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The Role of the Glycine Triad In Human Glutathione Synthetase

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Abstract

Experimental kinetics and computational modeling of human glutathione synthetase (hGS) support the significant role of the G-loop glycine triad (G369, G370, G371) for activity of this ATP-grasp enzyme. Enzyme kinetic experiments indicate that G369V and G370V mutant hGS have little activity (<0.7 and 0.3%, respectively, versus wild-type hGS). However, G371V retains ~13% of the activity of wild-type hGS. With respect to G-loop:A-loop interaction in hGS, mutations at Gly369 and Gly370 decrease ligand binding and prevent active site closure and protection. This research indicates that Gly369 and Gly370 have essential roles in hGS, while Gly371 has a lesser involvement. Implications for glycine-rich ensembles in other phosphate-binding enzymes are discussed.

Keywords

glutathione synthetase; glutathione; negative cooperativity

Introduction

Glutathione (GSH) [1], an essential tripeptide antioxidant, is present in most bacterial, plant and mammalian cells. It protects against reactive oxygen species and participates in metabolism of drugs and carcinogens. GSH helps maintain protein thiols in their reduced states [2,3]. GSH is synthesized in two consecutive ATP-dependent steps, catalyzed by first γ -glutamylcysteine synthetase and then glutathione synthetase (GS; EC 6.3.2.3). Decreased levels of GSH are observed in Parkinson's disease, Alzheimer's disease [4], HIV, *etc.* [5]. Low levels of GSH occur in 5-Oxoprolinuria [6], a disease caused by inborn errors in GS. Improved understanding of GS is thus important to developing new therapies for GSH deficiencies.

Glutathione synthetase catalyzes the second and final step in GSH biosynthesis: ATP-dependent ligation of glycine with γ -glutamylcysteine. This process involves formation of a putative enzyme-bound acyl-phosphate that is attacked by glycine to form an enzyme-product complex. The latter dissociates with release of GSH, ADP and phosphate (P_i). Glutathione synthetase [7] belongs to the Glutathione synthetase ATP-binding domain-like (ATP-grasp) superfamily [8]. With two exceptions, structurally characterized ATP-grasp superfamily members are prokaryotic [9].

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Analysis shows that the entire amino acid sequence of human GS (hGS) is only conserved in mammals, but identity remains between hGS and other eukaryotes. However, differences between human and prokaryotic GS sequences are much greater. Thus, prokaryotic GS sequences show little (<10%) sequence identity [10,11] when aligned against mammalian counterparts. However, there are specific regions (loops) of GS that are highly conserved in all species.

Structural data on hGS, co-crystallized with products [11], indicates three loops cover the active site: substrate-binding or S-loop (Phe266 - Ser276), glycine-rich or G-loop (Gln366 -Leu374) and alanine-rich or A-loop (Ile454 - Ala466). Our recent research [12] has shown that the amino acid sequence of the glycine-rich-loop (G-loop) is the most strictly conserved in eukaryotic GS.

Similar to eukaryotes, GS from *E. coli* has glycine-rich regions: small (164-GMGG-167) and large (226-IPQGGETRGNLAAGGR-241) loop. The structure indicates that the catalytically essential glycine-rich large loop of *E. coli* GS is very flexible and covers the substrate binding site [13]. Large loop residues are proposed to act as a “lid” to protect the acyl phosphate intermediate from hydrolysis during catalysis [13]. Mutagenesis studies suggested that two residues, Gly220 and Gly240, are important for substrate binding and catalytic activity [14, 15]. Research [14,15] shows that the glycine-rich large loop of *E. coli* GS is important for enzyme activity. However, given significant differences in sequence identity and structure between human (dimer) and *E. coli* (tetramer) GS, it is essential to study the G-loop of hGS.

