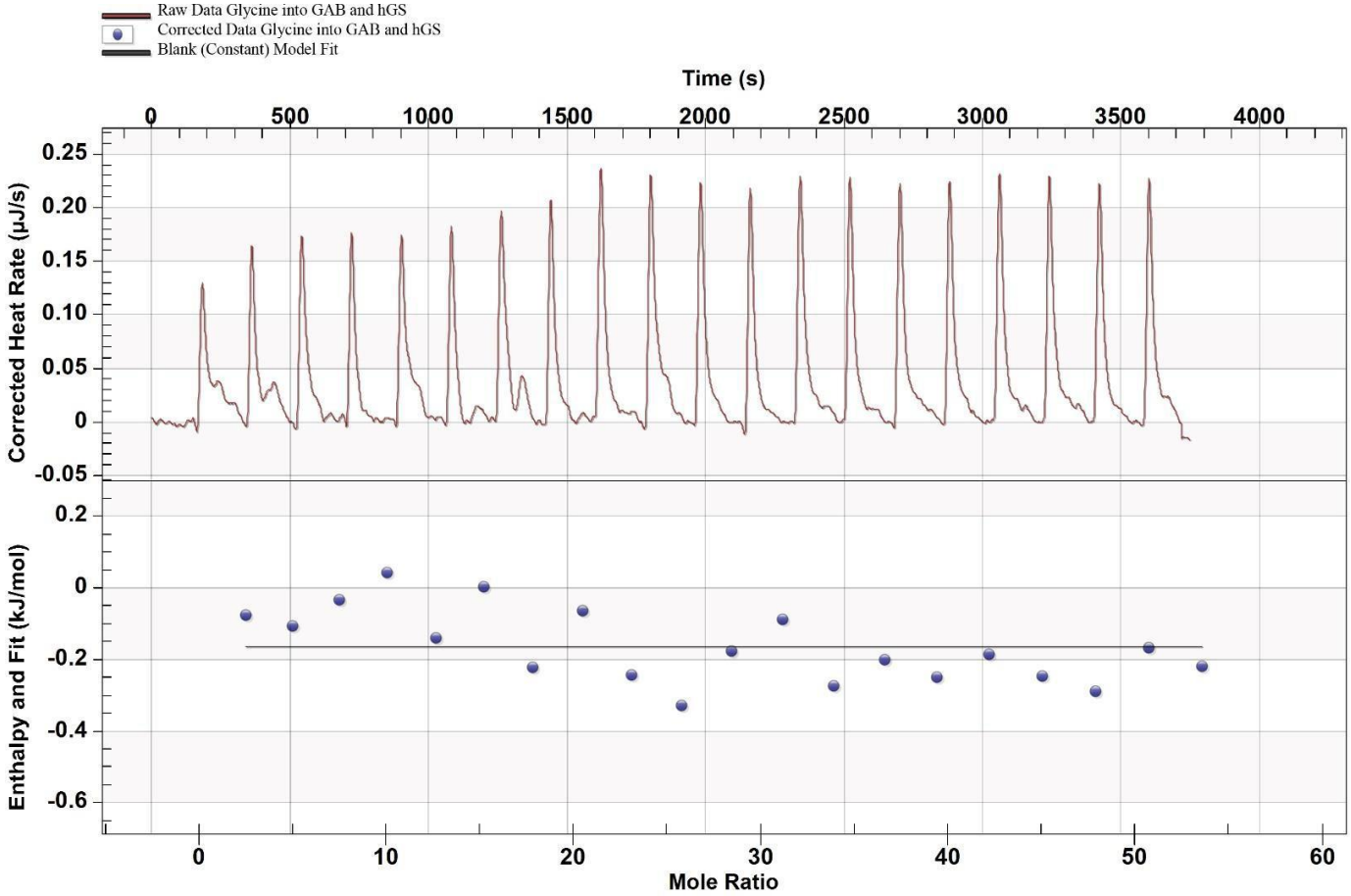
The final concentration of glycine in hGS was 6.58 mM (Top). Molar ratio vs enthalpy of glycine into ATP with Mg^{2+} and hGS (Bottom). *N* = 3.

GAB and hGS

When smaller amounts of glycine were added to GAB and hGS, no binding was found (see Figure 17). Indicating that under normal K_m conditions glycine does not bind when GAB is present with hGS.

