Human γ-Glutamyl Transpeptidase 1

STRUCTURES OF THE FREE ENZYME, INHIBITOR-BOUND TETRAHEDRAL TRANSITION STATES, AND GLUTAMATE-BOUND ENZYME REVEAL NOVEL MOVEMENT WITHIN THE ACTIVE SITE DURING CATALYSIS

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Background: γ-Glutamyl transpeptidase 1 (GGT1) plays a role in asthma, reperfusion injury, and cancer.

Results: We report four new crystal structures of human GGT1, including the free enzyme, inhibitor-bound transition states, and glutamate-bound enzyme.

Conclusion: Novel enzyme-substrate interactions and movement of the catalytic nucleophile, oxyanion hole, and lid loop were revealed.

Significance: This structural information is critical for developing GGT1 inhibitors.

γ-Glutamyl transpeptidase 1 (GGT1) is a cell surface, N-terminal nucleophile hydrolase that cleaves glutathione and other γ-glutamyl compounds. GGT1 expression is essential in cysteine homeostasis, and its induction has been implicated in the pathology of asthma, reperfusion injury, and cancer. In this study, we report four new crystal structures of human GGT1 (hGGT1) that show conformational changes within the active site as the enzyme progresses from the free enzyme to inhibitor-bound tetrahedral transition states and finally to the glutamate-bound structure prior to the release of this final product of the reaction. The structure of the apoenzyme shows flexibility within the active site. The serine-borate-bound hGGT1 crystal structure demonstrates that serine-borate occupies the active site of the enzyme, resulting in an enzyme-inhibitor complex that replicates the enzyme’s tetrahedral intermediate/transition state. The structure of GGSTop-bound hGGT1 reveals its interactions with the enzyme and why neutral phosphate diesters are more potent inhibitors than monoanionic phosphonates. These structures are the first structures for any eukaryotic GGT that include a molecule in the active site covalently bound to the catalytic Thr-381. The glutamate-bound structure shows the conformation of the enzyme prior to release of the final product and reveals novel information regarding the displacement of the main chain atoms that form the oxyanion hole and movement of the lid loop region when the active site is occupied. These data provide new insights into the mechanism of hGGT1-catalyzed reactions and will be invaluable in the development of new classes of hGGT1 inhibitors for therapeutic use.

# References

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γ-Glutamyl transpeptidase 1 (GGT1, also known as γ-glutamyl transferase) is a cell surface enzyme that is expressed on the apical surface of ducts and glands throughout the body (1). The highest level of enzyme activity is on the surface of the proximal tubules of the kidney, where GGT1 cleaves glutathione in the glomerular filtrate, preventing its excretion from the body and thereby conserving cysteine (2). GGT1 also cleaves the γ-glutamyl bond of any substrate in which the glutamate moiety is unaltered (Fig. 1). Such substrates include oxidized and reduced glutathione, glutathione S-drug conjugates, leuk-

2 The abbreviations used are: GGT1, γ-glutamyl transpeptidase (also known as γ-glutamyl transferase); hGGT1, human GGT1; GGSTop, 2-amino-(3-(carboxymethyl) phenyl) (methyl) phosphono)-butanoic acid; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; Bicine, N,N-bis[2-hydroxyethyl]glycine.
Human GGT1 Crystal Structures during Catalysis

at the N terminus of the small subunit acts as the catalytic nucleophile in Escherichia coli and Helicobacter pylori GGT (15, 16). Using mass spectrometry analysis of inhibitor-bound hGGT1, Castonguay et al. (17) identified Thr-381 as the catalytic nucleophile in the human enzyme. Our structures confirm that the side chain oxygen of Thr-381 is the catalytic nucleophile in hGGT1 and show the rotameric states of the side chain in the apoenzyme and the inhibitor-bound enzyme. These results advance the understanding of the interaction between hGGT1 and inhibitors that are bound in the active site. This knowledge is critical for the design and development of novel, more potent, less toxic hGGT1 inhibitors.

Experimental Procedures

hGGT1 Expression and Purification—For crystallization studies, the natural variant V272A of hGGT1 (P19440) was expressed in Pichia pastoris strain X-33, purified, and deglycosylated as described previously (12).

Thermofluor Study—The protein sample consisted of 0.1 mg/ml hGGT1 alone or complexed with GGsTop (Waco Chemicals, Richmond, VA) in 10 mM HEPES buffer, pH 7.5, 150 mM NaCl, and 5× SYPRO Orange. To each well of a 96-well plate, 12 μl of the protein sample and 4 μl of 0.1 M screening buffer were added. We used nine buffers at 12 different pH levels. The plate was spun for 5 min at 1000 rpm to remove air bubbles and was then placed in an Applied Biosystems thermocycler 7500 RT-PCR. The temperature of the samples was increased from 25 to 95 °C at a rate of 1 °C/min. At each degree, the fluorescence of the protein-bound SYPRO Orange was measured.

