Published in final edited form as:
Anal Biochem. 2011 July 15; 414(2): 208–214. doi:10.1016/j.ab.2011.03.026.

Gamma-Glutamyl Compounds: Substrate Specificity of Gamma-Glutamyl Transpeptidase Enzymes

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Abstract

Gamma-glutamyl compounds include antioxidants, inflammatory molecules, drug metabolites and neuroactive compounds. Two cell surface enzymes have been identified that metabolize gamma-glutamyl compounds, gamma-glutamyl transpeptidase (GGT1) and gamma-glutamyl leukotrienase (GGT5). There is controversy in the literature regarding the substrate specificity of these enzymes. To address this issue, we have developed a method for comprehensive kinetics analysis of compounds as substrates for GGT enzymes. Our assay is sensitive, quantitative and is conducted at physiologic pH. We evaluated a series of gamma-glutamyl compounds as substrates for human GGT1 and human GGT5. The K_ms for reduced glutathione were 11μM for both GGT1 and GGT5. However, the K_m for oxidized glutathione was 9μM for GGT1 and 43μM for GGT5. Our data show that the K_ms for leukotriene C_4 are equivalent for GGT1 and GGT5 at 10.8μM and 10.2μM, respectively. This assay was also used to evaluate serine-borate, a well-known inhibitor of GGT1, which was 8-fold more potent in inhibiting GGT1 than inhibiting GGT5. These data provide essential information regarding the target enzymes for developing treatments for inflammatory diseases such as asthma and cardiovascular disease in humans. This assay is invaluable for studies of oxidative stress, drug metabolism and other pathways that involve gamma-glutamyl compounds.

Keywords

Glutathione; gamma-glutamyl substrates; gamma-glutamyl transpeptidase; gamma-glutamyl leukotrienase; glutamate assay

Introduction

Many extracellular gamma-glutamyl compounds have been identified in humans and they serve a variety of functions. Glutathione (GSH), which is present in human serum at 10 to 15 μM, is an antioxidant and provides a method to transport cysteine from the liver to other

[1]This work was supported by National Institutes of Health Grant RO1CA57530 and R56CA57530 (M.H.H.).

[2]This work was supported by the Grayce B. Kerr endowment to the University of Oklahoma to support the research of P.F.C.

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tissues [1]. Metabolites of both endogenous and exogenous compounds form GSH S-conjugates and are excreted from the cell. Leukotriene C_4 (LTC_4), a GSH conjugate, is a potent inflammatory compound [2]. S-nitroso-GSH is a transport form of nitric oxide [3; 4]. For some drugs, such as MDMA, conjugation to GSH is an intermediate metabolite in their activation to potent toxins [5]. For other compounds, such as aflatoxin B1, conjugation to GSH and transport out of the cell are steps in the detoxification and excretion of the compound [6]. Additional gamma-glutamyl compounds have also been identified including gamma-glutamyl taurine, an endogenous modulator of excitatory aminoacidergic neurotransmission in the brain [7]. Studies with GGT knockout mice indicate that there are other gamma-glutamyl compounds yet to be discovered that play a role in osteogenesis and other physiologic processes [8; 9; 10; 11; 12; 13; 14].

GSH cannot be taken up intact by most cells and there is no evidence that GSH S-conjugates can be transported into the cell [9; 15]. Therefore, GSH and GSH-S-conjugates must be metabolized by extracellular enzymes [9; 15]. The initial step in their metabolism is cleavage of the gamma-glutamyl bond [16; 17; 18]. GGT1 and GGT5 are the only two extracellular enzymes that have been shown to cleave gamma-glutamyl bonds [19; 20; 21]. Both enzymes are expressed in mice and in humans and data regarding the substrates for these two enzymes is largely derived from GGT1 and GGT5 knockout mice [2; 19; 20; 21; 22; 23]. GGT1 knockout mice cannot breakdown GSH into its constituent amino acids as it passes through the proximal tubules of the kidney [9]. They excrete large amounts of GSH in their urine, which leads to a fatal cysteine deficiency [9]. Supplementing the mice with N-acetylcysteine restores cysteine levels, but does not correct deficiencies in bone resorption [12]. No gamma-glutamyl compound has been shown to modulate osteoclasts. These data suggest that the gamma-glutamyl compound that is essential in this process has yet to be identified. Studies with GGT1 knockout mice have shown reduced activation of cisplatin to a nephrotoxin, implicating a cisplatin-S-GSH conjugate as a metabolite in its activation [8].

Gamma-glutamyl leukotrienase (GGT5) is the only other member of the GGT family of enzymes that has been shown, to date, to have catalytic activity [2; 21]. GGT5 knockout mice are phenotypically normal, but in most organs have only 10% of the wild type levels of gamma-glutamyl leukotrienase activity [23]. The remaining activity was attributed to GGT1 and was absent in GGT1/GGT5 double knockouts [23; 24]. Carter and colleagues reported that GSH and GSSG were not substrates for murine GGT5 [2]. However, Heisterkamp and colleagues reported that GSH was a substrate for human GGT5 [21]. To date, there has not been any method to evaluate the substrate specificity of gamma-glutamyl enzymes. We have developed a novel assay and done a comprehensive kinetic analysis of the metabolism of a series of gamma-glutamyl compounds by two human enzymes gamma-glutamyl transpeptidase (GGT1) and gamma-glutamyl leukotrienease (GGT5).

