VALINE 44 AND VALINE 45 OF HUMAN GLUTATHIONE SYNTHETASE ARE KEY FOR SUBUNIT STABILITY AND NEGATIVE COOPERATIVITY

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Abstract

It was hypothesized that residues Val44 and Val45 serve as important residues for human glutathione synthetase (hGS) function and stability given their location at the dimer interface of this enzyme. Computational studies suggest that mutation at Val45 has more impact on the structure and stability of hGS than does mutation at Val44. Experimentally, enzymes with mutations at the 44 and or 45 positions of hGS were prepared, purified and assayed for initial activity. Val45 position mutations (either to alanine or tryptophan) have a greater impact on enzyme activity than do mutations at Val44. Differential scanning calorimetry experiments reveal a loss of stability in all mutant enzymes, with V45 mutations being less stable than the corresponding Val44 mutations. The γ-GluABA substrate affinity remains unaltered in V44A and V45A mutant enzymes, but increases when tryptophan is introduced at either of these positions. Hill coefficients trend towards less negative cooperativity with the exception of V45W mutant hGS. These results imply that residues V44 and V45 are located along the allosteric pathway of this negatively cooperative dimeric enzyme, that their mutation impacts the allosteric pathway more than it does the active site of hGS, and that these residues (and by extension the dimer interface in which they are located) are integral to the stability of human glutathione synthetase.

Keywords

glutathione synthetase; glutathione; negative cooperativity

INTRODUCTION

Proteins fold into intricate three-dimensional structures held together by chemical interactions among amino acid residues, such as hydrogen bonding, van der Waals interactions, and hydrophobic effects. Any interruption of these interactions can lead to loss of protein stability and/ or function. How function and stability are interrelated, particularly for multimeric enzymes, is still not fully understood. Hydrophobic interactions have been proposed to make a major contribution in stabilizing the native structure of proteins in an
aqueous environment\textsuperscript{11}. Efforts to use site-directed mutagenesis to investigate the stabilizing forces in multimeric enzymes have been carried out\textsuperscript{12}, but still have not fully answered the question: Does a change in enzyme stability directly affect the activity and/or allosteric properties, such as cooperativity, of the protein? In order to address this question, human glutathione synthetase (hGS) was chosen for study, not only for its importance as a member of the ATP-grasp superfamily of enzymes\textsuperscript{3, 4}, but also due to its dimeric structure\textsuperscript{5} and the negative cooperativity\textsuperscript{6} it displays in substrate binding\textsuperscript{7-9}. Human glutathione synthetase is the enzyme responsible for the ligation of \(\gamma\)-glutamylcysteine and glycine to form the antioxidant tripeptide glutathione (GSH)\textsuperscript{10}.

Wild-type human glutathione synthetase (WT-hGS), a homodimer, displays negative cooperativity towards the \(\gamma\)-glutamylcysteine substrate\textsuperscript{7-9}. As a consequence, the binding of \(\gamma\)-glutamylcysteine in one subunit of WT-hGS reduces the binding affinity of this substrate in the second subunit. Analysis of the crystal structure of WT-hGS reveals the presence of a dimer interface containing \(\alpha\)-helices \(\alpha1\) and \(\alpha2\), \(\beta\)-sheets \(\beta1\) and \(\beta2\) and a hydrophobic core (residues 3 – 48) acting as an interface between the two monomers\textsuperscript{4}, Figure 1. Negative cooperativity and the presence of a dimerization unit leads to the hypothesis that communication occurs between each subunit's active site via the dimer interface. Furthermore, we hypothesized that the hydrophobic core of hGS at the dimer interface is responsible for maintaining the stability and activity of the hGS enzyme. The present research illuminates the roles that the hydrophobic residues Val44 and Val45 play in the function and stability of human glutathione synthetase.

