GLUTATHIONE SYNTHETASE: CONSERVED RESIDUES OF THE SUBSTRATE LOOP

A THESIS

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COLLEGE OF ARTS AND SCIENCES

BY

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MAY 2013

TEXAS WOMAN'S UNIVERSITY
DENTON, TEXAS

April 3, 2013

To the Dean of the Graduate School:

I am submitting herewith a thesis written by Bisesh Shrestha entitled "Glutathione Synthetase: Conserved Residues of the Substrate Loop." I have examined this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Chemistry.

Mary E. Anderson, Ph.D., Major Professor

We have read this thesis and recommend its acceptance:

Department Chair

Accepted:

Interim-Dean of the Graduate School

DEDICATION

For my wife Sumanjali Shrestha, thank you
for your love and support.

ACKNOWLEDGEMENTS

Dr. Mary Anderson, thank you for all your support through the years that has helped me achieve my goal. Your insights and ideas have been very helpful and have allowed me to learn. I would not have been able to complete my research without help from my lab mates. Thank you, Kerri Slavens and Teresa Brown for teaching me all the new biochemistry techniques. I am grateful to Margie DeJesus and Brandy Ingle for all the volunteered and requested support during the intense research years. Finally, I would like to thank my spouse for all her patience, love and support.

ABSTRACT

BISESH SHRESTHA

GLUTATHIONE SYNTHETASE: CONSERVED
RESIDUES OF THE SUBSTRATE LOOP

MAY 2013

The important antioxidant tripeptide glutathione (GSH) is synthesized in two ATP-dependent steps; the second enzyme in the biosynthetic pathway, glutathione synthetase (GS), ligates glycine to γ-glutamylcysteine (γ-GC). Human glutathione synthetase (hGS) deficiency causes hemolytic anemia, metabolic acidosis, 5-oxprolinuria and a total deficiency may be lethal. Three flexible loops (A, G and S) surround the substrates (ATP, glycine and γ-GC). Human glutathione synthetase is negatively cooperative to one substrate, γ-GC. The Substrate- or S-loop is proximal to γ-GC and thought to participate in γ-GC binding. The S-loop (266-FRDGYMPRQYS-276) contains 11 residues, some of which are highly conserved (F266, R267, G269, Y270, P272 and Y275). Site directed mutagenesis was used to change these highly conserved S-loop residues, and then their roles in substrate binding, enzyme activity and stability were assessed.

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CHAPTER I

INTRODUCTION

GLUTATHIONE

Glutathione (GSH) is an antioxidant tripeptide (L-γ-glutamyl-L- cysteinylglycine), with an unusual gamma peptide linkage between glutamate and cysteine. GSH is the most abundant cellular thiol in aerobic cells. Intracellular GSH concentration ranges from 1 - 10 mM, with the highest concentrations observed in liver cells [Meister, 1983]. GSH is found in all prokaryotic and eukaryotic cells, with the exception of a few anaerobes. GSH carries out many important biological functions: serves as a cofactor for several enzymes, maintains protein sulfhydryl groups, participates in amino acid transport and cellular protection against reactive oxygen species and toxic compounds. GSH is an essential antioxidant for many cellular functions.

GLUTATHIONE BIOSYNTHESIS

Cellular GSH biosynthesis is catalyzed in two ATP-dependent steps [Anderson, 1998]. In the first step, γ-glutamylcysteine synthetase (GCS) ligates glutamate to a cysteine and forms γ-glutamylcysteine (γ-GC); since cysteine levels are normally low, it is likely rate-

limiting. In the second step, glutathione synthetase (GS) (Scheme 1) ligates γ-glutamylcysteine to glycine and forms GSH which is a feedback inhibitor of the first enzyme (GCS). Thus deficiency of GS leads to an overproduction of γ-GC which is then converted into cysteine and 5-oxoproline; the latter is an organic acid, and results 5-oxprolinuria, a potentially life threatening disease.

$$\text{L-Glutamate + L-Cysteine+ } Mg^{2+}\text{-ATP} \xrightarrow{GCS} \text{L-}\gamma\text{-Glutamyl-L-cysteine + ADP + } P_i$$

$$\text{L-}\gamma\text{-Glutamyl-L-cysteine + Glycine + } Mg^{2+}\text{-ATP} \xrightarrow{GS} \text{Glutathione + ADP + } P_i$$

Scheme 1 Biosynthesis of glutathione

HUMAN GLUTATHIONE SYNTHETASE DEFICIENCIES

The gene for human glutathione synthetase (hGS) is on chromosome 20q11.2 [Gali, 1995]. Deficiency of hGS is a rare autosomal recessive disorder, and as of 2011, only 77 patients in 65 families are identified [Winkler, 2011]. In these patients, mutation within the hGS gene result in truncated protein, no protein or protein with lowered stability. Phenotypically hGS deficiency is grouped into three forms: mild, moderate and severe [Larsson, 2000]. All hGS deficient patients have hemolytic anemia. In the mild form, the deficiency is limited to erythrocytes and patients present with symptoms as hemolytic anemia and neonatal jaundice as cellular levels of GSH in non-nucleated erythrocytes are sufficient to prevent accumulation of 5-oxoproline [Polekhina, 1999]. In the moderate form, additional symptoms may include metabolic acidosis and/or 5-oxoprolinuria, likely

due to change in mutant hGS substrate binding i.e. V_{max} and K_m or lowered enzyme stability. In the more severe cases of hGS deficiency, patients often suffer from various neurological problems including motor retardation, results of mutations that lead to completely non-functional enzyme [Ristoff, 1998]. The mutations in loops (S) near the binding site of hGS need further study to shed light on the molecular basis of hGS deficiencies.

HUMAN GLUTATHIONE SYNTHETASE CATALYTIC LOOPS

The active sites of the homodimeric hGS are 40 Å apart [Polekhina, 1999]. Three substrates: γ-glutamylcysteine, glycine and ATP are bound in the active site and covered by the G-, A- and S- loops [Dinescu, 2007]. The loops of hGS play an important role in catalysis, G-loop contains a rare glycine triad that assists in ATP binding [Dinescu, 2010] and the A-loop residues, closer to the glycine substrate, plays an equally important catalytic role [Brown, 2011]. Patients with hGS deficiencies have mutations within these active site loops; none are found in the G-loop, one in the A-loop and several in the S-loop. Further study of the S-loop is necessary to describe its role in catalysis.

Binding of substrates in protein active sites facilitated by loops have been previously observed in numerous enzymes [Kempner, 1993]. Streptavidin binds its cofactor biotin via movement of a 6 residue flexible loop [Weber, 1989] and triosephosphate isomerase (TIM) has an 11 residue loop that binds the substrate and covers the active site [Joseph, 1990]. Loops serve several roles in facilitating substrate

binding and orientation and in covering active sites. Thus, the loops (G, A and S) of hGS are thought to serve a similar function in the binding of γ-glutamylcysteine [Dinescu, 2007].