The initial steps in the reaction catalyzed by GGT1 are the cleavage of the gamma-glutamyl bond of the substrate and the formation of a transient ES complex [16; 17; 18]. All but the gamma-glutamyl group of the substrate is released. In the ES complex, the gamma-carbon of the gamma-glutamyl substrate forms an acyl bond with the side chain of the Thr in the active site of the enzyme [16; 17; 18; 25; 26] (Fig.1). Hydrolysis of the esterified enzyme can occur, releasing glutamate and regenerating free enzyme [25; 26]. In the presence of a high concentration of an acceptor amino acid or dipeptide, a transpeptidation reaction occurs in which the ES complex is resolved when the gamma-glutamyl group is transferred to the amine of the acceptor, thereby forming a new gamma-glutamyl compound [27]. The transfer of the gamma-glutamyl group to a dipeptide acceptor, occurs by a modified Ping-Pong mechanism [17; 28]. The degree to which GGT1 catalyzes the hydrolysis versus transpeptidation reaction is influenced by both pH and the presence of an acceptor amino acid or peptide [27; 29; 30].

The standard assay for GGT1 activity uses gamma-glutamyl p-nitroanalide (GpNA), a non-physiologic compound as a substrate, includes high concentrations of the acceptor glycylglycine (glygly), and is conducted at pH 8.0 or higher [16; 27; 29; 30; 31]. GpNA is colorless, but the cleavage product, pNA, is yellow and can be monitored spectrophotometrically. Measuring the release of pNA, does not distinguish between the hydrolysis and transpeptidation reactions (Fig. 1A). In addition, the L-GpNA substrate can also act as a weak acceptor resulting in autotranspeptidation of the substrate yielding gamma-glutamyl-gamma-glutamyl-p-nitroanalide [27; 30; 32]. In the absence of glygly, with L-GpNA as the substrate, the reaction is a mixture of hydrolysis and transpeptidation reactions which can not be distinguished by measurement of the pNA product that is released [27; 30; 32]. D-GpNA, a stereoisomer of the standard L-GpNA substrate, does not act as an acceptor and has been used as a substrate to specifically evaluate the hydrolysis reaction [32]. However, neither L-GpNA nor D-GpNA is a physiologic compound. Under physiologic conditions, hydrolysis is the predominate reaction catalyzed by GGT1 [28; 33]. Measuring the release of glutamate from gamma-glutamyl compounds can be used to measure the hydrolysis reaction. Therefore, we have developed a quantitative assay to measure glutamate release by GGT from gamma-glutamyl compounds at physiologic pH (Fig. 1B). To date, there is no assay for developed for the kinetic analysis of GGT5. Several investigators have reported that neither human nor mouse GGT5 can cleave GpNA, the substrate in the standard assay for GGT1 [2; 20; 21]. However, an assay that measures glutamate release can be used to measure the hydrolysis of physiologic substrates by GGT5.

The assay that we have developed is based on a L-glutamate detection system [34]. Our assay detects the release of glutamate from any gamma-glutamyl compound and is conducted at physiologic pH. With this assay, we have found that the K_m for physiologic substrates of GGT1 and GGT5 are in the concentration range at which they are present in human serum. The assay can also be used to study inhibition of GGT1 and GGT5 under *in vivo* conditions, with physiological substrates.

Methods and Materials

Enzyme Isolation

Human GGT1 (P19440), lacking the transmembrane domain, was expressed in *Pichia pastoris* and isolated as previously described [35]. The deletion of the transmembrane domain and short cytoplasmic tail does not alter the enzymatic activity of human GGT [35; 36; 37; 38]. Human GGT5 Isoform b cDNA was purchased from American Type Culture Collection (ATCC, Manassas, VA). cDNA for GGT5, with a His6 tag and TEV cleavage site but lacking the transmembrane domain, was cloned into a *Pichia pastoris* expression vector. *Pichia pastoris* were transformed and induced to secrete a soluble form of GGT5 into the media. GGT5 was purified as previously described for GGT1 [35].

GGT Hydrolysis Reaction Quantified with the Glutamate Release Assay

Solution A contained the compound to be evaluated as a substrate in the assay buffer (100 mM Na_2HPO_4, 3.2 mM KCl, 1.8 mM KH_2PO_4, and 27.5 mM NaCl, pH 7.4). In experiments in which inhibitors were evaluated, the compound to be evaluated as an inhibitor was included in Solution A. Solution B contained the components of the L-glutamate detection system: 238 mM triethanolamine (TEA), 30.1 mM K_2HPO_4 pH 7.4, Triton X-100 (1.5% v/v), sodium azide (0.013% w/v), 3.85 mM NAD^+, 275.1 mM iodonitrotetrazolium (INT), 6.25 mUnits/μL diaphorase, and 12.5 mUnits/μL glutamate dehydrogenase (GDH) (Megazyme, Wicklow, Ireland). The assay was performed in 96-well plates. Ninety μL of solution A, and 40 μL of solution B were added to each well. The reaction was initiated by the addition of 10 mU GGT1 (1.91 nM) in 10 μL assay buffer or 10 mU GGT5 (56.39 nM)

in 10 μL assay buffer. The reaction was carried-out at 37°C and monitored continuously at 490 nm using a Bio-Rad model 680 microplate reader with Microplate Manager 5.2 software (Bio-Rad, Hercules, CA). In each experiment all samples were run in triplicate. Two or more independent experiments were performed for each set of data reported.