Crystallization Conditions—Crystals of hGGT1 were grown at room temperature by vapor diffusion with the hanging drop method. The protein stock solution contained 4.3 mg/ml hGGT1 in 50 mM HEPES, pH 8.0, 0.5 mM EDTA, and 0.02% sodium azide. Crystallization drops contained 2 μl of protein solution, 1.7 μl of H2O, and 2 μl of reservoir solution. Drops were equilibrated against 500 μl of one of two reservoir solutions. Solution A contained 20−25% PEG 3350, 0.1 mM sodium cacodylate buffer, pH 6.0, and 0.1 mM ammonium chloride. Reservoir solution B contained 16% PEG 6000, 0.1 mM MES buffer, pH 6.3, and 0.1 mM ammonium chloride. Two days after setting the drops, microcrystals of previously grown crystals were added to the drops to facilitate crystal growth. Crystals appeared in 1 or 2 days after seeding. After an additional week, the crystals grew to a final size of ~0.05 × 0.1 × 0.5 mm.

Crystals of the apoenzyme were grown against reservoir solution A or B. Crystals of GGT1 with serine-borate were prepared by soaking crystals of the apo-form of hGGT1 (grown against reservoir solution A) for 15 min in reservoir solution A supplemented with 10 mM L-serine-borate. The stock serine-borate solution contained 0.5 M Tris borate, pH 7, and 0.5 M L-serine. Crystals of hGGT1-bound GGsTop were prepared with hGGT1 preincubated in 1 mM GGsTop. Two μl of 0.1 M GGsTop in 0.1 N HCl was added to 100 μl of the protein solution. The mixture was incubated overnight at 4 °C prior to preparing the crystallization drops against reservoir solution B. Crystals with glutamate were prepared by growing the crystals in 2.5 mM glutamate against reservoir solution A and soaking the crystals in reservoir solution A containing 10 mM glutamate.

Oxidative C4, and glutathione S-nitric oxide (3, 4). In a variety of diseases, GGT1 is induced and mislocalized, resulting in cleavage of substrates in serum and in interstitial fluid, thereby contributing to the disease pathology. GGT1 activity has been shown to potentiate tissue damage in ischemia-reperfusion injury, to contribute to airway hyperreactivity in asthma, to initiate the metabolism of a series of glutathione-S-drug conjugates to nephrotoxins, and to increase resistance of tumors to alkylating agents (5–10).

GGT1 inhibitors that have been evaluated clinically, including 6-diazo-5-oxo-norleucine, azaserine, and acivicin, are all glutamate analogs and are extremely toxic in humans (11). We recently reported the crystal structure of glutamate-bound human GGT1 (hGGT1), the first structure reported for any eukaryotic GGT (12). To better understand the interaction between hGGT1 and inhibitors that are structurally related to glutamate, we have extended our structural characterization to include the analysis of the crystal structures of the apoenzyme and inhibitor-bound hGGT1 complexes. The structures of the hGGT1-bound serine-borate and 2-amino-4-[(3-carboxymethyl)phenyl] (methyl)phosphonono)butanoic acid) (GGsTop) are the first structures reported for these inhibitors bound to GGT1. Comparison of the apo-structure with the inhibitor-bound structures has revealed multiple regions within the active site where there is movement upon inhibitor binding. In addition, we have obtained new structural data for glutamate-bound hGGT1 that include clear density for the side chain atoms of the bound glutamate, which was lacking in our initial glutamate-bound structure.

Published kinetic analyses of the GGT1 reaction indicated that during cleavage of γ-glutamyl substrates, a bond forms between the δ-carbon of the γ-glutamyl substrate and a nucleophile within the active site (Fig. 1) (13, 14). Affinity labeling and structural studies of bacterial GGTs revealed that the threonine moieties, and the γ-glutamyl bond (green arrow) is cleaved. The nucleophile in hGGT1 is the side chain oxygen of Thr-381, which forms an acyl bond with the substrate in the enzyme-substrate complex.
Human GGT1 Crystal Structures during Catalysis

and 1 mM OU749 for 2.5 h prior to cryopreservation. OU749 (N-(5-(4-methoxybenzyl)-1,3,4-thiadiazol-2-yl)benzenesulfonamide) is an uncompetitive inhibitor of hGGT1 (18).

Crystal Cryopreservation—Crystals were quickly dragged through a cryoprotectant solution consisting of the reservoir solution with 15% PEG 1500 and the corresponding inhibitor molecule. The crystals were vitrified in liquid nitrogen.

Data Collection—Crystals were screened at the Macromolecular Crystallography Laboratories at the University of Oklahoma (Norman, OK) and the University of Oklahoma Health Sciences Center (Oklahoma City, OK). The majority of the x-ray diffraction data were collected at 100 K at beam line X25 at the National Synchrotron Light Source (Brookhaven, NY). The beam line was equipped with a Pilatus 6M detector, and data were collected at λ = 1.1 Å. Data for the serine-borate-bound GGT1 were collected at Stanford Synchrotron Radiation LightSource (Menlo Park, CA), at beam line BL14-1, λ = 0.979 Å. The diffraction data were processed with the HKL-2000 suite (19).