There are several examples of glycine-rich loops within enzyme catalysis that modify important conformational changes and ligand binding. For instance, X-ray crystallographic and kinetic studies of cytosolic aspartate aminotransferase containing pyridoxal 5'-phosphate (PLP) cofactor showed that mutation of a loop glycine residue (Gly38) significantly reduced enzyme activity and impaired loop structural flexibility, preventing small domain closure [16]. Triosephosphate isomerase (TIM) contains a glycine-rich flexible loop that closes the active site via interactions with the peripheral phosphate of the substrate [17]. The proposed role of the glycine residues in the loop of TIM is to bind and protect the enediol phosphate intermediate. Aminolevulinate synthase (ALAS), a PLP-containing enzyme in heme biosynthesis, has a conserved glycine-rich loop. Mutagenesis studies indicate that replacement of Gly142 or of Gly144 considerably decreases the binding affinities of PLP cofactor and also the catalytic efficiency of ALAS [18]. Dihydrofolate reductase (DHFR) [19], an important anticancer target, has a highly mobile loop with a conserved glycine residue. When Gly121 in the flexible loop is mutated to valine, binding of NADPH (nicotine adenine dinucleotide phosphate) and enzyme activity are significantly reduced [20]. Finally, there is a glycine-rich loop in the catalytic subunit of cAMP-dependent protein kinase (PKA) [21]. Several studies on PKA conclude that the glycine-rich loop positions the nucleotide triphosphate and also prevents water molecules from accessing the active site [22,23,24]. In summary, glycine residues are important in enzyme catalytic loops for controlling flexibility, ligand orientation and binding, and loop closure to protect reaction intermediates.

Based on the structure of hGS and the aforementioned literature precedents, we propose that glycine residues in the G-loop of human GS maintain loop flexibility essential for ligand binding and active site closure. We further hypothesize that glycine residues in the G-loop facilitate γ -phosphate transfer to substrate and protect the acyl-phosphate intermediate. We have constructed and characterized, by the use of experimental kinetics and modeling, mutant hGS enzymes that support the vital role of the glycine triad (G369, G370, G371) of the G-loop for hGS.

Materials and Methods

Computational Methods

Calculations were performed with MOE [25]. The AMBER94 force field [26] and PEOE atomic charges [27] were used. As in a previous theory-experiment study of hGS [12], the X-ray structure [11] for hGS (product form, PDB ID = 2HGS) [28] was used to initiate a molecular dynamics (MD) search for lowest energy conformations. Enzyme models were subjected to the MD protocol described previously [12,29]. Modeling was performed on product and reactant forms of hGS; only the latter are discussed here as the modeling results are similar and both corroborate experimental analyses.

Materials

Oligonucleotide primers for mutagenesis and sequencing were from Integrated DNA Technologies. Restriction enzymes were from New England Biolabs. Isopropyl-1-thio- β -D-galactopyranoside (IPTG) and lactate dehydrogenase (LDH) were from Amresco. The Quickchange™ site-directed mutagenesis kit was obtained from Stratagene. L- γ -Glutamyl-L- α -aminobutyrate (GAB) was synthesized as described [1,2]. All other reagents were obtained from Sigma.

Recombinant DNA methods

N-terminal His tag (His₆) was added to wild-type hGS by subcloning it from plasmid pT7-7 into pET-15b vector (Novagen) at BamHI and NdeI sites (hGS_{pET-15b}) [29]. Mutants of hGS were generated using the Quickchange™ kit (Stratagene). The internal primers used for synthesizing the mutants are shown in Table 1. The mutations were confirmed by sequencing hGS.

Enzyme purification

The growth and purification protocol adopted for the recombinant wild-type and mutant hGS was the same, as previously described [29]. All purification steps were performed at 4 °C [29]. Purified hGS proteins, wild-type and mutants, were apparently homogeneous by SDS-PAGE.