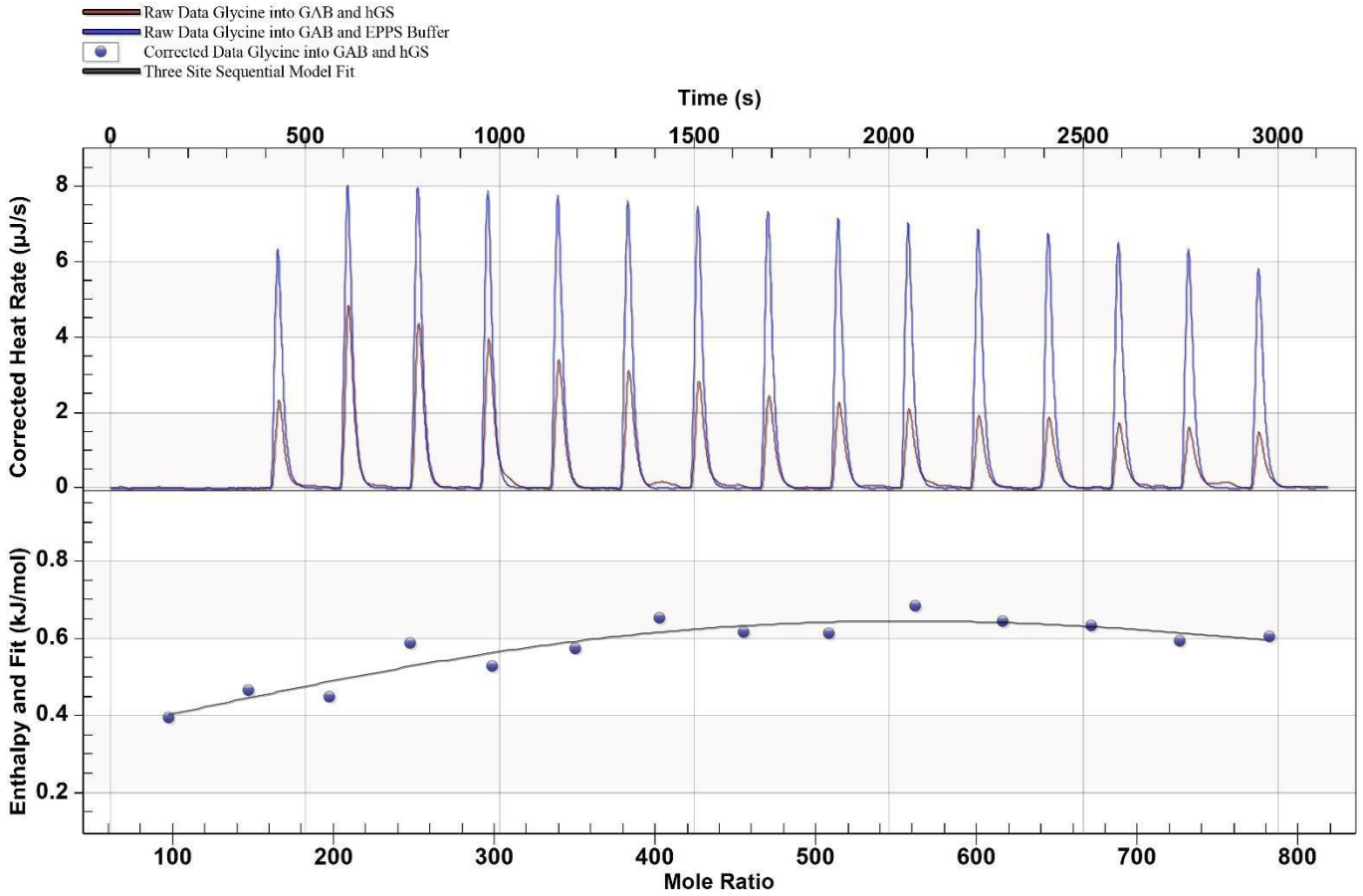
When higher concentrations of glycine were injected into a solution in which GAB was already mixed with hGS the resulting isotherms showed erratic heats of reaction instead of distinctive binding (see Figure 18). The heat of the raw data for the control experiment for the reaction exceeded the raw heat of the experimental data. This indicates that no binding occurs when glycine is added to hGS when only in the presence of GAB and any reaction observed is due to heat of dilution. Additionally, it is possible that unbound GAB in the control experiment was free to interact slightly with glycine whereas it was bound to hGS in the experimental injections and therefore showed a lower heat per injection.