Structural Determination, Refinement, and Analysis—The unit cell parameters of crystals were isomorphous to those from our published structure (Protein Data Bank code 4GDX) of hGGT1 in complex with glutamate. Rigid body refinement was used for positioning of the new structures in the unit cell. Weighted difference 2Fo − Fc and Fo − Fc maps were used for detection of bound inhibitor molecules. The 4GDX structure without alternative conformations, water, and cofactor molecules served as a starting model. The structures were manually corrected with the computer graphics program COOT and refined using REFMAC 5 (20, 21). In the last stages of refinement, cofactor atoms (chlorine and sodium) and water molecules were added to the structure using ARP/wARP, COOT, or both programs. The refinement statistics and Protein Data Bank accession codes are listed in Table 1. The structures were analyzed and compared using programs from CCP4 and COOT (20, 22). The figures were made with Molmol, Chimera, and LIGPLOT (23–25).

Results

Structure of the Apoenzyme—Our previously reported crystal structure was glutamate-bound hGGT1 (4GDX) (12). We have now solved the structure of apo-hGGT1 (Protein Data Bank code 4Z9O). Purified hGGT1 was crystalized against solution B, complete x-ray diffraction data were collected, and the structure of the apoenzyme was determined (Table 1 and Fig. 2). The structure was isomorphous to the glutamate-bound apo-hGGT1 crystal structure. Therefore, the structure was solved by means of difference Fourier maps. The coordinates of the glutamate-bound hGGT1 with glutamate, water molecules, and Cl− and Na+ ions removed were used as a starting model. Residues with alternative positions were modeled in the highest occupancy conformation. Initial maps were calculated after 20 cycles of rigid body refinement by REFMAC 5 and a cycle of simulated annealing with slow cool protocol by CNC. The weighted Fo − Fc maps at the position of Thr-381 contained positive and negative electron density when we tried to model this residue in only one of the two conformations detected in our high resolution glutamate-bound structure (4GDX) (12). Therefore, the Thr-381 was modeled in two conformations similar to ones in structure 4GDX. The structure was refined to 2.37 Å resolution with a Rwork = 15.05% and Rfree = 19.93%.

The overall structures of apo- and glutamate-bound hGGT1 were similar. When the structures of the apo- (4Z9O) and glutamate-bound (4GDX) forms were superimposed, the root mean square deviation between 500 CA atoms with a maximal distance cut-off of 1.5 Å was 0.241. The crystal structure of the apo-form of hGGT1 showed that the side chain of Thr-381 adopted two rotameric states (χ1 = −165° and −75°; Fig. 2B). The side chain oxygen of Thr-381 is the catalytic nucleophile in the GGT1 reaction (Fig. 1). The observation of two rotameric states suggests unrestrained movement of the Thr-381 side chain when the active site is not occupied. The oxyanion hole-forming loop in the glutamate-bound hGGT1 structure (4GDX) was modeled in two conformations (12). In the apo-form, this loop occupies only one orientation, similar to one of two forms detected for the complex structure, but was shifted toward Thr-381. The largest shift of 1.1 Å was between CA atoms of Gly-474. Other small but noticeable shifts were detected in the loops composed of residues 428–439 (lid loop) and 505–514 and in residues 323–330 of helix α14. The shift in these loops relative to other structures is shown under “Discussion.”

Two molecules of the MES buffer were observed within the crystal structure of the apo-hGGT1 (4Z9O). One was bound near, but not in, the active site of the enzyme. The MES molecule was almost parallel to the side chain of Tyr-403. One of three oxygen atoms of the SO3 group of the MES molecule formed a hydrogen bond with the main chain nitrogen of Tyr-403 (2.87 Å) and with the NZ atom of Lys-407 through a bridging water molecule. The oxygen in the six-membered ring of MES interacted with the side chain OG oxygen atom of Thr-539. The second MES molecule was bound at a location remote from the active site.