Enzyme assays and kinetic analysis

All kinetic assays were carried out in duplicate using at least two different recombinant hGS enzyme preparations. The enzyme activity was measured at 37 °C using a spectrophotometric assay that couples ADP production to NADH oxidation with continuous monitoring at 340 nm [29]. The Michaelis-Menten kinetic equation was used for glycine and ATP kinetic data analysis. For GAB kinetic analysis, the initial velocity (v), concentration (S) and $V_{\max}$ are substituted into the Adair equation for negative cooperativity, and GAB K_m and α (α), or interaction factor are calculated. The degree of negative cooperativity was assessed via a Hill plot ($\log[(V/V_{\max} - V)]$ versus $\log[S]$) to obtain a Hill coefficient, h . Kinetic data was plotted and non-linear regression analysis was performed using SigmaPlot 8.0 (SPSS Science Inc.). A unit of enzyme catalyzes 1 μmol of product min^{-1} at 37 °C. Protein concentration was determined by the Lowry method [30] using bovine serum albumin as the standard.

Circular Dichroism

Circular dichroism was performed using a Jasco715. Enzymes in sodium phosphate buffer (10 mM; pH 7.5) were run for a total of three accumulations from 320 – 190 nm at 20 °C. Baseline was subtracted from each sample and subsequently underwent three Savitzky-Golay 25 point smoothings. The circular dichroism spectra of wild-type and mutant hGS were superimposable.

Results and Discussion

In Vitro Experiments

Our hypothesis that highly conserved glycine residues (Gly369, Gly370, Gly371) of the hGS G-loop are important for activity was evaluated by site-directed mutagenesis. Glycine triad residues were individually mutated to valine (to give G369V, G370V and G371V mutant GS), a bulky amino acid expected to hinder G-loop flexibility [20]. Kinetics indicate that purified G369V and G370V have little activity (<0.7 and 0.3%, respectively, versus WT-hGS) (Table 2). Interestingly, however, G371V retains ~13% of the activity of the wild-type enzyme. Thus, individual mutations of glycine residues in the G-loop (Gly369, Gly370, Gly371) substantially reduce hGS activity.

Highly purified wild-type hGS (WT-hGS) does not have activity unless all substrates (glycine, Mg-ATP, γ -glutamyl- α -aminobutyric acid (GAB), a non-thiol and non-oxidizable analog of γ -glutamylcysteine) are present. ATP hydrolysis activity is not observed in the absence of any of its substrates individually; notably WT-hGS does not hydrolyze ATP when glycine is absent, i.e., hGS not glycine-independent (Table 3). Similar to wild-type GS, the G371V mutant displays only 5% glycine-independent activity (Table 3). In contrast, both the slightly active G369V and G370V mutant hGS enzymes hydrolyze ATP in the presence of GAB, but absence of glycine (Table 3, GAB + ATP; Gly-Independent). Mutations G369V and G370V thus lead to a GAB-dependent ATPase activity (Table 3) (or “glycine-independent” GS activity). Since WT-hGS does not show ATPase activity, mutagenesis experiments indicate that highly conserved glycine residues of the G-loop have a substantial impact on enzyme function.

The Michaelis constant (K_m) of mutant hGS enzymes was determined for substrates (Table 4). The K_m for ATP increased substantially over wild-type for both G369V and G371V mutant hGS enzymes (37- and 10-fold, respectively). The K_m for glycine increased five-fold for G371V. The activity of the G370V mutant was too low for determination of K_m . The little activity present for G370V was only dependent on GAB, not glycine. As previously observed [31,32], mammalian GS, a homodimer, displays negative cooperativity or non-linear double reciprocal plots for GAB. A measure of negative cooperativity is given by the Hill coefficient (h), which ranges from 0.5 to 1 for a homodimer. The G371V mutant is similarly negatively cooperative ($h = 0.88$) versus WT-hGS ($h = 0.80$). However, G369V no longer displays negative cooperativity for GAB binding. Mutations of conserved G-loop glycine triad residues thus substantially affect the Michaelis constant for the substrates of hGS.

Based on the structure of hGS, mutations of glycine triad residues, which are near the enzyme surface, are not expected to greatly affect hGS secondary structure. Circular dichroism spectra of mutant and wild-type hGS are virtually identical (data not shown), supporting this contention, as does similarity of structures obtained from MD simulations (*vide infra*).