MATERIALS AND METHODS

Materials

Expression vector pET-15b and \textit{E. coli} XL1 Blue competent cells were from Novagen, Inc. The primers were synthesized by IDT, Inc. The QuickChange\textsuperscript{TM} Site-Directed Mutagenesis Kit was from Stratagene. Isopropyl-1-thio-\(\beta\)-galactopyranoside was from American Bioanalytical. \(\gamma\)-L-Glutamyl-L-\(\alpha\)-amino butyrate (\(\gamma\)-GluABA), was synthesized as described previously\textsuperscript{8, 11}. All other reagents, unless noted, were purchased from Sigma and were of the highest purity.

Preparation of hGS mutant hGS enzymes

Recombinant human Glutathione Synthetase (hGS) cDNA is in pET-15b expression vector containing an N-terminal Histidine Tag (6x histidine residue)\textsuperscript{12, 13}. GS mutant enzymes were made using the QuickChange\textsuperscript{TM} Site-Directed Mutagenesis Kit using the internal primers (IDT) shown in Table 1. After mutagenesis, the plasmid DNA was transformed into \textit{E. coli} XL1 Blue cells, and purified (Wizard Plus Midiprep, Promega). Each mutant hGS mutant cDNA sequence was confirmed by sequencing (Gene Wiz). The mutant hGS plasmids were transformed into \textit{E. coli} BL21(DE3) cells for protein expression as previously described\textsuperscript{12, 13} and summarized below.

Enzyme Purification: Induction

The cells containing the pET-15b GS plasmids were grown (37 °C, 1 L LB media with 100 \(\mu\)g/ml ampicillin, 275 RPM) to an \(OD\textsubscript{600}\) of 0.8 to 1.0, for about 4 hrs. IPTG (Isopropyl-1-thio-\(\beta\)-galactopyranoside) \(-0.80\) mM (American Bioanalytical) was then added, chilled on ice (\(-30\) min) and incubated further at 18 °C (275 RPM, 2 – 4 hours).

Purification

All procedures were carried out at 4 °C. After induction, the cells were centrifuged (5 min at 5,000 \(\times\) g) and washed with cold saline (0.85% NaCl, 15 mL). The cells were then
centrifuged (15 min at 5,000 × g), and the ~3.0 gram cell pellet was lysed using a constant cell disruptor (Constant Cell Disruption System model: 01/40/BA at 15,000 PSI) in MCAC-0 buffer (20 mM Tris-Cl, 0.5 M NaCl, 10% glycerol, 5mM). The lysed cells were then centrifuged (20 min at 10,000 × g) and the supernatant was applied to a metal chelate affinity chromatography column (1.5 × 15 cm) with Ni-NTA His-Bind® resin (Novagen). Once the lysate washed through, MCAC-0 buffer was continued until the OD280 returned to baseline, and then repeated the same for the MCAC-55 buffer (MCAC-0 buffer with 55 mM imidazole). Finally, the recombinant hGS was eluted in MCAC-100 (MCAC-0 buffer with 100 mM imidazole, 4 mL fractions collected). The purified GS enzyme was dialyzed (2 × 4 L) overnight against Tris Buffer (20 mM Tris-Cl and 1 mM EDTA, pH 8.6). The purity of the GS enzyme was confirmed using SDS-PAGE.

Enzyme Assays

All kinetic assays were carried out with purified recombinant hGS enzymes as previously described[12, 13] and summarized in brief. Enzyme activity was measured using a pyruvate kinase (PK)/lactate dehydrogenase (LDH) coupled assay. γ-L-Glutamyl-L-α-aminobutyrate (γ-GluAβA), was used in place of γ-gluamylcysteine to avoid oxidation of the thio[8]. For standard assays, the reaction was initiated by the addition of the purified recombinant hGS into a preincubated (37 °C, 5 min) mixture containing 100 mM Tris (pH 8.2, 25 °C), 50 mM KCl, 20 mM MgCl2, 5 mM PEP, 10 Units/assay LDH (type II Rabbit muscle) and PK (type II rabbit muscle) and 0.3 mM NADH to a final volume of 0.2 mL. For specific activity measurements the concentration of γ-GluAβA, ATP and glycine were each 10 mM. The rate of the reaction was continuously monitored at 340 nm. A unit of activity is defined as the amount that catalyzes 1 μmole of product per min.