The A and G loop have been previously studied by our lab, however less is known about the S-loop (266-FRDGYMPRQYS-276), which is near to the γ-glutamyl moiety of the substrate γ-glutamylcysteine and likely participates in γ-glutamyl moiety binding and orientation.

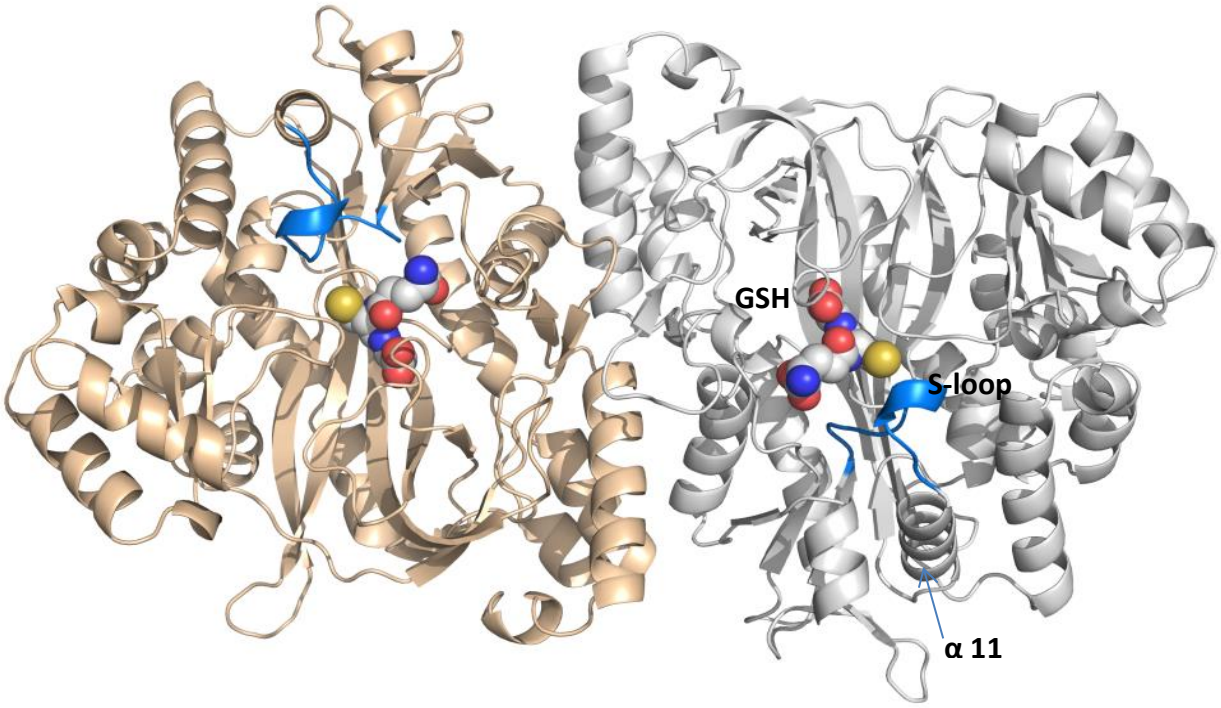

Figure 1 Homodimeric human glutathione synthetase

NEGATIVE COOPERATIVITY IN HUMAN GLUTATHIONE SYNTHETASE

The double reciprocal (Lineweaver-Burk) plots for γ-glutamyl substrate in rat GS kinetic analysis shows that GS is negatively cooperative towards its γ-glutamyl substrate, as initially noted by Oppenheimer for rat GS (K_m 200 µM / 20 µM) [Oppenheimer, 1979]. The Anderson group later showed that human GS also has biphasic double reciprocal plots for the γ-glutamyl substrate, indicating negative cooperativity [Njalsson, 2001]. Although rare, several other negatively cooperative enzymes have been identified as glycoprotein hormone receptors [Urizar, 2005], G-protein coupled receptors [Suzuki, 2004], and lysine 2, 3-aminomutase [Ruzicka, 2010] and negative cooperativity is more appreciated.

More studies are required to further explain the mechanism of negative cooperativity in hGS. The proximity of the S-loop of hGS to the γ-glutamyl binding site makes it a good candidate to study enzyme-substrate interactions and cooperativity.

CHAPTER II

METHODOLOGY

MATERIALS

The His tag expression vector pET-15b™ and *Escherichia coli* (*E. coli*) XL1 Blue competent cells were purchased from Novagen, Inc. The QuickChange™ Site-Directed Mutagenesis Kit was purchased from Stratagene. Ampicillin, bovine serum albumin, lactate dehydrogenase (Type II Rabbit muscle) and pyruvate kinase (Type II Rabbit muscle) were purchased from Sigma-Aldrich. Primers were purchased from IDT, Inc. Isopropyl-1-thio-β-galactopyranoside (IPTG) was purchased from American-Bioanalytical. γ-L-Glutamyl-L-α-aminobutyrate (GAB) was purchased from Bachem, Inc. Concentrators (20K MWCO / 7ml) were from Pierce Inc®. All other reagents were purchased from Sigma-Aldrich, US Biological, Fisher Scientific or Amresco and were of the highest purity.

METHODS

Preparation of hGS recombinant enzymes

Recombinant human glutathione synthetase (hGS) cDNA was subcloned in pET-15b expression vector containing an N-terminal histidine tag (6 x histidine residue) [Dinescu,

2010]. Human GS mutant enzymes were made using the QuickChange™ Site-Directed Mutagenesis Kit using the internal primers listed (IDT). The plasmid DNA (mutants/wild type) was transformed into *E. coli* XL1 Blue competent cells, grown and purified (Wizard plus Midiprep, Promega). The mutant hGS mutant cDNA were confirmed by sequencing (Gene Wiz, Inc.). The mutant hGS plasmids were transformed into *E. coli* BL21 (DE3) cells for protein expression as previously described [Dinescu, 2010] and summarized below.

Enzyme purification

Cells containing the pET-15b hGS plasmids with a 6x His-tag were grown in Luria Broth (LB) (1 L, 37˚C, containing 100 µg/mL ampicillin) to an OD_{600} of 0.8–1.0 (log phase) (~ 4 hrs). Enzymes were induced by addition of IPTG (0.80 mM, with shaking 275 rpm), at 19˚C (unless noted).