Figure 17. Representative raw data of glycine (3.95 mM) injected into GAB (1.42 mM) and hGS (approximately 0.012 mM).



The final concentration of glycine in hGS was 0.520 mM (Top). Molar ratio vs enthalpy of glycine into GAB and hGS with a two site sequential model fit and multisite fit (Bottom). $N = 2$.

Figure 18. Representative raw data of glycine (50 mM) injected into GAB (7.57 mM) and hGS (0.01 mM) shown in red, buffer control with glycine into GAB and buffer in blue.



The final concentration of glycine in hGS was 6.58 mM (Top). Molar ratio vs enthalpy of glycine into GAB and hGS with a two site sequential model fit (Bottom). $N = 2$.

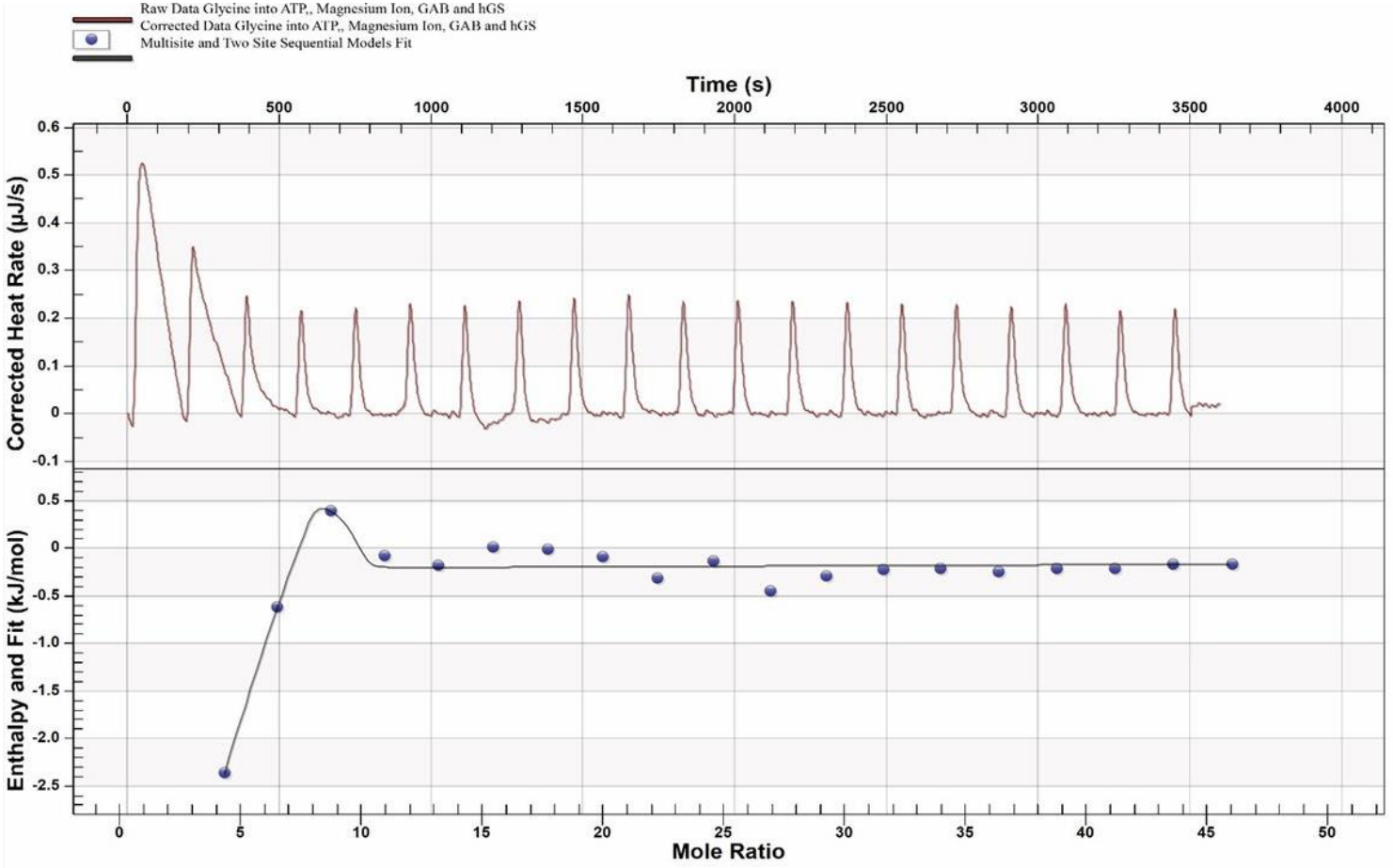
All Substrates

Glycine into ATP, Mg^{2+}, GAB and hGS