Modeling

We analyzed the geometry of reactant hGS to gain atomic-level insight into the effects of mutation on the G-loop glycine triad. Comparative structure analysis for WT-hGS and the three hGS mutants was performed.

Ligand Binding

Molecular dynamics of wild-type hGS (reactant) indicate that the G-loop has a flexible backbone that aids in substrate access to the active site. The backbone length (distance between nitrogen of Gly369 and carbonyl carbon of Gly371) is 7.92 Å – an extended conformation (Fig. 1.a). Structural analysis of the wild-type reactant shows all three glycine triad residues form hydrogen bonds with ATP γ -P_i (the phosphate lost to form acyl-phosphate intermediate). The

amide of Gly369 and Gly370 each hydrogen bond (2.37 and 2.74 Å, respectively) with the same oxygen of γ -P_i, while the G371 amide hydrogen binds (2.94 Å) a different oxygen of the γ -P_i of ATP. Glycine triad residues in reactant hGS are thus oriented so that the backbone amides position the γ -P_i of ATP by forming hydrogen bonds during ligand binding.

G369V Mutant hGS—Investigation of the modeled structure of hGS mutant G369V (reactant) reveals altered orientations of residues and ligands (Figure 1.b) versus WT-hGS. While the γ -P_i of ATP has a position similar to that of the wild-type structure, backbone amides of Val369, Gly370, and Gly371 are quite distinct in G369V. As a result of structural changes in G369V, the three backbone amide groups of the glycine triad now have an unfavorable orientation that prevents hydrogen bonding to the phosphates of ATP in G369V mutant.

G370V Mutant hGS—For reactant G370V, the orientations of ATP, the backbones of the remaining glycine residues (G369, G370), and mutated residue (G370V) are all altered. The distance between the N of Gly369 and the amide N of Asn372 in the reactant is shortened to 6.79 Å (wild-type distance = 7.24 Å). (Figure 1.c). Hence, the G-loop in G370V is in a less extended conformation than the G-loop for wild-type hGS. In G370V, γ -P_i has both a different orientation and altered hydrogen bonding arrangement vis-à-vis WT-hGS. Instead of each of the glycine residues forming a hydrogen bond with ATP as in wild-type hGS (Figure 1.a), there is only one hydrogen bond (2.32 Å, Figure 1.c) with ATP made by G371. This hydrogen bond is to the bridging oxygen between γ -P_i and β -P_i, rather than the γ -P_i of ATP. Interestingly, one of the γ -phosphate oxygens now forms a hydrogen bond (2.69 Å) with a glycine residue (Gly459) in the A-loop (*vide infra*).

G371V Mutant hGS—Analysis of G371V shows less drastic changes in loop orientation and ATP binding (vs. G369V and G370V) relative to WT-hGS. Even though the orientation of ATP is relatively unaffected (Figure 1.d), the valine side chain prevents Val371 from hydrogen bonding to ATP. The phosphate groups of ATP retain almost the same orientation in the active site of G371V as in the wild-type enzyme. The two remaining glycines, 369 and 370, form hydrogen bonds (2.07 Å and 2.90 Å, respectively) to the γ -P_i of ATP akin to those in wild-type hGS. The G371V mutant is thus the most structurally similar to WT-hGS of the three G-loop mutations studied in this research.

G-loop: A-loop Interaction

Our previous research [12,29] indicates that in addition to loop-ligand interactions, there are important loop-loop interactions in hGS. Interaction between the G-loop and A-loop of wild-type hGS occurs only in the reactant conformation (Figure 2.a). The carbonyl oxygen of Gly369 (G-loop) hydrogen bonds to the amide of both Val461 (2.99 Å) and Ala462 (2.44 Å) in the A-loop that lead to loop closure.