Kinetic parameters were determined using the standard assay as described above with varying substrate concentrations. For the K_m value determination, two substrates were held constant at saturation, while the third was varied by at least 10-fold around the approximate K_m. Reactions were run in the absence of γ-GluAβA as controls to ensure the ATP hydrolysis was indeed γ-GluAβA dependent. Sigma Plot 11.0 was used to calculate and determine Hill coefficients[12]. Protein concentration was determined by the Lowry method using bovine serum albumin as the standard[14].

Differential Scanning Calorimetry

Enzymes were dialyzed (2 × 4 L) overnight against Tris buffer (20 mM Tris-Cl and 1 mM EDTA, pH 8.6). Samples were concentrated (~1.0 – 2.0 mg/mL) using Centricon 10 microconcentrators (W.R. Grace Co., MA). DSC scans were performed on a Calorimetry Sciences Nano Series III instrument at 1.0 atm and were baseline corrected with a run against the dialysate. Scan rate was 1.0 °C/min.

Computational Methods

Computational methodology followed the precedents employed in our previous studies of human GS[13, 15], and are summarized here.

The crystallographic coordinates from the X-ray structure analysis of dimeric human glutathione synthetase (hGS, glutathione synthetase, product form) (PDB code = 2HGS)[14] were visualized and analyzed with DeepView [16, 17]. DeepView was used to find amino acid residues within a chosen distance of the dimer interface of hGS. These are defined as amino acids that have interchain interactions between chain A and chain B of 2HGS. Analyses of the protein-contacts were carried out for both wild type and mutant 2HGS. Contact types (i.e., hydrogen bonds, hydrophobic contacts, etc.) and pertinent atomic distances were computed with the MOE program[18].
To explore dimer interaction energies (i.e., $\Delta E_{\text{dimer}}$), the energy of interaction between chain A and chain B) of mutant hGS, these were constructed from wild-type hGS and modeled with the MOE software\[18\] using the Amber94 force field\[19\]. Starting with the crystal structure coordinates of hGS, hydrogen atoms were first added, and with heavy atoms fixed in position, the resulting hydrogen-decorated enzyme structure was energy minimized with the Amber94 force field. Subsequently, the fixed heavy atoms were then allowed to move and then the resulting enzyme structure was fully geometry optimized, again with the Amber94 force field.

Mutants were built by altering individual residues of the energy minimized wild-type dimer hGS. Subsequently, the appropriate hydrogens were added, followed by Amber94 energy minimization. Water molecules were removed from the final optimized structures prior to dimer interface analysis so that the calculated $\Delta E_{\text{dimer}}$ are solely the energy between the amino acids that comprise chain A and the amino acids that comprise chain B of the hGS dimer or its mutants.

**RESULTS**

**Experimental Activity Studies**

Human glutathione synthetase mutant enzymes were prepared, purified and assayed for initial activity (Table 2). When Val44 or Val45 was changed to the small nonpolar alanine residue (V44A or V45A) activity decreases only slightly (a 3 – 11% loss is measured). When Val44 or Val45 was changed to the large bulky nonpolar residue tryptophan (V44W or V45W) mutant enzymes show a more significant loss in activity (16 and 30%, respectively, reduction in activity versus WT-hGS). Interestingly, doubly mutated hGS (V44A-V45A) shows initial activity that is comparable to WT-hGS. Further activity studies reveal that the double mutation (V44A-V45A) shows a 50% loss of activity within 1 month of purification, whereas the remaining mutant enzymes show only slight loss of activity after 3 months (data not shown). For comparison, WT-hGS retains activity over a period of years.