Analysis of the Transpeptidation Reaction

A stock solution of glycylclycine (glygly, Fisher) was prepared in assay buffer and included in solution A of the assay. The final concentration range of glygly in the assay was 0.01 to 40 mM.

GGT Substrates and Inhibitor

Stock solutions of glutathione (GSH, Sigma), oxidized glutathione (GSSG, Sigma), S-(4-Nitro-benzyl)glutathione (Sigma), S-methylglutathione (Sigma), glutathionesulfonic acid (Sigma), gamma-glutamyl leucine (Sigma), and leukotriene C_4 (LTC_4, Cayman Chemical) were prepared in assay buffer, adjusted to pH 7.4 and evaluated as substrates for GGT1 and GGT5. A stock solution of equimolar L-serine (Sigma) and boric acid (Sigma) was prepared in assay buffer and adjusted to pH 7.4. Inhibition of GGT1 (10 mU/reaction) or GGT5 (10 mU/reaction) in the presence of serine-borate was evaluated.

Data Analysis

Double reciprocal plots of initial velocities versus substrate concentrations were generated to assess data quality and determine the appropriate rate equation for data fitting. The data were fitted with the appropriate rate equations using the Marquardt-Levenberg algorithm (Enzfitter program, BIOSOFT, Cambridge, UK). Kinetic parameters with standard errors were estimated using a simple weighting method and graphed using Prism GraphPad Software (San Diego, CA).

Data adhering to Michaelis-Menten Kinetics were fitted to eq. 1. Data for the dependence of V_{max} and $V_{max}/K_{D\text{-}GpNA}$ on the concentration of inhibitor were fitted using eq. 2 [39].

$$v = \frac{V_{\max} A}{K_a + A} \tag{1}$$

$$v = \frac{V_{\max} A}{K_a \left(1 + \frac{I}{K_{is}}\right) + A} \tag{2}$$

In eqs 1 and 2, v and V_{max} are the measured initial and maximum rates, respectively, **A** and **I** are concentrations of substrate and inhibitor, respectively, K_a is the Michaelis constant for the varied substrate, and K_{is} is the slope inhibition constant.

Results

Development of a Quantitative L-Glutamate Release Assay

The assay to detect the hydrolysis of gamma-glutamyl bonds is a coupled assay in which the glutamate released by the hydrolysis of the gamma-glutamyl bond is quantified. The series of reactions are shown below with GSH as the gamma-glutamyl substrate (reactions 1-3).

$$GSH + H_2O \xrightarrow{GGT} GGT + L\text{–}Glu + Cysteinylglycine \quad \text{(reaction 1)}$$

$$L\text{–}Glu + NAD^+ + H_2O \xrightarrow{GDH} 2\text{–}oxoglutarate + NADH + NH_4+ \quad \text{(reaction 2)}$$

$$INT + NADH + H^+ \xrightarrow{Diaphorase} NAD^+ + INT\text{–}formazan \quad \text{(reaction 3)}$$

In reaction 1, the gamma-glutamyl bond of GSH is hydrolyzed. The glutamate that is released from GSH is quantified by a modified version of an assay developed by Beutler [34]. The glutamate is oxidized by glutamate dehydrogenase (GDH), reducing NAD^+ to NADH (reaction 2). The concentration of L-glu is the rate-limiting component of reaction 2. NADH in the presence of diaphorase reduces iodonitrotetrazolium (INT) to INT-formazan (reaction 3). NADH is the limiting component in reaction 3. INT-formazan is a colored product, which is detected at 490 nm (Fig 1B). The production of glutamate in reaction 2 can be monitored continuously. The amount of INT-formazan produced in reaction 3 is stoichiometric with the amount of L-glutamate (Fig. 2A).

A time course of the rate of glutamate release from GSH by human GGT1 shows dose-dependence with concentration of the substrate (Fig. 2B). There is an initial lag in the velocity of the reaction as the glutamate concentration builds to a detectable level, followed by a steady state rate that is constant for more than 5 min at all concentrations tested (Fig. 2B). The rate at which glutamate is released from GSH by GGT1 follows Michaelis-Menten kinetics (Fig. 2C). The initial velocity of glutamate release from GSH is linear versus enzyme concentration (Fig. 2D).