Based on substrate orientations and loop positions, we propose that G-loop:A-loop interaction [12] covers the active site, leading to loop closure, thus protecting from hydrolysis the acyl-phosphate intermediate (or the related transition state) in peptide bond formation. Hydrogen bonding patterns show that loop-loop interactions orient both the γ -P_i of ATP and the glycine substrate near (~10 Å) of the γ -glutamylcysteine substrate. In the reactant form, Gly369, Gly370, and Gly371 bind the γ -P_i while Gly460 of the A-loop binds glycine by forming two hydrogen bonds. Thus, loop closure and glycine residues in the G-loop, particularly Gly369 and Gly370, substantially impact γ -P_i transfer from ATP to γ -glutamylcysteine while A-loop residues (Gly460, Val461, Ala462) together with Gly369 presumably facilitate glycine transfer to the acyl-phosphate. Nevertheless, further experiments are needed to confirm the role of A-loop residues.

G369V Mutant—Mutation of glycine 369 to valine alters the G-loop orientation of G369V (reactant) so that G and A loops no longer close (Figure 2.b). In the reactant G369V structure, the carbonyl oxygen of Val369 now points in the opposite direction to that found for WT-hGS, which prevents hydrogen bonding with both Val461 and Ala462. Hence, there is no longer A-loop:G-loop interaction in G369V, and thus no loop closure.

G370V Mutant—When Gly370 is mutated to valine (Figure 2.c), the distance between the Gly369 (G-loop) and A-loop residues increases significantly, from ~3 to ~8 Å! Hence, this mutation significantly disrupts loop-loop interaction in hGS.

G371V Mutant—Analysis of G371V shows that the geometry (Figure 2.d) for both ligand binding and for G-loop:A-loop interaction is similar to that of WT-hGS. In G371V, the oxygen atom of Gly369 still hydrogen bonds with the amide of Val461 (3.02 Å) and of Ala462 (2.64 Å) of similar length to those calculated for the wild-type enzyme. Similarly, Gly460 hydrogen bonds the glycine substrate, albeit less effectively because now there is only one hydrogen bond of 1.96 Å between the oxygen of G460 and the amide of glycine substrate in G371V instead of the two hydrogen bonds in wild-type hGS.

Conclusions

Analyses of conformational changes and hydrogen bonding variations elucidate experimentally observed decreased enzyme activity and altered binding affinity of glycine-triad mutant hGS relative to the wild-type enzyme. As a result, this research provides new and valuable insight into the function of Gly369-Gly371 residues of the hGS G-loop. Rotations around backbone C-C and C-N bonds in proteins are known to generate more flexibility for glycine than other amino acid residues [33]. Both G369V and G370V mutants have extremely low experimental activities, while G371V is less affected. However, the latter still shows a ~10-fold decrease in k_{cat} versus wild-type hGS. Single-point mutations (G369V, G370V, and G371V), analyzed via experiment and modeling, clarify the role of the three glycines in the G-loop of hGS. Simulations indicate that upon mutation of any of these residues, binding of γ -P_i of ATP is altered. Structural differences in mutant hGS (Figure 3) are manifested by local conformational changes that affect ligand orientation and presumably also impact transition state stabilization. In the reactant conformation, all three glycines bind the γ -phosphate of ATP. These favorable interactions detected in wild-type hGS, when combined with experimental results from site-directed mutagenesis, indicate that G369 and G370 are responsible for γ -P_i transfer to γ -glutamylcysteine, while G371 has only a minor contribution to the correct orientation of γ -P_i.

Although a glycine-rich loop motif is found in many nucleotide binding enzymes, hGS displays a rare conserved Gly-Gly-Gly sequence. In contrast, the glycine-rich loop sequence in ALAS [18], DHFR [19], and the protein kinase family [34] is Gly-X-Gly-X-X-Gly, where X is a variable amino acid. Other phosphate binding loops [35] that interact with the γ -phosphate of ATP have the consensus sequence Gly-X-X-X-X-Gly, including the large loop of *E. coli* GS.