Though mutations at the Val44 position show a decrease in activity, changes seen at residue 45 were more significant, of the activity of V45A versus V44A and V45W versus V44W, Table 2. Crystal structure analysis of hGS shows the V44 residues across the dimer interface are further apart (as measured by Cα-Cα distance), 6.6 Å, than the V45 residues (5.7 Å), Figure 2. Hence, one may reasonably expect there to be greater structural impact from mutation at V45 than V44 given their positions relative to the dimer interface.

**Experimental Stability Studies**

Differential scanning calorimetry (DSC) was used to examine the temperature of unfolding (Tm), i.e., stability, of the mutant hGS enzymes. Compared to WT-hGS (60 °C), the V44 mutant enzymes (V44A and V44W) are the most stable (57 and 51 °C, respectively), Table 2. The V45 mutant enzymes (V45A and V45W) show a more dramatic loss in stability (48 and 41 °C, respectively) than their V44 mutant congeners. The double mutation (V44A-V45A) approximates the stability experimentally measured for the V45W mutant enzyme (43 °C). While all Val44 and Val45 mutant enzymes show a decrease in stability compared to WT-hGS, the double point V44A-V45A mutant enzyme seems to express an additive decrease in stability vis-à-vis the Val44A and the V45A single point mutant enzymes.

The instability observed for V44 and V45 mutants is proposed be a direct reflection in the loss of local interactions at or around the dimerization unit. Note that the molecular dynamics simulations (vide supra) do not indicate a major structural reorganization (RMSD < 0.6 Å for each mutant enzymes as compared to WT-hGS). Given that the inter-chain interaction is less for Val45A***Val45B (5.7 Å) than for Val44A***Val44B (6.6 Å) one would
expect the former to have a greater impact on the overall stability of hGS, as is observed experimentally from the differential scanning calorimetry.

**Experimental Michaelis Constant (Km) and Cooperativity Studies**

Michaelis constants (Km) of the mutant hGS enzymes were also determined (Table 2). When compared to WT-hGS, V44A, V45A, and V44A-V45A mutant enzymes are relatively unchanged. The remaining mutant enzymes (V44W and V45W) show a decrease in the Km value for the γ-GluABA substrate. The resulting data suggest that the introduction of a large bulky residue such as tryptophan promotes a conformational change that impacts the active site causing the γ-GluABA substrate to bind more tightly.

Hill coefficients were experimentally determined for all hGS mutant enzymes (Table 2). The V44A mutant enzyme was comparable to WT-hGS with a Hill Coefficient (H) equal to 0.73. Mutant enzymes V45A, V44A-V45A, and V44W show a trend toward lower negative cooperativity (H = 0.90, H = 0.86, and H = 0.83, respectively). Interestingly, the V45W mutant enzyme became more cooperative (H = 0.61) than the WT form of hGS.

**Structural and Energetic Analysis of Dimeric hGS Mutants**

The aforementioned experiments have indicated several profitable targets for site directed mutagenesis simulations. The data accumulated thus far indicate that the interchain interactions can be prioritized as follows in terms of their expected impact on the structure and activity of hGS: Val45A→Val45B > Val44A→Val44B. To this end, an in silico site directed mutagenesis “alanine scan” was performed. Note that all mutations are symmetric, e.g., in the case of the V44A mutation, the Val44 from chain A and chain B are both mutated to alanine.

The contact distances and calculated RMSD values (vide supra) indicate little structural change on the other significant dimer interface contacts when a single point mutation to V44 or V45 is made. Thus, given these structural similarities, analysis of the chain A:chain B interaction with molecular mechanics methods (Amber94 force field) was deemed prudent. To further enhance the reliability of the simulations, the dimer interface interaction energies (ΔE_dimer) are indexed relative to that of the wild-type enzyme (ΔE_dimer). The data are summarized in Table 3.