All procedures were at 4˚C unless noted. Cells were harvested by centrifugation (5,000 x g, 10 min); the pellet was washed with saline (0.85% NaCl, 15 ml) and after centrifugation (5,000 x g, 10 min), the pellet was lysed (15 kPsi, One Shot Cell Disruption System model: 01/40/BA, Constant Systems, Inc.) in MCAC-0 buffer (20 ml; 20 mM Tris-Cl, 0.5 M NaCl, 10% Glycerol, pH 8.0) and sonified (Branson sonifier D-450, CE converter 102C tip) (2 min, 35% amplitude, 0.5 s pulse, 0.5 s rest). After centrifugation (10,000 x g, 20 min), the supernatant, containing hGS was applied to a metal chelate affinity column (1.5 x 15 cm) Ni-NTA His-Bind® resin (Novagen Inc.). The

column was developed sequentially with buffer (until OD_{280} returned to baseline): MCAC-0, MCAC-55 (MCAC-0 + 55mM imidazole) and followed by MCAC-100 (MCAC-0 + 100 mM imidazole). Human GS was eluted in the MCAC-100 fraction and collected (2-4 ml fractions). Fractions with hGS were dialyzed overnight (Tris buffer, 2 x 4 L, 20 mM Tris-Cl and 1 mM EDTA, pH 8.6). Protein purity was assessed by SDS-PAGE. Protein concentration was quantified using the Lowry assay using bovine serum albumin as standard [Lowry, 1951].

Enzyme activity assays

Human GS activity was determined using a pyruvate kinase (PK)/lactate dehydrogenase (LDH) coupled assay as previously described [Adriana, 2007]. γ-L-Glutamyl-L-α-aminobutyrate (GAB; a γ-glutamylcysteine analog with similar properties) was used as an alternative to γ-glutamylcysteine to prevent the oxidation of thiol groups [Oppenheimer, 1979]. The reaction was initiated by addition of hGS enzyme to a pre-incubated (37º C, 11 min) standard reaction mixture (0.2 ml final volume consisting of: 100 mM Tris-HCl (pH 8.2), 50 mM KCl, 20 mM $MgCl_2$, 5 mM phospho(*enol*)pyruvate, 10 mM Na-ATP, 10 mM glycine, 10 U/ml LDH, 10 U/ml PK, 0.3 mM NADH, and GAB (10 mM for specific activity assays). Specific activity assays were conducted with concentration of 10 mM of each GAB, ATP and glycine. The rate of reaction was measured by monitoring rate of NADH oxidation (340 nM). Activity is defined as the amount that catalyzes 1 µmol of product per min at 37 °C.

Enzyme kinetics

Kinetic parameters were determined using the standard assay procedure but varying one of the substrates while maintaining the other two constant. The Michaelis constant (K_m) value was determined by keeping two substrates at constant saturation while varying the third substrate 10-fold of the approximate K_m. Data analysis software Sigma Plot 11.0 was used to analyse data and calculate V_{max}, K_m and Hill coefficient [Dinescu, 2010]. A hill coefficient value of <1 indicates negative cooperativity, 1 indicates no cooperativity and >1 indicates positive cooperativity.

Differential scanning calorimetry

Differential scanning calorimetry was performed after dialysis in buffer (20 mM Tris-Cl and 1 mM EDTA, pH 8.6) on a Calorimetry Sciences Nano Series III instrument at 1.0 atm at a 1.0 °C/min scan rate (10 – 90 °C). Enzyme, if below the required concentration of 1 mg/ml, was concentrated in a Pierce Concentrator (20 K MWCO / 7 ml, Thermo Scientific) to 1-2 mg/ml. Data was baseline corrected against the buffer (20 mM Tris-Cl and 1 mM EDTA, pH 8.6).

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Enzyme was dialysed in phosphate (10 mM, pH 7.0, 4 °C) buffer overnight (2 X 4 L). Circular dichroism measurements were conducted in an Ollis RSM 1000 with DSM CD

attachment from 190–280 nm. Data was smoothed (Digital filter 15, RC filter 13) following acquisition and analysis. Data is represented as molar elipticity against wavelength.

CHAPTER III

GLUTATHIONE SYNTHETASE:

γ-GLUTAMYLCYSTEINE BINDING RESIDUES IN THE SUBSTRATE LOOP

B. Shrestha[1], B. Ingle[2], M. DeJesus[1], K. Slavens[1], T. Cundari[2], M. E. Anderson[1].

A Paper To Be Submitted For Publication

SUMMARY

Glutathione synthetase catalyzes the second step of glutathione biosynthesis. Human glutathione synthetase is homodimeric negatively cooperative enzyme. Human glutathione synthetase binds three substrates, γ-glutamylcysteine, glycine and ATP. The active site of hGS is covered by several loops. The substrate or S-loop is at the γ-glutamylcysteine binding site and is postulated to play a role in substrate binding and enzyme activity and stability. Using site directed mutagenesis the substrate binding residues of the S-loop were changed to alanine and other amino acid residues. Analyses of these mutant enzymes show that the substrate binding residues of the S-loop have an effect on enzyme activity and γ-glutamylcysteine affinity.

Keywords: Glutathione synthetase, negative cooperativity, ATP-grasp enzymes, substrate binding, cooperativity, γ-glutamylcysteine, glutathione

INTRODUCTION

Glutathione (GSH) is an antioxidant tripeptide (L-γ-glutamyl-L-cysteinylglycine), with an unusual gamma peptide linkage between glutamate and cysteine. GSH is the most abundant cellular thiol in aerobic cells. Intracellular GSH concentration ranges from 1 - 10 mM, with the highest concentrations observed in liver cells [1]. GSH is found in all prokaryotic and eukaryotic cells, with the exception of a few anaerobes. GSH carries out many important biological functions: serves as a cofactor for several enzymes, maintains protein sulfhydryl groups, participates in amino acid transport and cellular protection against reactive oxygen species and toxic compounds. GSH is an essential antioxidant for many cellular functions.

Cellular GSH biosynthesis is catalyzed in two ATP-dependent steps [2]. In the first step, γ-glutamylcysteine synthetase (GCS) ligates glutamate to a cysteine and forms γ-glutamylcysteine (γ-GC); since cysteine levels are normally low, it is likely rate-limiting. In the second step, glutathione synthetase (GS) (Scheme 1) ligates γ-glutamylcysteine to glycine and forms GSH which is a feedback inhibitor of the first enzyme (GCS). Thus deficiency of GS leads to an overproduction of γ-GC which is then converted into cysteine and 5-oxoproline; the latter is an organic acid, and results 5-oxprolinuria, a potentially life threatening disease.

The gene for hGS is on chromosome 20q11.2 [3]. Deficiency of human glutathione synthetase (hGS) is a rare autosomal recessive disorder, and as of 2011, only 77 patients in 65 families are identified [4]. In these patients, mutation within the hGS

gene result in truncated protein, no protein or protein with lowered stability. Phenotypically hGS deficiency is grouped into three forms: mild, moderate and severe [5]. All hGS deficient patients have hemolytic anemia. In the mild form, the deficiency is limited to erythrocytes and patients present with symptoms as hemolytic anemia and neonatal jaundice as cellular levels of GSH in non-nucleated erythrocytes are sufficient to prevent accumulation of 5-oxoproline [6]. In the moderate form, additional symptoms may include metabolic acidosis and/or 5-oxoprolinuria, likely due to change in mutant hGS substrate binding i.e. V_{max} and K_m. or lowered enzyme stability. In the more severe cases of hGS deficiency, patients often suffer from various neurological problems including motor retardation, results of mutations that lead to completely non-functional enzyme [7]. The mutations in loops (S) near the binding site of hGS need further study to shed light on the molecular basis of hGS deficiencies.