Injecting glycine into ATP, Mg^{2+}, GAB, and hGS at approximate K_m values exhibited a distinct isotherm in which a large amount of exothermic reaction occurred and was quickly followed by a small endothermic reaction in which presumably product or substrates released before the isotherm flattened and exhibited a lack of binding (see Figure 19).

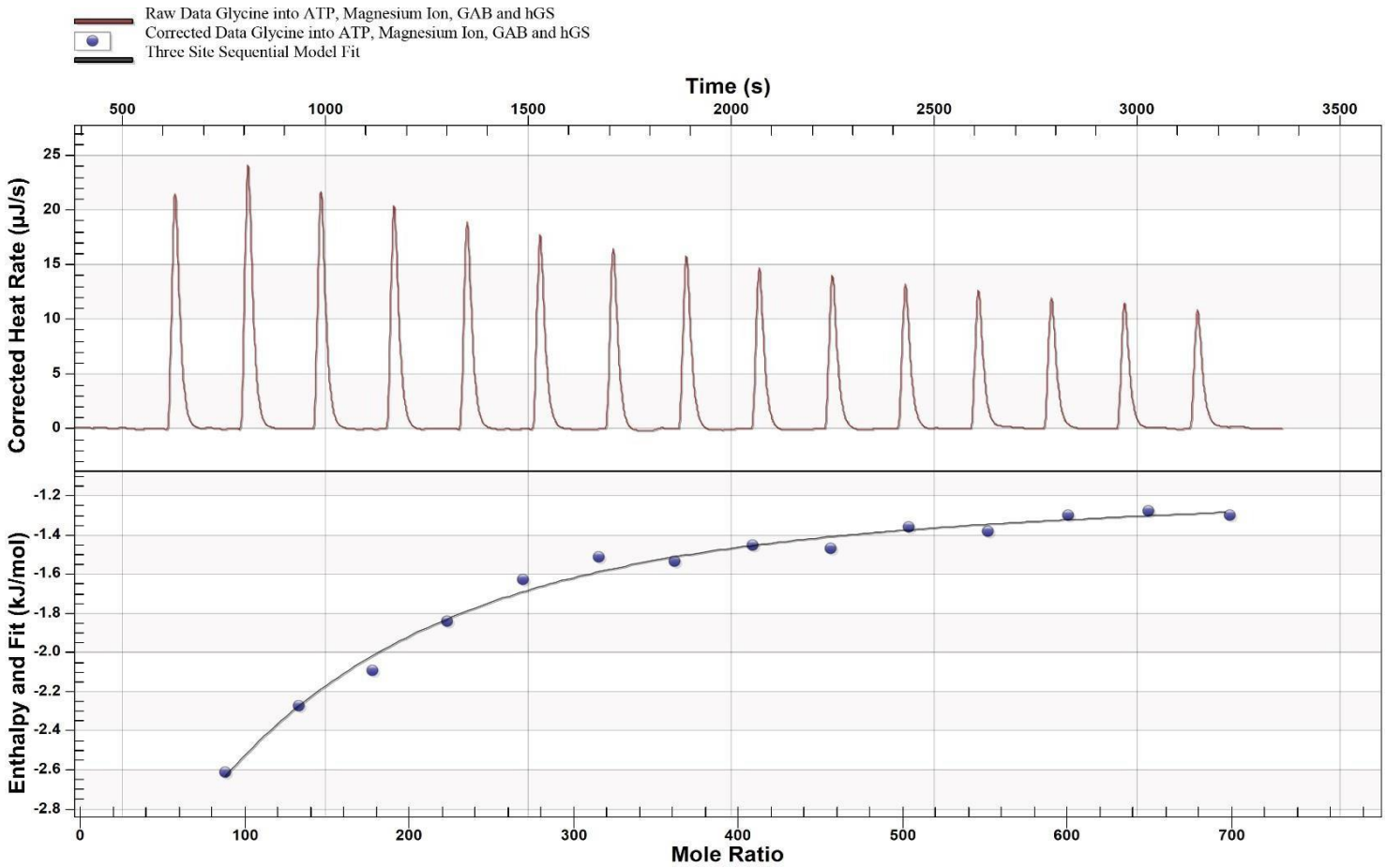
When glycine was injected into a solution containing hGS as well as ATP, Mg^{2+} and GAB already present, in higher concentrations, a large amount of heat was released (see Figure 20). A binding model of a three site sequential binding fit the data. The resulting isotherm showed that once glycine is added into a solution with the other substrates it clearly leads to binding and the reaction creating the production of glutathione (ophthalmic acid in laboratory conditions).