Structure of the hGGT1-Serine-Borate Complex—In 1959, Revel and Ball (26) showed that serine in the presence of borate buffers inhibited mammalian GGT1, but neither serine nor borate buffer alone inhibited the enzyme. Tate and Meister (27) proposed that in solution, a serine-borate complex forms and that this complex would mimic γ-glutamyl-bound glutamate when bound in the active site of GGT1. Moreover, they suggested that the serine-borate complex would interact with the hydroxyl group of the nucleophile in the active site of the enzyme, forming an enzyme-inhibitor complex that replicates the tetrahedral transition state of the enzyme. Indeed, our structure of the serine-borate-bound hGGT1 (Protein Data Bank code 4ZCG) solved at 2.1 Å resolution (final Rwork = 17.5 and Rfree = 21.7) confirmed their hypothesis (Table 1 and Fig. 3). The difference Fourier electron density calculated using coordinates of the apo-form structure (only protein and carbohydrate atoms) clearly showed a serine-borate complex bound in the active site of hGGT1 (Fig. 3B). The serine-borate complex was formed by a covalent bond between the side chain OG atom of serine and the boron atom of the serine-borate. The backbone atoms of serine formed hydrogen bonds and salt bridges with atoms within the active site of the enzyme (Fig. 3C). The α-carboxyl group of the serine formed hydrogen bonds with Arg-107 NH1, Ser-451 OG, and Ser-452 nitrogen and bound to Ser-452 OG via a water molecule. The α-amino
group of serine formed bonds with Asn-401 OD1, Glu-420 OE1, and Asp-423 OD2. The complex was linked to the enzyme by an ester bond between boron and the side chain oxygen of Thr-381. The conformation of the side chain of Thr-381 is similar to one of two conformations of this residue in the apo-form structure (with $\chi = -165^\circ$), with a slight shift of 0.19 Å for the CA atom away from the inhibitor molecule. The boron was tetrahedral, with the two additional oxygens forming hydrogen bonds with the enzyme stabilizing the serine-borate complex in the active site of hGGT1. One oxygen atom (oxyanion) formed hydrogen bonds with the $\alpha$-amino groups of Gly-473 and Gly-474, two of the three amino acids that form the oxygen hole.

### TABLE 1

| Name                  | Apo-hGGT1 | Serine-borate-bound hGGT1 | GGSTop-bound hGGT1 | Glutamate-bound hGGT1 |
|-----------------------|-----------|---------------------------|-------------------|----------------------|
| **Data collection**   |           |                           |                   |                      |
| Protein Data Bank code| 4Z9O      | 4ZC6                      | 4ZBK              | 4ZCG                 |
| Unit cell (Å)         | 105.6, 125.3, 104.0 | 105.5, 122.0, 103.6 | 105.7, 123.6, 104.2 | 105.2, 125.2, 104.1 |
| Resolution (Å)        | 50–2.37 (2.45–2.37) | 50–2.1 (2.14–2.1) | 47–2.18 (2.22–2.18) | 50–2.22 (2.26–2.22) |
| No. of reflections    | 27,118 (2270) | 38,155 (1511) | 34,573 (1490) | 34,071 (1661) |
| Completeness (%)      | 96.2 (81.1) | 95.5 (77) | 96.6 (84.1) | 98.2 (83.7) |
| Redundancy            | 5.7 (3.7) | 6.3 (4.8) | 5.8 (3.7) | 10.7 (4.8) |
| $R_{merge}$ (%)       | 18.7 (4.2) | 9 (1.7) | 24 (2.7) | 21.9 (3.3) |
| $R$ factor from Wilson plot (Å²) | 37.4 | 24.9 | 37.5 | 32.1 |
| **Refrinement**       |           |                           |                   |                      |
| Resolution high (Å)   | 2.37 (2.43–2.37) | 2.1 (2.15–2.1) | 2.18 (2.24–2.18) | 2.22 (2.28–2.22) |
| No. of reflections    | 24,340 (1545) | 34,240 (2090) | 31,113 (2035) | 30,360 (2046) |
| Free set              | 1545 (68) | 1939 (113) | 1715 (99) | 1704 (114) |
| Overall               | 25,885 (1613) | 36,179 (2203) | 32,828 (2134) | 32,064 (2160) |
| $\sigma$ cut-off      | None | None | None | None |
| Completeness (%)      | 96.12 (77.92) | 95.27 (75.57) | 96.35 (81.39) | 98.42 (85.48) |
| $R_{work}$ (%)        | 15.05 (21.0) | 16.2 (27.6) | 16.2 (27.6) | 15.7 (23.7) |
| $R_{free}$ (%)        | 9.97 (19.93) | 9 (1.7) | 24 (2.7) | 21.9 (3.3) |
| $R_{overall}$ (%)     | 15.3 | 16.4 | 19.5 (24.7) | 15.9 |
| Figure of merit       | 86.6 | 83.6 | 86.6 | 88.06 |
| Correlation coefficient | 0.97 | 0.97 | 0.97 | 0.97 |
| Estimated coordinate error based on likelihood (Å) | 0.139 | 0.136 | 0.136 | 0.106 |
| No. of atoms          | 4574 | 4532 | 4570 | 4624 |
| Protein               | 4176 | 4184 | 4195 | 4183 |
| Ligand                | 24 | 10 | 35 | 10 |
| Anions                | 3 | 3 | 3 | 3 |
| Water molecules       | 371 | 335 | 337 | 428 |
| Mean B (Å²)           | 40.35 | 29.26 | 41.26 | 35.84 |
| Subunit A             | 38.14 | 25.87 | 37.76 | 33.35 |
| Mean B (Å²)           | 77.92 | 18.12 | 65.5 | 39.7 |
| Subunit B             | 51.76 | 32.29 | 50.39 | 45.2 |
| Mean B (Å²)           | 47.25 | 36.29 | 49.97 | 44.78 |
| Water                 | 97.4, 2.4, 0.2 | 97.4, 2.4, 0.2 | 97.2, 2.6, 0.2 | 97.6, 2.2, 0.2 |
| Ramachandran plot favored outliers (%) | 97.4, 2.4, 0.2 | 97.4, 2.4, 0.2 | 97.2, 2.6, 0.2 | 97.6, 2.2, 0.2 |
| Rotamer outliers (%)  | 0.8 | 0.19 | 0.7 | 0.19 |
| Root mean square deviation from ideal values | 0.009 | 0.011 | 0.01 | 0.011 |
| Bonds (Å)             | 1.38 | 1.45 | 1.40 | 1.41 |
| Angles (degrees)      | 4.00 | 28.0 | 40.0 | 35.0 |
| $B$ factor (Å²)       | 5.9 | 5.6 | 5.5 | 4.3 |
| Estimated $B$ value error (Å²) | 42.8 |