The active sites of the homodimeric hGS are 40 Å apart [6]. Three substrates: γ-glutamylcysteine, glycine and ATP are bound in the active site and covered by the G-, A- and S- loops [8]. The loops of hGS play an important role in catalysis, G-loop contains a rare glycine triad that assists in ATP binding [9] and the A-loop residues, closer to the glycine substrate, participates in catalysis [10]. Patients with hGS deficiencies have mutations within these loops, none are found in the G-loop, one in the A-loop and several in the S-loop. Further study of the S-loop is necessary to describe its role in catalysis.

Binding of substrates in protein active sites facilitated by loops have been previously observed in numerous enzymes [11]. Streptavidin binds its cofactor biotin via movement of a 6 residue flexible loop [12] and triosephosphate isomerase (TIM) has an 11 residue loop that binds the substrate and covers the active site [13]. Loops serve several roles in facilitating substrate binding and orientation and in covering active sites. Thus, the loops (G, A and S) of hGS are thought to serve a similar function in the binding of γ-glutamylcysteine [8].

The A and G loop have been previously studied by our lab [8, 9], however less is known about the S-loop (266-FRDGYMPRQYS-276), which is near to the γ-glutamyl moiety of the substrate γ-glutamylcysteine and likely participates in γ-glutamyl moiety binding and orientation.

The double reciprocal (Lineweaver-Burk) plots for γ-glutamyl substrate in GS kinetic analysis shows that GS is negatively cooperative towards its γ-glutamyl substrate, as initially noted by Oppenheimer for rat GS (K_m 200 μM / 20 μM) [14]. The Anderson group later showed that human GS also has biphasic double reciprocal plots for the γ-glutamyl substrate, indicating negative cooperativity [15]. Although rare, several other negatively cooperative enzymes have been identified as glycoprotein hormone receptors [16], G-protein coupled receptors [17], and lysine 2, 3-aminomutase [18] and negative cooperativity is more appreciated.

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Computational Studies

Computational studies as previously employed were used to [8, 9] create enzyme models. Hydrogen bonding distances were calculated using PyMOL and MOE program [20, 21].

Table 1 Primers for site directed mutagenesis

Enzyme	DNA sequence
R267A	5'-G GTT TAC TTC GCG GAT GGC TAC ATG C-3' 5'-G CAT GTA GCC ATC CGC GAA GTA AAC C-3'
R267K	5'-G GTT TAC TTC AAG GAT GGC TAC ATG CCT CG-3' 5'-CG AGG CAT GTA GCC ATC CTT GAA GTA AAC C-3'
R267W	5'-G GTT TAC TTC TGG GAT GGC TAC ATG C-3' 5'-G CAT GTA GCC ATC CCA GAA GTA AAC C-3'
D268E	5'-G GTT TAC TTC CGG GAA GGC TAC ATG C-3' 5'-G CAT GTA GCC TTC CCG GAA GTA AAC C-3'
D268A	5'-GTG GTT TAC TTC CGA GCT GGC TAC ATG CCT C-3' 3'-G AGG CAT GTA GCC AGC TCG GAA GTA AAC CAC-5'
Y270A	5'-G GAT GGC GCC ATG CCT CGT CAG TAC AG-3' 5'-CT GTA CTG ACG AGG CAT GGC GCC ATC C-3'
Y270F	5'-C CGG GAT GGC TTC ATG CCT CGT CAG-3' 5'-CTG ACG AGG CAT GAA GCC ATC CCG G-3'

RESULTS

S-loop structure

The S-loop is one of the three loops covering the active site of hGS (Figure 1, vide supra). The S-loop is part of the γ-GC binding site of hGS. The X-ray crystal structure of hGS shows that the S-loop is located between beta sheet β10 and alpha helix α11 (Figure 1) [6]. The beginning of the S-loop (266-270) is near to the γ-glutamyl moiety of γ-GC (Figure 2a), Arg267 and Asp268 are near the charged α-carboxyl and α-amino groups of the γ-glutamyl moiety of γ-GC and likely interact with γ-glutamyl moiety. The aromatic ring of Tyr270 is over the cysteine moiety of the γ-glutamyl substrate and may shield the active site from water [8]. The early residues of the S-loop (Arg267, Asp268 and Tyr270) are in close proximity to the substrate γ-GC.

Computational studies (molecular dynamics modeling to find the lowest energy confirmation) were performed on the X-ray crystal structure (product form with GSH) of hGS using the MOE software (Molecular Operating Environment). Three different forms of hGS were modeled and analyzed for interactions of S-loop with the substrate γ-GC and the product GSH (Table 2): the free form was modeled by removing all substrates, the reactant form was modeled with three substrates (γ-GC, glycine and ATP) and the product form was modeled with products (GSH, ADP and P_i). In the reactant form, Arg267 forms salt bridges through its guanidino nitrogens with both of the both α-carboxyl oxygens of the glutamyl substrate; Arg267 also forms backbone hydrogen bond

to the γ-GC peptide bond nitrogen (Figure 2a, Table 2a). Asp268 forms a single salt bridge via its side chain carboxyl to the α-amino nitrogen of the γ-glutamyl substrate. Tyrosine 270 interacts via its hydroxyl with the γ-GC peptide backbone oxygen. All but one interaction between γ-GC and the S-loop are lost in the product form, a salt bridge between Arg267 guanidino and α-carboxyl of the γ-glutamyl substrate (Figure 2b, Table 2b). Of the 5 interactions between the S-loop and the substrate γ-GC, during catalysis to product form, only one bond is retained with the product GSH.