The present research indicates that the unusual glycine triad in the hGS G-loop greatly enhances flexibility of the backbone in close proximity to the γ -P_i of ATP. These factors facilitate a favorable orientation of ATP in the binding site, guide the transfer of γ -P_i to the substrate, and allow access of glycine substrate followed by its nucleophilic attack on the acyl-phosphate intermediate. Although the rate-limiting step is still under debate, glycine backbone atoms could easily form hydrogen bonds with the γ -phosphate of ATP in the transition state, lowering the activation energy for glutathione biosynthesis.

***Research Highlights**

- G-loop glycine-triad mutations greatly reduce the activity of human Glutathione Synthetase.
- Mutations to glycine-triad residues decrease ligand binding and prevent active site closure.
- G-loop glycines Gly369 and Gly370 have essential roles in hGS, while Gly371 has lesser involvement.

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Abbreviations

hGS	human glutathione synthetase
GSH	glutathione
GAB	γ -glutamyl- α -aminobutyrate
IPTG	isopropyl-1-thio- β -D-galactopyranoside
MCAC	metal chelate affinity chromatography
P_i	phosphate

Figure 1. Hydrogen bonding between γ -P_i of ATP and G-loop residues (reactant). (a) wild-type; (b) G369V; (c) G370V; (d) G371V.

Figure 2.

G-loop: A-loop interaction and glycine binding (reactant). The Gly370 and Gly371 residues were omitted for clarity. (a) wild-type; (b) G369V; (c) G370V; (d) G371V.

Figure 3.

Distortion of G-loop backbone in reactant-complex for mutant enzymes. Front view (left) and side view (right) show G and A loops. Wild-type is rendered in blue, G369V in red, G370V in yellow, G371 in cyan.

Table 1
Primers for site directed mutagenesis of the G-loop of glutathione synthetase^a

Enzyme	DNA sequence
G369V	5 -CCAGAGAGAGG <u>T</u> TGGAGGTAAC-3
	5 -GTTACCTCCA <u>A</u> CCTCTCTGG-3
G370V	5 -CCAGAGAGAGGGT <u>G</u> TAGGTAACAACC-3
	5 -GGTGTTCCT <u>A</u> CACCCTCTCTGG-3
G371V	5 -CCAGAGAGAGGGTGGAG <u>T</u> TAACAACCTATATGGG-3
	5 -CCCATATAGGTTGT <u>A</u> CTCCACCCTCTCTGG-3

^a Underlined bases are changed nucleotide positions.

Table 2
Activity and turnover number (k_{cat}) of GS enzymes^a

Enzyme	k_{cat} (sec ⁻¹)	% Activity
Wild-type	18.83 ± 0.77	100
G369V	0.133 ± 0.001	0.69
G370V	0.052 ± 0.026	0.27
G371V	2.36 ± 0.35	13.1

^aResults are an average of 3 different enzyme preparation, each assayed in duplicate. Control rates (lacking GAB) were subtracted to obtain k_{cat} values. The lower limit of detection is 0.008 sec⁻¹.

Table 3
Substrate dependent GS activity (k_{cat})^a

Enzyme	GAB + Gly + ATP	GAB + ATP	% Gly Independent
Wild-type	18.83 ± 0.77	0	0
G369V	0.133 ± 0.001	0.114 ± 0.017	85
G370V	0.052 ± 0.026	0.057 ± 0.024	100
G371V	2.36 ± 0.35	0.116 ± 0.065	5

^aResults are averages of replicate assays of two or three different enzyme preparations. For these studies the background rate subtracted to obtain K_{cat} values is that of ATP-Mg alone (Enzyme+ATP-Mg). There is no activity with glycine plus ATP.

Table 4
K_m values for glutathione synthetase mutant^a

Enzyme	ATP (mM)	Gly (mM)	GAB (mM) ^b
Wild-type	0.012 ± 0.009	0.529 ± 0.009	1.26 ± 0.32
Gly369Val	0.44 ± 0.04	N.D.	0.89 ± 0.36
Gly371Val	0.12 ± 0.05	2.52 ± 0.30	0.100 ± 0.043

^aThe results are the averages of replicate assays of two or three different preparations. N.D.: not determined.

^bGAB is estimated.