Human GGT1 Transpeptidation Reaction

Previous studies have shown that addition of millimolar concentrations of a dipeptide acceptor to the GGT reaction shifts the reaction from a hydrolysis reaction to a transpeptidation reaction [29; 30]. In the transpeptidation reaction, the gamma-glutamyl group of the substrate is transferred to the amine of the dipeptide forming a new gamma-glutamyl bond and a new gamma-glutamyl compound is released as the product of the reaction. Free glutamate is not released when the gamma-glutamyl group is transferred to the acceptor in the transpeptidation reaction. Therefore in the presence of increasing concentrations of a dipeptide acceptor, the reaction would shift from a hydrolysis reaction to a transpeptidation reaction and one would expect to observe decreased amounts of free glutamate released. The rate of glutamate released by GGT1 did decrease as the concentration of the acceptor, glygly, was increased in the presence of a fixed concentration of GSH (Fig. 2F). The same result would have been observed if the glygly competed with GSH for binding to the substrate binding site and thereby reduced the rate of the hydrolysis reaction. Our data do not distinguish between these two possibilities. However, in a study of the kinetics of human GGT1, Castonguay and colleagues incubated the enzyme with GSH and glygly. They quantified the production of gamma-glutamyl-glygly by LC-ESI-TOF, demonstrating that in the presence of glygly, GSH serves as a gamma-glutamyl donor for the transpeptidation reaction [37]. In the presence of high concentrations of the acceptor glygly, the amount of glutamate released from GSH is decreased up to 90%, but some glutamate continues to be released (Fig. 2F). This low level of hydrolysis that occurs simultaneously

with the transpeptidation reaction is not detected by other GGT assays that measure the portion of the substrate released from the gamma-glutamyl group.

Kinetic Analysis of Human GGT1 Substrate Specificity

Three gamma-glutamyl compounds, GSH, GSSG, and LTC_4, were evaluated as substrates for human GGT1 with the glutamate release assay. The K_m for hydrolysis with GSH as a substrate was 10.60 ± 0.07 μM (Fig. 3A). This value is comparable to the K_m of 7.3 μM for GSH, which was determined by Castonguay and colleagues using an HPLC-based assay [37]. The K_m of GSSG was 8.80 ± 0.05 μM (Fig. 3A). The K_m for LTC_4 was 10.8 ± 0.1 μM (Fig 3A). The similar K_ms and similar V/K constants for all three physiologic compounds indicates that all have similar access to the substrate binding site with similar catalytic efficiency (Table I). Human GGT1 was also able to cleave several non-physiologic GSH conjugates, S-(4-Nitro-benzyl)glutathione and S-methylglutathione, with similar K_m values and similar second order rate constants (V_{max}/K_mE_t), while other gamma-glutamyl compounds, gamma-glutamyl leucine, exhibited a higher K_m and lower second order rate constant (Table 1). These data indicate that GSH and GSH conjugates are substrates of GGT1 with GSH and GSSG having the highest catalytic efficiency among the substrates we evaluated.

Kinetic Analysis of Inhibition of Human GGT1

Previous studies with the standard GGT assay have shown that an equimolar solution of serine and borate, inhibits GGT1 activity [17; 31; 40]. Data from our glutamate release assay showed serine-borate was a competitive inhibitor of GGT1 with a K_i 0.50 ± 0.06 mM (Fig. 3B). To ensure that the inhibitor was not inhibiting the reactions in the second half of the assay system, glutamate was added as the substrate, and its detection was monitored in the presence of the inhibitor. Under these conditions, serine-borate inhibited the substrate conversion enzymes of the glutamate detection system with a K_i of 41.6 ± 2.0 mM. These data demonstrate that inhibition of human GGT1 was the rate-limiting step and that the inhibition observed with serine-borate was due to inhibition of human GGT1. Maleate has been reported to stimulate the hydrolysis reaction [17; 31]. We were unable to evaluate maleate in this assay because maleate inhibited the glutamate detection portion of the assay (data not shown).

Kinetic Analysis of Human GGT5 in the L-Glutamate Release Assay

Previous reports and data from this laboratory confirm that human GGT5 cannot cleave GpNA, the substrate in the standard GGT1 assay ([21], data not shown). The only method for measuring GGT5 activity has been quantifying LTC_4 conversion to LTD_4 by HPLC [2; 21; 22; 23]. Analysis of human GGT5 in our glutamate release assay revealed that GSH is a substrate of human GGT5 (Fig.4A). The rate of release of glutamate from GSH by GGT5 shows Michaelis-Menten kinetics (Fig. 4A). The initial velocity of glutamate release from GSH versus enzyme concentration is linear (Fig. 4B). A comparison of the rate of glutamate release from GSH by GGT1 versus GGT5 shows that per μg protein, GGT1 cleaves GSH approximately 46 times faster than GGT5 (Fig 2D, Fig 4B).

GGT5 has never been evaluated for its ability to transfer the gamma-glutamyl group of a substrate to an acceptor. Kinetic analysis of glutamate release from GSH by GGT5 in the presence of an acceptor (glygly) is shown in Fig. 4D. As the acceptor concentration increases, the rate of glutamate release decreases as was observed for GGT1 (Fig. 2F). These data suggest that human GGT5 is able to transfer the gamma-glutamyl group to glygly similarly to human GGT1. This is the first data to reveal that GGT5 may catalyze a transpeptidation reaction in addition to the hydrolysis reaction; however, this is not

representative of the physiologic reaction in which high concentration of an acceptor would not be present.