From the calculated data in Table 3, two points are particularly germane to the current analysis of hGS. First, both mutants (V44A and V45A) are less stable than WT-hGS as indicated by dimer interaction energies (ΔE_dimer = −391 kcal/mol) that are more negative for the former. Second, the mutation of Val45 to alanine has nearly four times greater relative impact (ΔΔE_dimer, Table 3) than is calculated for the V44A mutant enzyme.

**DISCUSSION**

It was hypothesized that residues Val44 and Val45 serve as important residues for hGS function and stability given their location at the dimer interface of this dimeric enzyme. Several important conclusions relevant to the structure, stability and allostery of this homodimeric enzyme have been developed, the most important of which are summarized here.

The Val45 position mutant enzymes have a greater impact on enzyme activity than the corresponding mutations at Val44. Additionally, differential scanning calorimetry experiments show a loss of stability in all mutant enzymes when compared to wild type human glutathione synthetase. Interestingly, mutations at Val44 result in more stable enzymes than those mutated at Val45, for both alanine and tryptophan. Replacement of each
of these individual valines with tryptophan has a more substantial impact than does the valine to alanine mutation.

Molecular dynamics simulations suggest that mutation at Val45 has more impact on the structure and stability of hGS than the mutation at Val44. Analysis of the different energy contributions among WT-hGS, V44A and V45A imply that roughly half of the total difference is due to changes in the van der Waals contribution, making it reasonable that a tryptophan mutation would have more impact than an alanine mutation. The tryptophan mutations (V44W and V45W) are indeed shown via enzyme kinetics and differential scanning calorimetry experiments to have more of an effect on activity and stability, respectively, than alanine mutations (V44A, V45A and V44A-V45A).

The affinity of the γ-GluABA substrate is approximately equal in all alanine mutant enzymes and is shown to increase in the tryptophan mutant enzymes. This result may explain the resulting lower activity, i.e., greater stabilization of the reactant state versus the product state of hGS. Lack of significant loss in activity suggests changes in these dimer interface residues do not directly affect the active site. Cooperativity studies show Hill coefficients trend towards less negative cooperativity with the exception of V45W mutant hGS. The explanation of the V45W cooperativity has yet to be determined. The differences in trends among the hGS mutations suggest a loss of dynamic motion in the mutants, thus altering the cooperativity of the enzyme[20]. Taken together, the present results imply that residues V44 and V45 are located along the allosteric pathway of hGS and that mutation at these residues impact the allosteric pathway more than the enzyme active site. Moreover, the results also suggest that the dimer interface of hGS is intimately responsible for the stability of this enzyme.

- Mutations of Val45 have a greater impact on human glutathione synthetase activity and stability than the corresponding mutations at Val44.
- The $K_m$ values for the γ-GluABA substrate are approximately equal in V44A and V45A mutant hGS enzymes and decrease in V44W and V45W mutant hGS enzymes.
- Residues V44 and V45 are located along the allosteric pathway of hGS.
- Mutations at residues 44 and 45 impact the allosteric pathway more than the hGS active site.
- The dimer interface of hGS is intimately responsible for the stability of hGS.

**Acknowledgments**

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**Abbreviations**

- hGS: human glutathione synthetase
- GSH: glutathione
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>-GluABA</td>
<td>γ-glutamyl-α-aminobutyrate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-1-thio-β-D-galactopyranoside</td>
</tr>
<tr>
<td>MCAC</td>
<td>metal chelate affinity chromatography</td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
<tr>
<td>MOE</td>
<td>molecular operating environment</td>
</tr>
<tr>
<td>RMSD</td>
<td>root mean square deviation</td>
</tr>
</tbody>
</table>

**References**


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Figure 1.
Ribbon diagram depicting monomer orientation of the hGS homodimer with the purple showing the dimerization unit created between the two monomer subunits.
Figure 2.
Close up of dimer interface of diagram of human glutathione synthetase. Color-coded space filling models indicate amino acids that participate in the close contacts across the dimer interface.
### Table 1