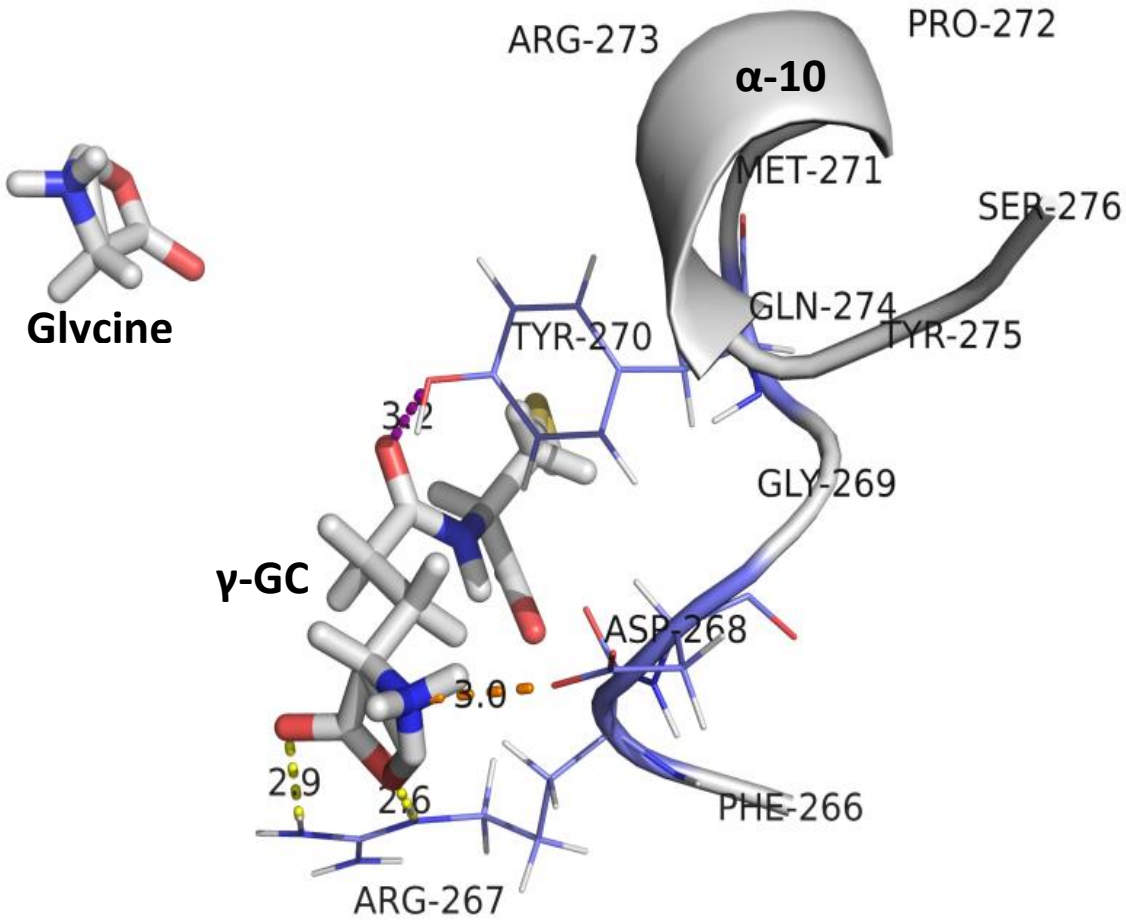


Figure 2a The S-loop of hGS (reactant form) with γ-GC

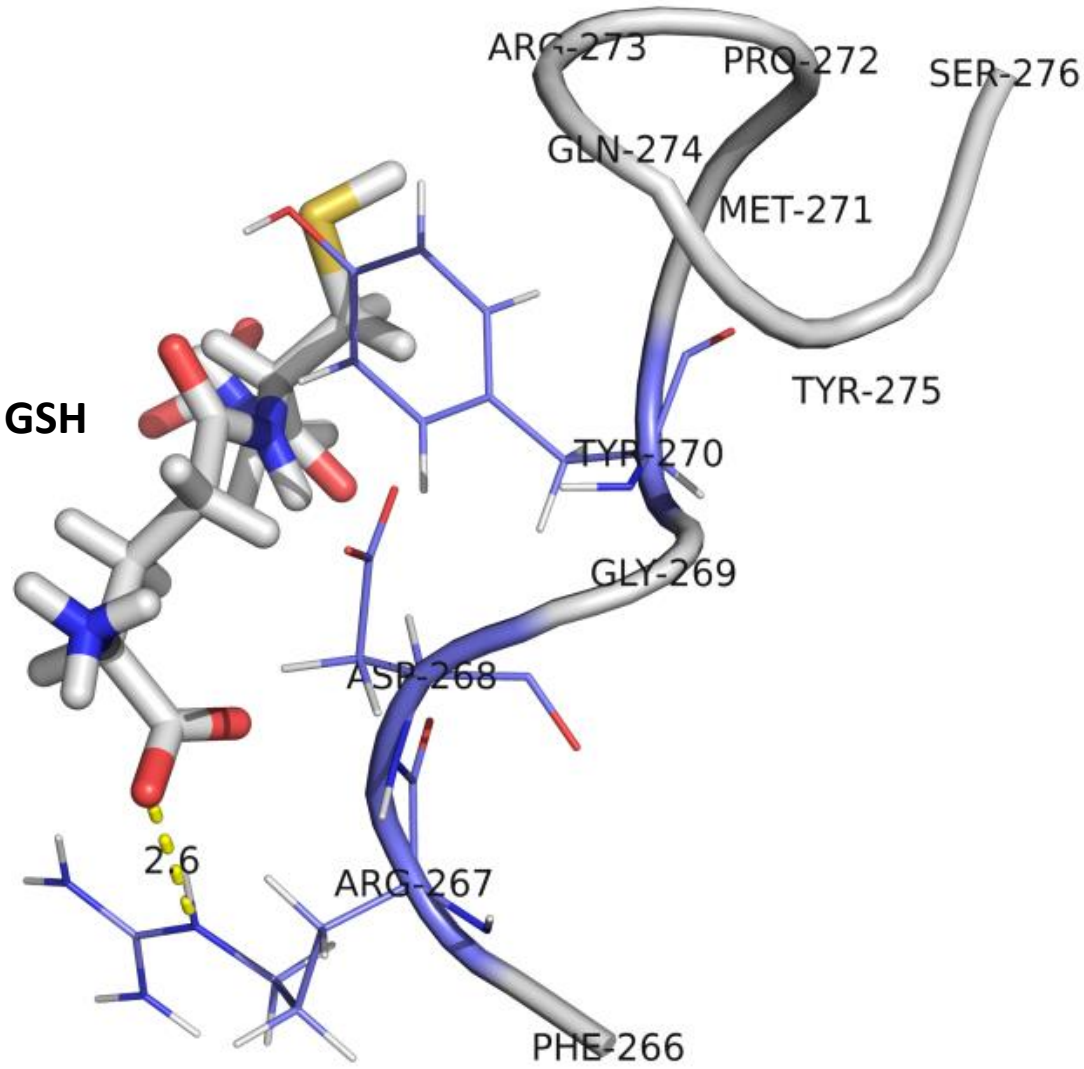
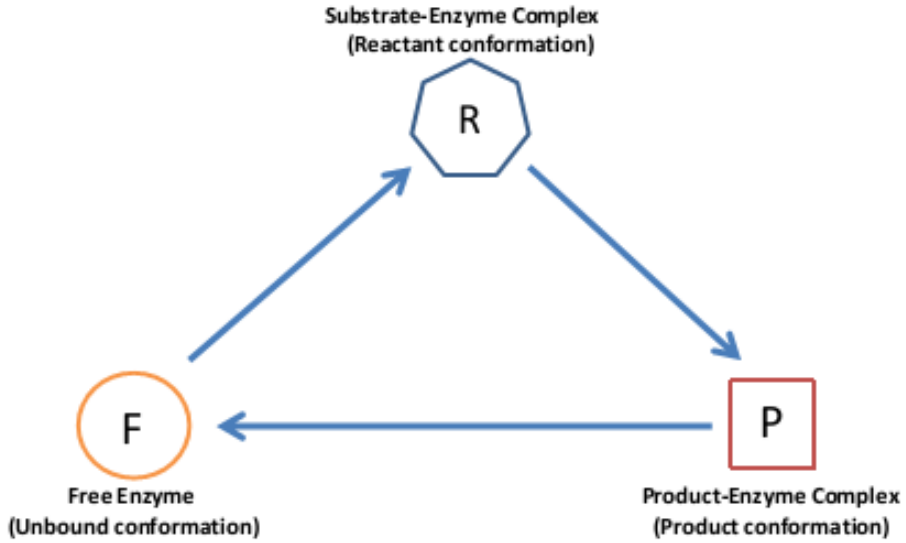