Kinetic Analysis of Human GGT5 Substrate Specificity

Kinetic analysis of human GGT5 revealed that the K_m for GSH is 10.50 ± 0.05 μM and for LTC_4 is 10.20 ± 0.1 μM (Fig. 5A). These values are similar to the K_ms of these same substrates with human GGT1. In contrast, the K_m of GSSG with GGT5 is 42.60 ± 0.06 μM, which is 7-fold higher than the K_m of GSSG with human GGT1 (Figs. 5A and 3A). Non-physiologic GSH conjugates, S-(4-Nitro-benzyl)glutathione and S-methylglutathione, had slightly elevated K_m values for human GGT5 compared to human GGT1 and had substantially lower second order rate constants (V_{max}/K_mEt) than those of GGT1 (Table 2 and Table 1). These data indicate that these compounds do not access the GGT5 binding pocket as well as they do the GGT1 binding pocket, but these compounds have similar catalytic efficiency. In addition, gamma-glutamyl leucine was not a substrate for human GGT5 although it was a substrate for GGT1 (Table 2 and Table 1). These data indicate that the substrate binding pockets of human GGT1 and human GGT5 both bind gamma-glutamyl substrates but have some unique characteristics.

Kinetic Analysis of Human GGT5 Inhibition by Glutamine Analogues

Serine-borate, a competitive inhibitor of GGT1, was evaluated for its ability to inhibit GGT5. Serine-borate was also a competitive inhibitor of human GGT5 with a K_i of 4.20 ± 0.31 mM (Fig. 5B). Serine-borate was 8-fold less effective as an inhibitor of GGT5 than GGT1 (Figs. 5A and 3A). These data indicate that, although human GGT1 and human GGT5 have some overlapping substrate and inhibitor specificities, they have unique kinetic properties.

Discussion

We have developed a novel, sensitive, and quantitative kinetic assay that provides a method for the in-depth analysis of the physiologic reactions catalyzed by GGT1 and GGT5. This assay allows for the continuous monitoring of L-glutamate release as GGT1 and GGT5 cleave gamma-glutamyl substrates. Within the assay, released L-glutamate is quickly metabolized, preventing it from acting as an acceptor in a transpeptidation reaction. This assay is conducted at physiological pH and can be used to evaluate physiological compounds as substrates of GGT1 and GGT5. We have found that the K_ms for several of the physiological substrates are in the range of 10 μM These conditions are in sharp contrast to the standard assay for GGT1, which uses GpNA, a non-physiological compound as the substrate, millimolar concentrations of a dipeptide acceptor, and is conducted at pH 8.0 or higher. In the standard assay, the K_m for GpNA is 1 mM [35]. Our glutamate release assay is also a major advancement over the HPLC-based assays that detect the relative amounts of substrate and product present at individual time points throughout the reaction [37]. In addition to being time consuming, the HPLC method results in an accumulation of free glutamate over time, which can act as an acceptor for the transpeptidase activity of the enzyme. As a result, newly formed gamma-glutamyl compounds released from transpeptidation would lead to inaccuracies in quantifying the concentration of the hydrolysis products. Vergauwen and colleagues described an assay for GGT that used gamma-glutamyl-D,L-phenylthioglycyl-glycine, a non-physiological compound, as the donor substrate [41]. The authors state that this assay measures only the hydrolysis reaction of GGT. But, the assay monitors the release of the phenylthioglycyl-glycine product which is released in both the hydrolysis and transpeptidation reactions and therefore the assay does not distinguish between the two reactions.

GSH is present in human serum at a concentration of 5-20 μM [1]. We determined the K_m of GSH with human GGT1 to be 10.6 μM. Castonguay and colleagues analyzed human GGT1 activity using a HPLC based method and determined the K_m for GSH to be 7.3 μM [37]. The K_m for GSH with human GGT1 determined in this study and by Castonguay and colleagues is very similar, despite the use of two different assays. The K_ms determined by both groups are in the physiologic range of serum GSH, which is consistent with data from GGT1 knockout mice that GSH is a physiological substrate of GGT1 [8; 9; 22; 24].

The ability of our L-glutamate detection assay to evaluate any compound as a substrate for GGT1 has lead to the confirmation that GSSG and LTC_4 are both substrates for human GGT1. In addition, several other non-physiological gamma-glutamyl compounds such as S-(4-Nitro-benzyl)glutathione, gamma-glutamyl leucine, S-methylglutathione, and glutathionesulfonic acid are substrates for human GGT1. The K_m values for GSSG and LTC_4 are similar, indicating that human GGT1 has similar catalytic efficiency for these GSH conjugates. These data are in contrast to data from the GGT1 and GGT5 knockout mice, which indicate that murine GGT1 has little to no activity in cleaving LTC_4 [2].

Several compounds have been shown previously to competitively inhibit the GGT1 transpeptidation reaction. We used our assay to evaluate inhibition of human GGT1 by serine-borate, a competitive inhibitor of rat GGT1. We determined the K_i for serine-borate under our assay conditions to be 0.50 mM for human GGT1, which is 50-fold higher than the K_i of 0.02 mM reported for serine borate in the standard GGT assay using rat GGT1 [40]. In both assays serine-borate acts as a competitive inhibitor of GGT1, but it is a much weaker inhibitor of human GGT1 when assayed in our physiological assay with GSH as the substrate, than reported previously for rat GGT1.