* $R_{merge} = \frac{\sum (\sum_{j} |I_{\ell_{j}} - \langle I_{\ell} \rangle|)}{\sum \sum_{j} |I_{\ell_{j}}|}$, where $I_{\ell_{j}}$ is the $j$th observation of reflection $H$.  

**FIGURE 2. Apo-Structure of hGGT1.** A, a stereo ribbon presentation of the apo-structure of hGGT1 (4Z9O). The large subunit is colored blue, and the small subunit is colored green. The N terminus of the large subunit (NL) and the C terminus of the large (CL) and small (CS) subunits are marked. The N terminus of the small subunit is Thr-381, the nucleophile in the GGT-catalyzed reaction, and its stick model in red is shown within the active site (orange oval). B, two conformations of Thr-381 observed in the apo-structure of hGGT1 (4Z9O). The oxygens are colored red, nitrogens are colored blue, and the carbons are colored yellow for one orientation and gold for the other.
(distances were 3.13 and 2.81 Å, respectively). The second oxygen interacted with two protein atoms: the main chain nitrogen of Thr-381, which is the N-terminal nitrogen of the small subunit of hGGT1 (distance 2.63 Å) and the main chain oxygen of Asn-401 (2.68 Å).

There were no significant changes in overall structure of the enzyme when CA atoms of the serine-borate-bound hGGT1 (4ZCG) were superimposed on the structure of the apoenzyme (4Z9O; root mean square deviation for 519 CA atoms /H11005/0.273). However, a shift was detected in the oxyanion-forming loop. It was moved up and back compared with its position in the apoenzyme structure, resulting in displacement of 1.45 Å for the CA atom of Gly-473, 1.64 Å for the CA atom of Gly-474, and 1.56 Å for CA atom of Thr-475. The second noticeable shift was detected in the lid loop composed of residues 427–438. The largest movement within the lid loop was 1.76 Å, which was observed as a downward shift of the CA atom of Asn-431. Smaller shifts were detected for the C-terminal part of helix α14 (residues 323–330) and the adjacent loop composed of residues 369–374. The shift in these loops relative to other structures is shown under “Discussion.”

Structure of hGGT1–GGsTop Complex—GGsTop (2-amino-4-((3-(carboxymethyl) phenyl) (methyl)phosphono)-butanoic acid) (Fig. 4A) is a phosphonate-based inhibitor of GGT1 that was synthesized as an active site probe (28). We conducted thermofluor studies to determine whether GGsTop would stabilize the structure of the enzyme in solution (Table 2). Analysis of hGGT1 at 11 different pH levels (nine buffers) showed that hGGT1 is most stable near neutral pH (boldface type in Table 2). Inactivating hGGT1 with GGsTop further stabilized the structure of the enzyme. It increased the melting temperature of the enzyme by ~20 °C at all pH levels tested independent of the buffer. As shown in the structure described below, the binding of GGsTop to hGGT1 resulted in the formation of a network of interactions, which stabi-
lized the structure and restricted movement within the active site of the enzyme.