Figure 2b The S-loop of hGS (product form) with GSH

Table 2 Hydrogen bonds and salt bridges between S-loop residues and γ-GC & GSH

Interaction distances are measured between heavy atoms (< 3.4 A)

	a. Reactant				b. Product		
	S-loop atom	Substrate atom	Length	Interaction	S-loop atom	Product atom	Length
R267	Guanidino ε-NH_2	γ-glutamyl α-carboxyl	2.9	Salt-bridge	-	-	-
	Guanidino η-NH^+	γ-glutamyl α-carboxyl	2.6	Salt-bridge	Guanidino η-NH^+	γ-glutamyl α-carboxyl	2.6
	Backbone Oxygen	Peptide backbone Nitrogen	3.2	H-bond	-	-	-
Y270	Hydroxyl	Peptide Oxygen	3.2	H-bond	-	-	-
D268	Carboxyl	γ-glutamyl α-amino	3.0	Salt-bridge	-	-	-



Scheme 2 Conformations of hGS during catalysis

Table 3 Intra-molecular interactions of S-loop residues

S-loop Residue	Interacting Residue	Interaction	Bond length
Wild Type Free			
Arg267	Asp219	Salt Bridge	2.79
Arg267	Asp219	H-bond	2.89
Asp268	Gly269	H-bond	3.16
Asp268	Arg273	Salt-bridge	3.10
Asp268	Gln274	H-bond	2.86
Wild Type Reactant			
Arg267	Ile 209	H-bond	3.6
Arg267	Asp219	Salt Bridge	2.71
Asp268	Ile 209	H-bond	3.51
Asp268	Gln211	H-bond	2.94
Asp268	Gln274	H-bond	2.86
Wild Type Product			
Arg267	Asp219	H-bond	3.94
Arg267	Asp219	Salt bridge	2.84
Asp268	Ile209	H-bond	3.02
Asp268	Gly269	H-bond	2.82
Asp268	Arg273	H-bond	2.60
Asp268	Arg273	Salt-bridge	2.86

Computational and structural

During the reaction cycle, hGS transitions from free to reactant to product form (Scheme 2). Alterations during the catalytic cycle were analyzed to assess changes in intra-molecular interactions of the γ-GC binding S-loop residues (Table 3). All three conformations exhibit a salt bridge between Arg267 and Asp219. An additional hydrogen bond is formed between Arg267 and Asp219 in the free and product form. In the reactant bound enzymes, Arg267 forms a hydrogen bond with Ile209. Overall, Arg267 shows moderate changes in bonding depending on the state of the enzyme.

Within the free structure, Asp268 forms three hydrogen bonds (Gly269, Arg273, and Gln274) and a salt bridge (Arg273) with neighboring residues. While in reactant structure, Asp268 loses the salt bridge with Arg273 and the Gly269 hydrogen bond. Reactant Asp268 forms two new hydrogen bonds with Ile209 and Gln211. The product structure exhibits some of the bonds of the free and reactant forms: two hydrogen bonds (Ile209, Gly269 and Arg273) and a salt bridge (Arg273). In contrast to Arg267, bonding with Asp268 shifts significantly between each form of the enzyme.

Tyr270 does not form interactions with other residues in the free, reactant and product states. The substrate binding residues of the S-loop (Arg267 and Asp268) forms interactions with neighboring residues which suggest a possible role in enzyme stability.

Table 4 Thermal stability of human glutathione synthetase

Differential scanning calorimetry was performed to analyze T_m (transition midpoint) or unfolding temperature on hGS enzymes. Results are duplicates of two different enzyme purifications

Enzyme		T_m
Wild-type		60.2±0.17
R267	A	52.8±0.00
	W	54.2±0.00
	K	54.2±0.01
D268	A	62.1±0.05
	E	54.4±0.05
Y270	A	59.7±0.10
	F	58.6±0.80

Intra-molecular interactions have been thought to play a role in stability of enzymes. The structural stability of wild type and hGS mutant enzymes were determined by measuring the transition midpoint or temperature of unfolding (T_m) using differential scanning calorimetry (Table 4) (Note: HGS unfolding is irreversible). All mutations of Arg267 have 10% lower T_m values. In D268, the addition of a methylene (D268E) yields enzyme with decreased T_m value by 10% (54.4℃), while the change to alanine gives a small but consistent change in T_m value (62.0℃). Tyr270 enzymes unfold at about the same temperature as wild type. Mutations of the early conserved S-loop residues (267, 268, 270) result in modest changes in T_m values.

Table 5 Conservation of human glutathione synthetase and the S-loop

Sequences from the NCBI (National Center for Biotechnology Information) database were compared against the hGS sequence (16 species including 4 mammals).

	Conservation hGS	Conservation of S-loop
Mammalian GS species	92%	89%
All GS species	57%	63%

S-loop sequence conservation

The S-loop (266-FRDGYMPRQYS-276) of hGS is part of the γ-GC binding site (Figure 1). The multiple sequence alignment (ClustalW) [24] of the S-loop was used to compare the human GS sequence against other GS sequences in the NCBI database. Among all species, hGS is conserved 57% (Table 5). While in all mammalian species, GS maintains a higher level of conservation (92%). The S-loop maintains slightly higher identity (63%) in all species. For mammals, the S-loop identity is the same as the whole enzyme (~90%). The S-loop, maintains similar identity as the overall enzyme among mammals and all species.

There is no reported cooperativity in prokaryotic glutathione synthetase species as of today. Only a few of the eukaryotic species including human, rat and *Arabidopsis thaliana* display negative cooperativity towards the γ-glutamyl substrate (GAB).

Table 6 ClustalW analysis of S-loop residues

Sequences from the NCBI (National Center for Biotechnology Information) database were compared against the hGS sequence (16 species including 4 mammals).