Figure 19. Representative raw data of glycine (3.95 mM) injected into GAB and ATP with Mg^{2+} (1.24mM, 0.243mM, and 0.487 mM) and hGS (approximately 0.012 mM).



The final concentration of glycine in hGS was 0.460 mM (Top). Molar ratio vs enthalpy of glycine into GAB, ATP with Mg^{2+} and hGS with multisite and two site sequential modes fit (Bottom). $N = 2$.

Figure 20. Representative raw data of glycine (50 mM) injected into GAB and ATP with Mg^{2+} (50 mM each) and hGS (0.0126 mM).



The final concentration of glycine in hGS was 5.81 mM (Top). Molar ratio vs enthalpy of glycine into GAB, ATP with Mg^{2+} and hGS with a three site sequential modes fit (Bottom). $N = 2$.

CHAPTER 4

DISCUSSION

Studying glycine interactions with its substrates and hGS shows a possible order of binding in the second step of glutathione production. When ATP or GAB is in the presence of hGS a heat of binding occurs regardless of what other substrates may or may not be present. However, glycine does not bind to hGS even in higher concentrations. This implies that glycine can only bind after one or more of the other substrates are present.

The addition of glycine to hGS when ATP was present exhibited a brief endothermic interaction. This could possibly be due to glycine binding loosely and releasing because not all the necessary substrates are present for it to bind tightly. In addition, this could be due to glycine interacting with the substrate (ATP) which has already bound and possibly altering the interaction of the bound substrate.

Glycine at higher concentrations in the presence of hGS and GAB resulted in a noticeable exothermic reaction but this was due to large heats of reaction from the control experiments. At K_m values, no binding was observed between glycine and the GAB and hGS solution.

When higher concentrations of glycine are added last to solution already containing ATP, GAB and hGS a large consistent isotherm was obtained which indicated that glycine was finally binding to the enzyme. Similar results were observed at lower glycine concentrations and an initial exothermic, then endothermic reaction occurred indicating a binding, and release happened prior to the reaction proceeding.

Comparing this isotherm to that of all of the substrates injected into the enzyme at once reveals that the two reactions look slightly different. The initial binding and quick release seen when glycine was added last to GAB, ATP and hGS was more subtle when all three were added simultaneously. This could be attributed to different interactions with the substrates with the ATP grasp lid when added simultaneously. In addition, it is possible that adding glycine after ATP and GAB require that some of the temporary structural bonds in the active site that already formed need to be broken prior to glycine binding to the active site.

Overall, these results indicate that the reaction of hGS with its substrates is semi-ordered. While further study of the binding order of ATP and GAB needs to be conducted, preliminary data from studying glycine provides a potential binding order for the reaction of substrates to hGS. The order of hGS may be different than the random order of reaction found in plant GS by Jez.[23] In fact, based on the data showing glycine will not bind with hGS alone and ATP or GAB may bind second, it is likely that the reaction is semi-ordered random A-B ordered C (see Figure 5).

If the production of glutathione is in fact semi-ordered, then this reaction may be regulated not only by the negative cooperativity of hGS but also by the availability of ATP and L-γ-Glu-Cys present in the body (GAB in lab settings). These results could reveal more about how specific mutations in hGS can be more severe if they affect the regions that need to bind ATP and L-γ-Glu-Cys, which are necessary for the first step of production of glutathione. In addition, patients suffering from excessive glutathione production may find relief if the binding sites for ATP and L-γ-Glu-Cys can be targeted to slow overproduction. Future studies confirming either ATP and or L-γ-Glu-Cys are required in specific

amounts to start the production of glutathione should confirm the exact binding order for the substrates of hGS and provide greater insight into the treatment of disorders related to glutathione as well as its role in regulation of key checkpoints in the human body through binding order.

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