Prior to crystallization, we inactivated the enzyme by incubating it with a 20-fold molar excess of GGsTop overnight at 4 °C. The data were collected at the National Synchrotron Lightsource, and the structure of the complex (4ZBK) was solved to 2.2 Å resolution (Rwork = 16.2% and Rfree = 21.9%). The 2Fo − Fc and Fo − Fc maps were calculated after 10 cycles of rigid body and restrained refinement by REFMAC5 with the coordinates of the apoenzyme (4Z9O) as a model. A clear density attached to Thr-381 was observed in the difference Fourier maps (Fig. 4B). We modeled GGsTop into the density and found that the density corresponded to a cleavage product of the GGsTop. Han et al. (28) had predicted the hGGT1-mediated hydrolysis of GGsTop, and our structure confirmed their hypothesis (Fig. 4B). GGsTop is a glutamate analog as is the serine-borate complex. The α-carboxyl group of the butanoic acid portion of the GGsTop molecule formed hydrogen bonds with Arg-107 NH1 and NH2, Ser-451 OG, and Ser-452 nitrogen and bound to Ser-452 OG via a water molecule (Fig. 4C).
The α-amino group of GGsTop formed bonds with Asn-401 OD1, Glu-420 OE1, and Asp-423 OD2. This is the same series of hydrogen bonds observed with the α-carboxyl group and α-amino groups of serine when the serine-borate complex is bound. The electron density in the GGsTop hGGT1 data indicated that upon GGsTop binding in the active site, there was a nucleophilic attack of the Thr-381 side chain oxygen on the phosphate atom of GGsTop, resulting in the formation of a covalent bond and cleavage of the phosphorus-oxygen bond of the GGsTop phenol. There was no electron density corresponding to the cleaved phenol of GGsTop. The crystal structure also showed that one of the phosphate-bound oxygens formed tight hydrogen bonds with the two main chain nitrogen atoms of Gly-473 and Gly-474; the distances were 2.66 and 2.61 Å, respectively. The loop containing Gly-473 and Gly-474 forms the oxyanion hole in the GGT1 catalytic reaction. The oxygen of the phosphate-bound methoxy group formed a hydrogen bond with N-terminal nitrogen of the small subunit (2.95 Å). The methyl group attached to this oxygen mimics the position of the cysteine in a molecule of glutathione. The methyl group protruded out toward the solvent and did not make any interactions with the enzyme (Fig. 4D). This observation is consistent with our knowledge of hGGT1 substrates, some of which have large substituents bound to the cysteine of glutathione yet are cleaved at the same rate as glutathione, indicating that the substituents do not impede binding of these substrates in the active site (3). GGsTop-bound hGGT1 was crystallized in MES-containing buffer, and two molecules of MES were observed in the structure, both localized as in the apo-hGGT1 structure.

**New Structure of Glutamate-bound hGGT1 with Complete Glutamate Molecule**—We collected new high resolution data for glutamate-bound hGGT1. In our previous structure of glutamate-bound hGGT1 (4GDX), significant electron density was present for the α-carboxyl and α-amino groups of the bound glutamate, but the structure provided poor electron density for the glutamate side chain atoms. As a result, the glutamate side chain atoms were not modeled in that structure (4GDX). Diffraction data for the new glutamate-bound hGGT1 (4ZCG) were obtained from a crystal that was soaked in a solution containing a higher concentration of glutamate than was used for our original structure. Data collected for the glutamate-bound hGGT1 extended to 2.1 Å resolution. The structure was refined to $R_{work}$ and $R_{free}$ values of 15.7 and 19.5, respectively. The initial phases for the structure factors were evaluated using only the coordinates of the atoms from protein and carbohydrate molecules within the original glutamate-bound structure (4GDX). We used coordinates from the 4GDX structure because at that point we did not have the structure of the apoenzyme. The new glutamate-bound structure (4ZCG) showed clear electron density for all atoms of the glutamate molecule bound in the active site of the enzyme (Fig. 5). In the new glutamate-bound structure, one of two oxygen atoms of the γ-glutamyl carboxyl group formed hydrogen bonds with the side chain OG atom of Thr-381 and the main chain nitrogen of Gly-474, whereas the second oxygen did not interact with the enzyme atoms (Fig. 5). Glutamate is the product of GGT1 hydrolysis of γ-glutamyl substrates (3). Therefore, a structure with a complete glutamate-bound molecule provides insight into the conformation of the enzyme after the cleavage of the γ-glutamyl bond of the substrate but prior to release of the glutamate, which is the second product of the reaction. The first product is the portion of the substrate released following cleavage of the γ-glutamyl bond. In comparing the structures of apoenzyme (4Z9O) and the new glutamate-bound hGGT1 structure (4ZCG), we observed minor displacement of the amino acid backbone of the enzyme and rotation of amino acid side chains upon glutamate binding. Displacements of the CA atoms of the lid loop region (residues 428–439) were 0.7 Å for Asn-431, 0.36 Å for Lys-326, and 0.24 Å for Gln-508. The diffraction data for the new glutamate-bound hGGT1 structure were collected from a crystal soaked in a solution containing 1 mM OU749, an uncompetitive inhibitor of the enzyme, but there was no electron density that could be interpreted as a bound inhibitor molecule.