The data from this study confirm an earlier report that LTC4 is a substrate for human GGT5 [21]. Human GGT5, has been referred to as gamma-glutamyl leukotrienase, based on its homology with murine GGT5 which cleaves LTC4 but is a weak glutathionase [20; 22]. However, our data demonstrate that human GGT1 is more active as a gamma-glutamyl leukotrienase than human GGT5. Therefore, in humans denoting GGT5 as the gamma-glutamyl leukotrienase is misleading. Our data are in agreement with those of Heisterkamp and colleagues, who reported that human GGT5 was capable of cleaving GSH, but not GpNA [21]. Among the substrates that we assayed with human GGT5 in the L-glutamate release assay, we determined that GGT5 can metabolize gamma-glutamyl compounds that are GSH conjugates (LTC_4 and GSSG), but not gamma-glutamyl compounds such as gamma-glutamyl leucine.

There are only limited data regarding inhibition of GGT5. Heisterkamp and colleagues reported that human GGT5 is inhibited by acivicin [21]. These data correlate with studies by Carter and colleagues in mice, demonstrating that acivicin inhibits murine GGT5 [2]. These have been the only attempts to characterize the inhibition of either murine or human GGT5, until the development of the L-glutamate detection assay. Initial studies of human GGT5 inhibition by acivicin utilizing the L-glutamate detection assay show that acivicin is capable of inhibiting GGT5 (data not shown), but further kinetic analysis of the inhibition have not been performed due to a lack of commercially-available acivicin. Data in this study show that serine-borate inhibits human GGT1 and human GGT5 with different potency, indicating that the binding pocket differs between human GGT1 and human GGT5.

Our novel L-glutamate release assay provides an unbiased method for the characterization of GGT enzymes under physiologic conditions. This assay provides an invaluable tool for the study of GGT1 and GGT5. The assay also provides a method for the evaluation of compounds as inhibitors of these enzymes. Such inhibitors are actively being sought out for

the treatment of a wide range of human diseases, including cancer, cardiovascular disease, allergies, and asthma [25; 35; 42].

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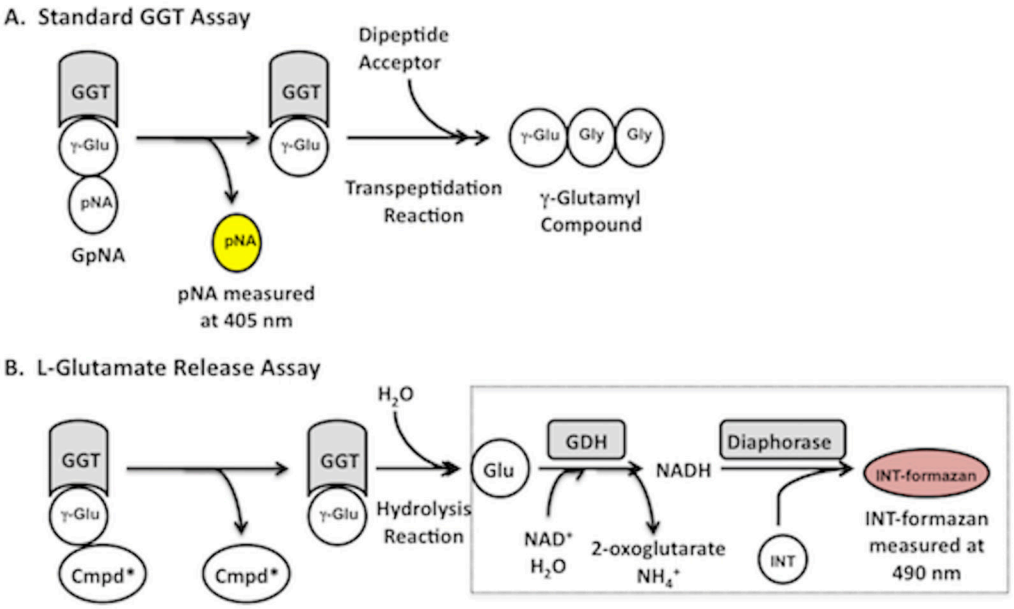

Fig. 1.

GGT1 reaction with Gamma-Glutamyl Substrates. The standard assay used to detect GGT1 activity (A) uses the synthetic substrate gamma-glutamyl para-nitroanilide (GpNA). The assay measures the release of p-nitroanaline (pNA) from the substrate. Glycylglycine is included in the reaction as an acceptor resulting in the formation of a new gamma-glutamyl bound between the glutamate and the acceptor, which accelerates the release of the free enzyme. The new glutamate release assay (B) is a coupled assay in which any gamma-glutamyl compound can be evaluated as a substrate. The rate-limiting step in the reaction is the hydrolysis of the gamma-glutamyl-enzyme intermediate releasing glutamate and free enzyme. The rate of glutamate release is quantified by the formation of iodonitrotetrazolium (INT)-formazan (see text for details).