	Phe F	**Arg R**	**Asp D**	Gly G	**Tyr Y**	Met M	Pro P	Arg R	Gln Q	Tyr Y	Ser S
	266	**267**	**268**	269	**270**	271	272	273	274	275	276
Mammalian GS species	100%	**100%**	**100%**	100%	**100%**	100%	100%	40%	80%	100%	60%
All GS species	94%	**100%**	**29%**	94%	**100%**	35%	94%	12%	24%	88%	18%

For GS from all mammalian species, eight of the eleven S-loop residues are completely identical, with only Arg273, Gln274 and Ser276 (Table 6) being different. For all non-predicted species, the S-loop residues of GS have a wider range of sequence identity (12-100%). A high level of sequence identity is retained for six of the eleven S-loop residues, Phe266, Arg267, Gly269, Tyr270, Pro272 and Tyr275 (≥ 88%). Most of the S-loop residues maintain a high level of sequence identity.

Of the three S-loop residues that bind the γ-glutamyl substrate, two, Arg267 and Tyr270, are completely identical within all species. The third S-loop residue that binds the γ-glutamyl substrate, Asp268, is completely identical in mammals, but is not conserved (29%) in all species. Overall, the three γ-glutamyl substrate binding S-loop residues that bind the γ-glutamyl substrate (Arg267, Asp268 and Tyr270) have complete identity in mammals, while, Arg267 and Tyr270 are completely identical within all species.

Table 7 Activity of human glutathione synthetase enzymes

Results are an average of duplicate assays of least two different enzyme preparations

Enzyme		k_{cat} (s^{-1})	% Activity	Glycine Independent hGS Activity
Wild-type		17.89±1.83	100	<1%
R267	A	0.36±0.01	2	14%
	W	0.05±0.01	0.3	80%
	K	2.70±0.30	15	5%
D268	A	11.8±0.05	66	<1%
	E	11.4±0.53	64	<1%
Y270	A	0.33±0.04	1.8	14%
	F	8.60±0.50	48	<1%

Experimental results

The spatial location, conservation and interaction of early S-loop residues (Arg267, Asp268 & Tyr270) warranted further analysis of the S-loop residues. To explore this site directed mutagenesis was used to prepare mutations of the early residues. The mutant hGS enzymes were expressed, purified and analyzed for changes in activity (Table 7). Activity was assessed by measuring the k_{cat} (turnover number). We hypothesize that changes to the early S-loop residues will have effects on enzyme activity, kinetics and cooperativity.

Arg267, which is near the α-carboxyl of the γ-glutamyl binding site, was investigated by mutating the bulky positively charged residue. When mutated to the small non-polar alanine, the resulting mutant enzyme (R267A) has little (2%) activity (Table 7). When

Arg267 was mutated to the bulky tryptophan, the enzyme (R267W) is not active (0.3%). When mutated to the charge-conserved lysine, the mutant enzyme (R267K) has 15% of wild type hGS activity. Mutation to alanine, tryptophan or lysine of Arg267 leads to loss of activity, with the conserved replacement (R267K) being the most active.

Asp268, which is near the α-amino group of the γ-glutamyl moiety, was examined by mutational analysis. The mutation of the negatively charged Asp268 to the non-polar alanine (D268A) or to the charge-conserved glutamate (D268E), yield enzymes with the same activity (65% of wild type). The mutations of Asp268 to either alanine (D268A) or to glutamate (D268E) result in active enzymes.

The aromatic Tyr270 that covers the γ-glutamyl binding site was investigated by mutating it to small alanine or to phenylalanine. The non-conserved mutation to the smaller alanine yields enzyme (Y270A) that has little (2%) activity. The conservative substitution to a phenylalanine gives enzyme (Y270F) that has about half of wild type activity. Both Tyr270 mutant enzymes lose activity, with good activity maintained by the conserved aromatic mutant enzyme.

The hydrolysis of ATP by wild type hGS requires the presence of all three substrates (γ-GC, glycine and ATP) to form product GSH. Mutant hGS enzymes (D268A, D268E and Y270F) with good activity (>20%) also require substrates, γ-glutamyl substrate (GAB) and glycine for ATP hydrolysis. However, mutant S-loop enzymes with low

activity (R267A, R267W and Y270A) display some ATP hydrolysis (Table 7) in the presence of GAB, but in the absence of glycine (glycine-independent activity).

Table 8 Kinetic properties of human glutathione synthetase

Results are an average of duplicate assays of least two different enzyme preparations. Not determined (n.d.) for enzymes with activity less than 5% of wild type

Enzyme		K_m GAB (mM)	Hill Coefficient (h)	V_{max} GAB
Wild-type		1.38±0.15	0.69±0.02	12.35±0.96
R267	A	n.d.	n.d.	n.d.
	W	n.d.	n.d.	n.d.
	K	13.33±0.98	1.03±0.06	13.33±1.00
D268	A	1.33±0.13	0.79±0.01	8.83±0.37
	E	0.19±0.06	0.68±0.05	7.12±0.04
Y270	A	n.d.	n.d.	n.d.
	F	8.73±0.70	0.80±0.03	11.23±0.70

Kinetic properties of the S-loop residues near the γ-glutamyl moiety binding site were studied using mutant hGS enzymes (Table 8). The mutant hGS enzymes were expressed, purified and analyzed for changes in activity, kinetic properties and stability (Table 7, 8). Kinetic properties (Michaelis constant, V_{max} and the hill coefficient) were analyzed for the γ-glutamyl substrate (GAB). V_{max} is the maximum velocity at substrate saturation; K_m is substrate concentration at half of V_{max} or the apparent substrate affinity. Hill coefficient is used as a measure of cooperativity of hGS towards the γ-glutamyl substrate (GAB). For a dimer like hGS, no cooperativity is shown with a Hill coefficient $h = 1$, negative cooperativity with hill coefficient $0.5 \leq h < 1$ and positive cooperativity with a Hill coefficient $h > 1$.

The role of the positively charged guanidino group of Arg267 in γ-glutamyl substrate binding was studied by mutating it to alanine, tryptophan or lysine. Kinetic studies were carried on the flexible charged lysine (R267K) mutation only because the non-conserved mutant enzymes (R267A, R267W) were inactive. The R267K mutant enzyme has a V_{max} value similar to wild type. Notably, the R267K mutant enzyme has a 10 fold higher K_m value for GAB and it loses negative cooperativity (h = 1.03) towards GAB. Any alteration of the Arg267 side chain has dramatic effect on enzyme kinetic properties.

The effects of mutations of Asp268, to conserved glutamate (D268E) and to non-conserved alanine (D268A) were examined. Both mutant enzymes were active with a lower V_{max} (60%) than wild type. The alanine mutant (D268A) enzyme has the same GAB K_m value (1.33 mM) as wild type and is slightly less negatively cooperative (h = 0.79) towards GAB. The conserved glutamate mutation (D268E) has a very low GAB K_m value (0.19 mM), but its cooperativity toward GAB was the same as wild type. In Asp268, D268A mutant enzyme has GAB substrate affinity similar to wild type; however, D268E mutant enzyme has a 7 fold reduction in GAB affinity.