Primers for site directed mutagenesis of V44 and V45 of glutathione synthetase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>DNA sequence</th>
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<tr>
<td>V44A</td>
<td>5'-CCTTCCCTCAGGATGAGGCTATGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCTATAGCTCACCCACCTCCAGAAGTG-3'</td>
</tr>
<tr>
<td>V45A</td>
<td>5'-CCTCCAGGATGAGGCTATGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCTATAGCTCACCCACCTCCAGAAGTG-3'</td>
</tr>
<tr>
<td>V44W</td>
<td>5'-CCTTCCCTCAGGATGAGGCTATGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCTATAGCTCACCCACCTCCAGAAGTG-3'</td>
</tr>
<tr>
<td>V45W</td>
<td>5'-CCTCCAGGATGAGGCTATGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGGCTATAGCTCACCCACCTCCAGAAGTG-3'</td>
</tr>
<tr>
<td>V44A-V45A</td>
<td>5'-CCTTCCCTCAGGATGAGGCTATGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCTATAGCTCACCCACCTCCAGAAGTG-3'</td>
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*aUnderlined bases are changed nucleotide positions.*
**Table 2**

Kinetic properties and stability of human glutathione synthetase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>Hill Coefficient (H)</th>
<th>$K_m$ -Glu ABA (mM)</th>
<th>$T_m$ (°C)</th>
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<tr>
<td>Wild type</td>
<td>19.5 ± 1.65</td>
<td>0.70 ± 0.04</td>
<td>1.42 ± 0.12</td>
<td>60.4 ± 0.17</td>
</tr>
<tr>
<td>V44A</td>
<td>18.9 ± 1.11</td>
<td>0.73 ± 0.01</td>
<td>1.60 ± 0.28</td>
<td>51.1 ± 0.04</td>
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<tr>
<td>V45A</td>
<td>17.4 ± 0.11</td>
<td>0.90 ± 0.06</td>
<td>1.54 ± 0.30</td>
<td>47.8 ± 0.11</td>
</tr>
<tr>
<td>V44A-V45A</td>
<td>21.7 ± 2.41</td>
<td>0.86 ± 0.001</td>
<td>1.42 ± 0.11</td>
<td>42.8 ± 1.47</td>
</tr>
<tr>
<td>V44W</td>
<td>16.4 ± 1.93</td>
<td>0.83 ± 0.07</td>
<td>0.95 ± 0.25</td>
<td>57.0 ± 0.05</td>
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<tr>
<td>V45W</td>
<td>13.6 ± 2.26</td>
<td>0.61 ± 0.003</td>
<td>0.32 ± 0.01</td>
<td>41.3 ± 0.04</td>
</tr>
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Table 3

Chain A: Chain B interaction energies: Wild-type versus mutant hGS

<table>
<thead>
<tr>
<th>Enzyme&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$E_{dimer}$&lt;sup&gt;b&lt;/sup&gt; (kcal/mol)</th>
<th>$E_{dimer}$&lt;sup&gt;c&lt;/sup&gt; (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$-391.5$</td>
<td>$0$</td>
</tr>
<tr>
<td>V44A</td>
<td>$-388.9$</td>
<td>$2.6$</td>
</tr>
<tr>
<td>V45A</td>
<td>$-381.3$</td>
<td>$10.2$</td>
</tr>
</tbody>
</table>

<sup>a</sup>WT = wild-type hGS; for others, the single point mutation is designated by the original residue then the residue number and finally the residue (alanine) to which the residue was mutated to.

<sup>b</sup>Interaction energy (kcal/mol) between chain A and chain B of dimeric hGS calculated with the Amber94 force field.

<sup>c</sup>The quantity $\Delta E_{dimer}$ is $E_{dimer}$ (kcal/mol) for the mutant hGS relative to $E_{dimer}$ for wild-type hGS (viz. $-391.5$ kcal/mol).