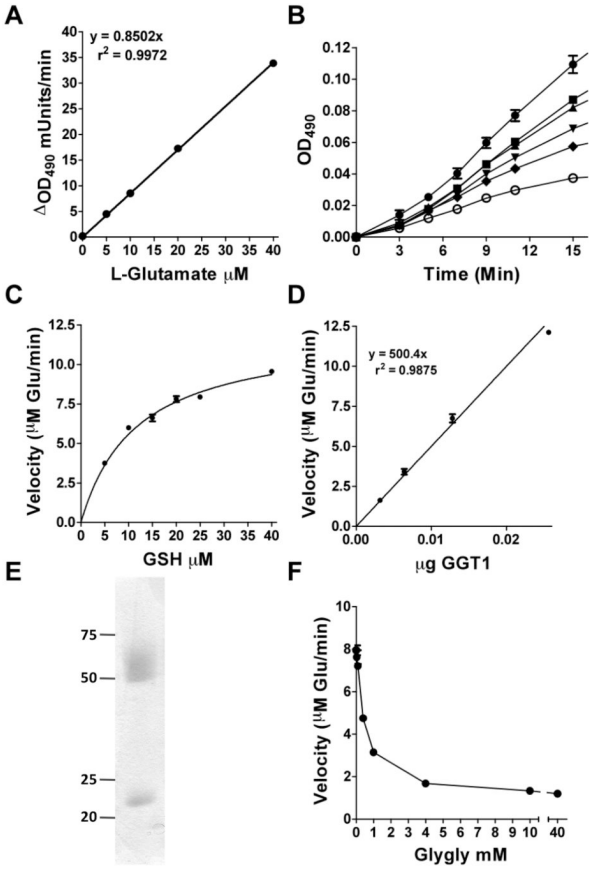

Fig. 2.

Characterization of L-Glutamate Release Assay. Initial velocity versus L-glutamate concentration is linear (A). Time course for the release of glutamate from glutathione by GGT; GSH concentrations used are as follows: of 5 μM (○), 10 μM (◆), 15 μM (▲), 20 μM (▲), 25 μM (■), and 40 μM (●), shown in (B). Steady state rate versus GSH concentration demonstrates the sensitivity of the assay; data adhere to eq. 1. (C). Steady state rate versus GGT1 concentration is linear. The substrate was 40 μM GSH (D). Silver stained SDS-PAGE gel demonstrating the purity of GGT1 and that GGT1 comprised of a large subunit migrating at 64 kDa and a small subunit migrating at 22 kDa (E). Initial maximum velocity versus glygly concentration in the presence of 40 μM GSH. In the transpeptidation reaction the glutamate is transferred to the acceptor and the amount of free glutamate released from the enzyme-substrate complex is decreased (F). Data shown are average of triplicate values ± S.D. (For many points, the error bars are smaller than the symbol.)

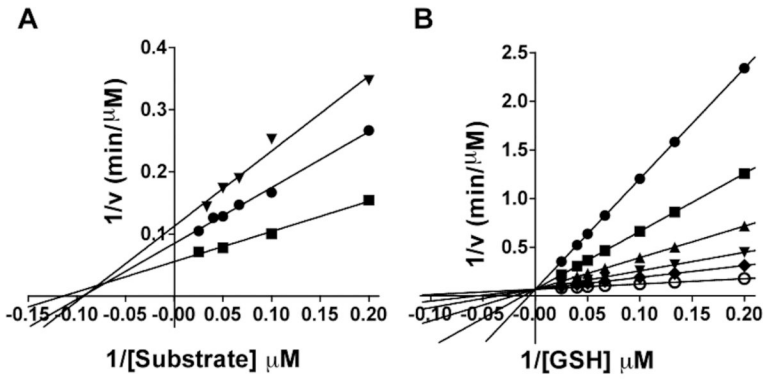

Fig. 3.

Kinetic Analysis of GGT1 Substrate Specificity and Inhibition. (A) Double reciprocal plot of the initial velocity of GGT1 in the presence of GSH (●), GSSG (■), and LTC_4 (▼). (B) Double reciprocal plot of the initial velocity versus substrate concentration in the presence of 0 mM (○), 0.625 mM (◆), 1.25 mM (▼), 2.5 mM (▲), 5 mM (■), and 10 mM (●) serine-borate. Data shown are average of triplicate values ± S.D. (For many points, the error bars are smaller than the symbol.) Enzyme was maintained at 10 mU.