**Discussion**

**Movement within the Active Site**—Four new hGGT1 crystal structures are reported here. These structures provide an important set of information suggesting likely movement within the active site as the enzyme progresses from the free enzyme through structures in which the active site is occupied with glutamate analogs covalently bound to the catalytic nucleophile and finally to the conformation of the enzyme prior to release of the final product. The structures of the covalently bound glutamate analogs are models for the enzyme-substrate intermediate. All substrates of GGT are free glutamate bound only by a carbon-nitrogen γ-glutamyl bond (Fig. 1). All of the structures reported here, with the exception of the apoenzyme, have the glutamate portion of the active site occupied. In each of the three structures, serine-borate bound hGGT1, GGsTop-bound hGGT1, and glutamate-bound hGGT1, we saw the same network of interactions between the hGGT1 and the α-carboxyl and the α-amino group of the bound glutamate/glutamate analog. The α-carboxyl group of the bound glutamate formed hydrogen bonds with Arg-107, Ser-451, and Ser-452. These interactions were also observed in our initial glutamate-bound structure (4DGX) (12). The α-amino group of the bound glutamate formed bonds with Asn-401, Glu-420, and Asp-423. In our initial structure of glutamate-bound hGGT1 (4DGX),
the α-amino group of the bound glutamate formed bonds with Glu-420 and Thr-399 (12). In comparing the two glutamate-bound hGGT1 structures (4DGX and 4ZCG) the difference in the interactions with the α-amino group of the bound glutamate is due to a rotation of the bound glutamate molecule in the new structure (4ZCG) around an axis going through an oxygen atom of the COOH group (the oxygen bound to Ser-452) and perpendicular to its plane about 10° and shifted 0.50 Å away from Arg-107. The initial glutamate-bound hGGT1 structure (4DGX) did not include the side chain atoms of the bound glutamate, and these data indicate that this shift occurs when the glutamate is stabilized in the active site.

The binding mode of the α-carboxyl and α-amino groups of glutamate in the active site of GGT observed in this study is almost identical to that observed in E. coli and H. pylori GGT structures (29, 30). However, the conformation of the side chain of the bound glutamate differs. In E. coli (Protein Data Bank code 2DBX), the plane of the γ-glutamyl carboxyl is rotated about −45° around the CG_CD bond when compared with our human enzyme structures (29). The χ1 angles differ by 20°, whereas there is no difference between χ2 angles. In H. pylori GGT (Protein Data Bank code 2QM6), the γ-glutamyl carboxyl is shifted away from the threonine toward the oxyanion hole and is wedged deeper into the active site (30).

Our structures of hGGT1 confirm that the side chain oxygen of Thr-381 is the catalytic nucleophile in the enzyme reaction. In the crystal structure of the apo-form of hGGT1 (4Z9O), the side chain of Thr-381 adopted two rotameric states, indicating unrestricted movement of the Thr-381 side chain when the active site is unoccupied (Fig. 2B). In both the serine-borate and GGsTop-bound hGGT1 crystal structures, there is a covalent bond between the side chain oxygen of Thr-381 and the inhibitor molecule. In both of these inhibitor-bound crystal structures, the side chain of Thr-381 is observed in only one orientation, and it resembles one of the conformations (χ = −169°) in the apo-form structure (Fig. 2B). In the serine-borate-bound

FIGURE 5. Glutamate-bound hGGT1 (4ZCG). A, structure of glutamate, which is the second product of the GGT1 reaction. B, stereo presentation of the model of Glu fitted into an Fo − Fc density map calculated after initial rigid body refinement and contoured at 3σ level. Enzyme carbon atoms are colored yellow; glutamate carbon atoms are colored orange, oxygens are red, and nitrogens are blue. C, LIGPLOT diagram of the interactions between hGGT1 and a glutamate molecule in the active site of the enzyme. Colors of the atoms are the same as in A with the exception that the carbon atoms are black. The water molecule is colored cyan.
hGGT1, the CA atom of Thr-381 is additionally shifted about 0.19 Å away from the inhibitor molecule (Fig. 6). The structure also revealed that the tetrahedral state of the boron is further stabilized by the interaction of two other boron-bound oxygen atoms with the enzyme. The second oxygen (oxyanion) interacts with nitrogen atoms of both Gly residues of the oxyanion hole-forming loop (Gly-473 and Gly-474), whereas the third oxygen atom forms hydrogen bonds with the main chain nitrogen of Thr-381, which is the N-terminal nitrogen of the small subunit of hGGT1, and the main chain oxygen of Asn-401. In the GGsTop-bound hGGT1, the phosphonate group of GGsTop is stabilized by interactions between one of the phosphonate oxygens and the α-nitrogens of Gly-473 and Gly-474 (the oxyanion hole) and a second phosphonate oxygen interacting with the N-terminal nitrogen of the small subunit. The tetrahedral borate and phosphonate are probably similar to the substrate’s tetrahedral transition state during cleavage. These data provide insight into the interactions within the active site as the enzyme reaction progresses.

In the new glutamate-bound structure (4ZCG), the catalytic residue Thr-381 was also detected in only one conformation (χ = −165°) despite the lack of a covalent bond between the enzyme and the substrate. There was a hydrogen bond between the side chain oxygen of Thr-381 and one of the OE oxygens of glutamate. The configuration of the OE1 and OE2 oxygens suggests that they may be aligned in the same orientation as they would be following the hydrolysis of the acyl bond of a γ-glutamyl substrate.