When Tyr270 was mutated to a small alanine, the mutant enzyme (Y270A) activity is too low to assess kinetic properties. However, the conserved phenylalanine mutant enzyme (Y270F) retains 50% activity and has a slight trend toward less negative cooperativity (h = 0.80). The V_{max} value of Y270F is about same as wild type hGS. Interestingly, the K_m value for GAB is increased by 6 fold. The non-conserved change to

alanine (Y270A) loses activity; while the charge-conserved change to phenylalanine (Y270F) maintains half activity with a 6 fold higher K_m value.

The three early residues (Arg267, Asp268 and Tyr270) of the S-loop of hGS were examined. All three residues interact with the γ-glutamyl moiety of the substrate γ-GC. Two residues (Arg267 and Tyr270) maintain complete identity in all species, while the Asp268 residue has low identity (29%). Mutations of Asp268, with lower sequence identity, to either charge conserved (Glu) or to non-conserved (Ala) yield enzymes with good activity. When the completely identical residues (Arg267, Tyr270) were mutated with non-conserved mutations (R267A, R267W, Y270A), most of the hGS activity was lost. However, the charge conserved mutant enzymes (Y270F, R267K) retain activity. The V_{max} values for all S-loop mutations prepared are about the same (60% to 110%) as wild type. Substantial differences in K_m values are observed, e.g. R267K is increased 10 fold, while D268E is decreased 7 fold decrease. A trend to a slight loss of negative cooperativity is observed with some mutations (D268A & Y270F); however the conserved mutation R267K has a complete loss of negative cooperativity toward the γ-glutamyl substrate. In summary, mutations of S-loop residues of hGS that bind the γ-glutamyl substrate have small effects on V_{max} but large changes are seen in enzyme activity (0.3-66%) and K_m values for GAB (0.19-13.33 mM) and one (R267K) has a complete loss of cooperativity.

DISCUSSION

The ionic interactions of Asp268 with the α-amino moiety of the γ-glutamyl substrate suggest participation of Asp268 in binding and orientation of the γ-glutamyl substrate. Previous studies of hGS demonstrated that hGS is less specific toward the γ-glutamyl moiety of the substrate γ-GC [14]. In agreement with its small effect on catalysis, Asp268 also maintains a low conservation in all hGS species. However, based on the position of Asp268, it is poised to bind the α-amino nitrogen of γ-GC substrate, suggesting that the residue plays a role in catalysis. The experimental results support both hypotheses as both alanine and glutamate mutation show modest effects on activity and hGS mutant enzymes still maintains activity with either mutation. Only the D268E mutation affects the kinetic properties of hGS, possibly due to increase in possibilities of hydrogen bonds with the longer glutamate closer to the γ-glutamyl substrate. With Asp268, the change in interactions with the γ-glutamyl substrate has small effect on activity but large impact on affinity. The large reduction of K_m value with the D268E mutation may be due to stabilization of the ES state. The large increase in γ-glutamyl substrate affinity and slight changes in activity of Asp268 mutant enzymes suggest that Asp268 helps maintain maximum enzyme activity and γ-GC substrate orientation.

Tyr270 of hGS is identical in all known species suggesting a crucial role in hGS. Since the alanine mutant shows no change in T_m, the role of Tyr270 is unlikely to be structural. Tyr270 covers the γ-GC substrate when bound to hGS and forms one hydrogen

bond between the hydroxyl of Tyr270 and the carbonyl oxygen of γ-GC. This suggests that Tyr270 likely participates in orienting the γ-GC substrate while shielding the reaction intermediate from hydrolysis. Both Y270A and Y270F mutant enzymes have decreased activity, however, the Y270F mutant enzyme has high activity suggesting that the H-bond of Tyr270 participates in γ-glutamyl substrate binding and, therefore, increases activity. That the conserved aromatic mutation Y270F is active, while Y270A is not, supports the thesis that the aromatic tyrosine shields the binding site. The alanine mutant enzyme (Y270A) has glycine-independent hGS activity (reactive intermediate hydrolysis), and it cannot protect the reaction intermediate, supporting the role of Tyr270 in shielding. The aromatic requirement of Tyr270 suggests possible anion-π interactions of the aromatic ring with the carbonyl oxygen of the γ-glutamyl substrate [22], whereby Tyr270 electron withdrawing capacity aids in catalysis. In conclusion, Tyr270 aromatic and phenol are both required for complete enzyme function.

The ionic interaction and salt-bridges of Arg267 with the γ-glutamyl substrate suggest participation of Arg267 in binding and orientation of the γ-glutamyl substrate. Arg267 acts to anchor the α-carboxyl of the glutamyl moiety of γ-GC through salt bridges. The complete identity of Arg267 in all species and its location suggests an important role in hGS activity and stability. The positively charged guanidino of Arg267 is essential in enzyme function because mutants that decrease the interactions with the γ-glutamyl substrate also decrease enzyme activity. The alanine mutant enzyme (R267A) loses all interactions, and hence almost all activity. The mutation to the smaller alanine

may allow water into the active site and thus, hydrolyze reaction intermediate (glycine-independent ATP hydrolysis). The larger bulky tryptophan (R267W) possibly blocks the active site resulting in minimal activity; while allowing hydrolysis of reaction intermediate. The positive charge-conserved lysine mutant enzyme (R267K) maintains a single charge and retains some activity.

The positive charge-conserved lysine mutation with a single charge maintains some activity but loses some interactions, leading to increase in K_m value for the γ-glutamyl substrate. The change in binding interactions with R267K also results in loss of negative cooperativity. Earlier studies in the Anderson lab of the D458 residue in the A-loop displayed similar loss of negative cooperativity as a result of disruption in substrate binding. These results suggest that the positively charged guanidino of Arg267 is necessary for hGS activity and γ-glutamyl substrate affinity; furthermore, changes to substrate binding can alter enzyme cooperativity.

From the present data, we can conclude that the S-loop substrate binding residues play a critical role in activity, γ-glutamyl substrate binding and enzyme cooperativity. The effect on activity is observed in all S-loop mutations. Changing interactions with the substrate have a direct effect on binding where reducing interactions (R267K, Y270F) decreases γ-glutamyl substrate affinity and increasing interactions (D268E) leads to higher γ-glutamyl substrate affinity. With R267K enzyme the alterations in interactions with the substrate also leads to loss of enzyme cooperativity. Further studies are needed

to completely assess the role of the S-loop substrate binding residues in activity, γ-glutamyl substrate binding and enzyme cooperativity.

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CHAPTER IV

FUTURE DIRECTION

Future research is focused on several other substrate binding residues of hGS that could be a cooperativity switch for the negatively cooperative enzyme. Further studies on the S-loop are needed to understand substrate binding with the use of an Isothermal Micro-calorimeter (ITMC) and also more studies are needed to better understand hGS reaction mechanism and order.

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