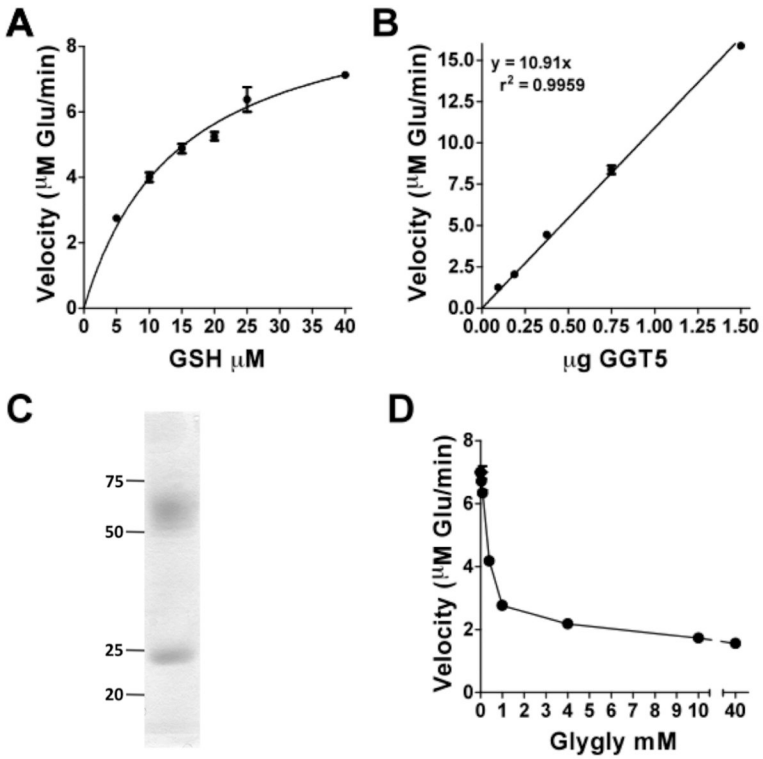

Fig. 4.

Kinetic Analysis of Human GGT5 with the L-Glutamate Release assay. Velocity versus substrate concentration with 10 mU GGT5 (A). Initial velocity versus GGT5 concentration where the substrate was 40 μM GSH (B). Silver stained SDS-PAGE gel demonstrating the purity of human GGT5 and that GGT5 comprised of a large subunit migrating at 64 kDa and a small subunit migrating at 25 kDa (C). Steady state rate versus glygly concentration in the presence of 40 μM GSH (D). In the GGT1 transpeptidase reaction, glutamate is transferred to the acceptor and the amount of free glutamate released from the enzyme-substrate complex is decreased, GGT5 exhibits the same decrease in glutamate released in the presence of the acceptor glygly (D). Data shown are average of triplicate values ± S.D. (For many points, the error bars are smaller than the symbol.)

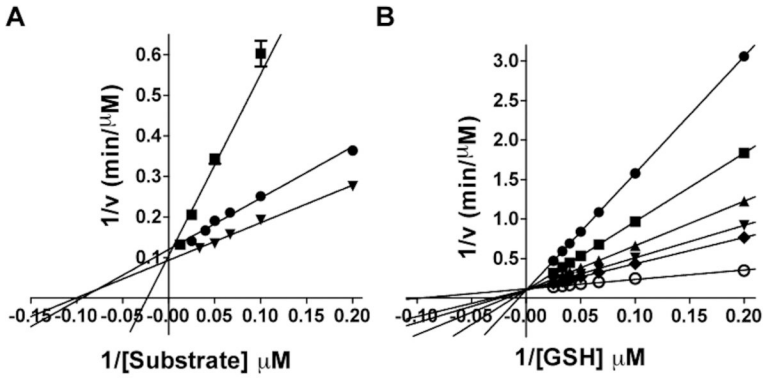

Fig. 5.

Kinetic Analysis of GGT5 Substrate Specificity and Inhibition. Double reciprocal plot of the initial velocities of human GGT5 versus substrate concentration: GSH (●), GSSG (■), and LTC_4 (▼). Double reciprocal plot of the initial velocity of 10 mU GGT5 versus substrate concentration with 0 mM (○), 1.25 mM (◆), 2.5 mM (▼), 5 mM (▲), 10 mM (■), and 20 mM (●) serine-borate (B). Data shown are average of triplicate values ± S.D. (For many points, the error bars are smaller than the symbol.)

Table 1

Summary of Human GGT1 Activity

Substrate	K_m (μM)	V_{max} (μM/min/nM)	V_{max}/K_mE_t ($min^{-1}nM^{-1}$)
GSH	10.60 ± 0.07	6.3 ± 0.3	0.60 ± 0.03
GSSG	8.80 ± 0.05	6.3 ± 0.5	0.71 ± 0.06
LTC_4	10.8 ± 0.1	5.3 ± 0.3	0.49 ± 0.03
S-(4-Nitro-benzyl)glutathione	13.10 ± 0.05	6.2 ± 0.4	0.47 ± 0.03
S-methylglutathione	9.90 ± 0.07	5.7 ± 0.2	0.57 ± 0.02
Glutathionesulfonic acid	34.60 ± 0.08	5.9 ± 0.1	0.169 ± 0.003
Gamma-glutamylleucine	33.4 ± 0.3	3.3 ± 0.3	0.099 ± 0.009

Table 2

Summary of Human GGT5 Activity

Substrate	K_m (μM)	V_{max} (μM/min/nM)	V_{max}/K_mE_t ($min^{-1}nM^{-1}$)
GSH	10.50 ± 0.05	0.17 ± 0.01	0.016 ± 0.006
GSSG	42.60 ± 0.06	0.28 ± 0.03	0.010 ± 0.006
LTC_4	10.2 ± 0.1	0.20 ± 0.01	0.019 ± 0.006
S-(4-Nitro-benzyl)glutathione	14.80 ± 0.06	0.27 ± 0.01	0.018 ± 0.007
S-methylglutathione	18.20 ± 0.08	0.22 ± 0.01	0.012 ± 0.007
Glutathionesulfonic acid	75.10 ± 0.08	0.18 ± 0.01	0.0024 ± 0.0007
Gamma-glutamylleucine	NA[a]	NA	NA

[a] Substrates with no enzymatic activity are represented by NA.