A second conformational change that was seen within the active site upon inhibitor and glutamate binding was movement of the loop containing residues 473–475, which constitutes the oxyanion hole. The displacement was similar in both of the inhibitor-bound and the glutamate-bound hGGT1 structures. The displacement was slightly larger when either serineborate or GGsTop was bound compared with the glutamate-bound hGGT1. Comparing the position of the loop in the apoenzyme (4Z9O) with its position in the glutamate-bound structure (4ZCG) revealed shifts of 0.98 Å for the CA atom of Gly-473, 1.03 Å for the CA atom of Gly-474, and 1.16 Å for the CA atom of Thr-475. In the serine-borate-bound hGGT1, the oxyanion-forming loop moved up and back compared with its position in the apoenzyme structure, resulting in a displacement of 1.45 Å for the CA atom of Gly-473, 1.64 Å for the CA atom of Gly-474, and 1.55 Å for the CA atom of Thr-475. Shifts in GGsTop-bound hGGT1 are similar to those for serine-borate-bound enzyme with following shifts: CA Gly-473, 1.50 Å; Gly-474, 1.56 Å; and Thr-475, 1.59 Å. These results demonstrate plasticity in the oxyanion hole-forming loop, switching from a closed conformation to an open conformation upon substrate binding and subsequent stabilization of intermediates/transition states as the reaction proceeds.

The lid loop region (residues 428–439), the loop connecting β16 and β17 strands (residues 505–514), and the C-terminal part of helix α14 (residues 323–330), and the loop connecting β16 and β17 strands (residues 505–514).

**FIGURE 6.** Least squares superposition of CA atoms of apo-hGGT1 (4Z9O, red), serine-borate bound hGGT1 (4ZCG, green), and glutamate-bound hGGT1 (4ZCG, blue) structures. The numbers show the first and last residues of the corresponding structure: the lid loop (residues 428–439), the oxyanion loop (residues 470–475), the C-terminal part of helix α14 (residues 323–330), and the loop connecting β16 and β17 strands (residues 505–514).
diester that reduces the negative charge on the phosphate oxygen, rendering the phosphate more favorable for nucleophilic attack. The second factor proposed by Han and colleagues (31) was that neutral phosphonates would have a higher binding affinity to the active site of hGGT1 than would anionic charged compounds (33). However, the structure of GGSTop-bound hGGT1 revealed that Gly-473 and Gly-474, which interact with the phosphonyl moiety and act as the oxyanion hole, have a partial positive charge. In addition, the N-terminal nitrogen of the small subunit, which also interacts with the phosphonyl moiety, is positively charged. Therefore, the negatively charged phosphate monoesters might be expected to have higher affinity for the active site of GGT1 than would more neutral phosphate diesters. The fact that the neutral diesters are better inhibitors of GGT1 than the phosphate monoesters demonstrates that the environment surrounding the phosphate is less important than its reactivity for inhibitory activity. The third proposed factor was a possible interaction of the added methyl group with the hGGT1 protein molecule. Our structure shows no such interaction between the methyl group and hGGT1, thus negating the hypothesis that such an interaction occurs and enhances inhibitory activity.

The four new structures reported in this study provided insight into flexibility within the active site of the enzyme and the molecular mechanism by which the catalysis of the substrate progresses. Serine-borate-bound hGGT1 confirmed the hypothesis that serine present in borate buffer forms a serine-borate complex that occupies the active site of the enzyme, resulting in an enzyme-inhibitor complex that replicates the tetrahedral intermediate/transition state of the enzyme. The structure of the GGSTop-bound hGGT1 revealed its interactions with the enzyme and provided data that showed that the neutral phosphate diesters are more potent inhibitors than monoanionic phosphonates, predominantly due to the fact that they are more favorable substrates for nucleophilic substitution. The glutamate-bound structure revealed novel information on the orientation of the side chain of Thr-381, displacement of the main chain atoms that form the oxyanion hole, and movement of the lid loop region when the active site is occupied by glutamate.

These data provide new insights into the mechanism of GGT-catalyzed reactions and will be invaluable in the development of new classes of GGT inhibitors for therapeutic use.

Author Contributions—S. S. T. isolated the enzyme, prepared and screened the crystals, determined all of the x-ray structures, designed and conducted the thermofluor study, co-wrote the paper, and prepared all of the figures. A. W. G. B. contributed important insights into the chemical nature of the interactions observed in this study and wrote sections of the “Results” and “Discussion” pertaining to those interactions. A. H. collected the x-ray diffraction data and processed the data for the apoenzyme, the GGSTop-bound hGGT1, and the glutamate-bound hGGT1. C. A. S. collected the x-ray diffraction data and processed the data for the serine-borate-bound hGGT1. B. H. M. M. provided the initial crystals for seeding and was actively involved in the study, providing technical advice. M. H. H. conceived and coordinated the study, contributed to isolating the enzyme and preparing the crystals, and co-wrote the paper. All authors contributed to the editing of the manuscript and approved the final version of the manuscript.

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