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REVIEW

Gamma-glutamyl transferases: A structural, mechanistic and physiological perspective

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Abstract **Gamma glutamyl transferases (GGT) are highly conserved enzymes that occur from bacteria to humans. They remove terminal γ-glutamyl residue from peptides and amides. GGTs play an important role in the homeostasis of glutathione (a major cellular antioxidant) and in the detoxification of xenobiotics in mammals. They are implicated in diseases like diabetes, inflammation, neurodegenerative diseases and cardiovascular diseases. The physiological role of GGTs in bacteria is still unclear. Nothing is known about the basis for the strong conservation of the enzyme across the living system. The review focuses on the enzyme's physiology, chemistry and structural properties of the enzyme with emphasis on the evolutionary relationships. The available data indicate that the members of the GGT family share common structural features but are functionally heterogenous.**

Keywords Gamma glutamyl transferase, Ntn hydrolase, structure, catalysis, function

Introduction

Gamma glutamyl transferases (GGT; E.C.2.2.3.2) are highly conserved enzymes that occur in bacteria, yeast, plants and in animals from nematodes to humans (Rawlings et al., 2006). It is a two substrate enzyme that removes the terminal γ-glutamyl residue from a molecule of the general form Glu-γCO-NH-R by breaking the amide bond and transfers it to a receptive molecule. Some of the common physiological γ-glutamyl substrates are glutathione (Elce and Broxmeyer, 1976), γ-poly glutamic acid (Kimura et al., 2004) and glutamine (Minami et al., 2003). Three types of reactions are possibly based on the destination of the γ-glutamyl moiety: (a) hydrolysis – when the γ- glutamyl moiety is transferred to water, (b) transpeptidation – when the γ-glutamyl moiety is transferred to an 'acceptor' molecule like amino acids or peptides and (c) auto-transpeptidation – when transferred to a second substrate molecule. In further discussion, the γ-glutamyl substrate will be referred to as the acyl-donor; and, the peptides and amino acids that accept the γ-glutamyl moiety during transpeptidation will be referred to as the acyl-acceptor. Glutathione is the physiological substrate of mammalian GGTs (Zhang et al., 2005). Little is known about the physiological substrate of the GGTs from plants and bacteria. The reactions catalyzed by GGT are depicted below:

$$\gamma\text{GluCONHR} + \text{H}_2\text{O} \leftrightarrow \text{Glu} + \text{RNH}_2$$

(Hydrolysis)

$$\gamma\text{GluCONHR}^1 + \text{R}^2\text{CONH}_2 \leftrightarrow \gamma\text{Glu-COR}^2 + \text{R}^1\text{NH}_2$$

(Transpeptidation)

$$2\gamma\text{GluCONHR} \leftrightarrow \gamma\text{Glu-GluCONHR} + \text{RNH}_2$$

(Autotranspeptidation)

The Nomenclature Committee of International Union of Biochemistry and Molecular Biology recommended that this enzyme, E.C.2.3.2.2, (5-L-glutamyl) peptide: amino-acid 5-glutamyl transferase, be referred to as 'γ-glutamyl transferase' (Webb, 1992). However, many authors continue to use the older name 'gamma-glutamyl transpeptidase'.

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Structural properties

Expression and organization of the polypeptide chain

GGT is a heterodimeric protein. The enzyme is expressed as a single polypeptide chain which subsequently undergoes autocatalytic proteolysis. The proteolytic site is sub-centric and disposed toward the C terminus. Thus, the N-terminal fragment forms the larger subunit while the C-terminal fragment forms the smaller subunit. The two polypeptide chains do not occur as discrete domains but are tightly intertwined to form a compact structure. Both the polypeptide chains are necessary for the enzyme activity. UniProt KB database search shows that GGTs are mostly composed of 550 ±100 amino acids. Length of the two proteolytic fragments of some representative GGTs in terms of amino acid content are as follows: humans (380 + 189), rat (253 + 188), *Culex* mosquito (390 + 193) *Arabidopsis* (364 + 204), yeast (469 + 190), *E. coli* (364 + 189), *B. subtilis* (366 + 184); respective polypeptide chains of these GGTs are shown in Fig. 1.

Crystal structure

The crystal structures of GGT from *E. coli* (Okada et al., 2006), *H. pylori* (Boanca et al., 2007), *B. subtilis* (Sharath et al., 2010; Wada et al., 2010) and humans (Mathew et al., 2013) have been determined at high resolution. Representative crystal structures are shown in Fig. 2. The tertiary structure is characterized by a tetralamellar αββα core. The enzyme is kidney-shaped and encloses a shallow groove in the region below the β sheets. The active site is represented by a finger-like depression that occurs toward the middle of the groove. The composition of the active site has been analyzed from crystal complexes with glutamate and γ-glutamyl-enzyme intermediate in *E. coli* GGT and with glutamate and *S*-(nitrobenzyl) glutathione in *H. pylori* GGT. The substrate is bound to the active site by a number of hydrogen bonds and salt bridges formed between the enzyme and the α-amino and α-carboxylate groups of the substrate. The interactions are represented in Fig. 3. The glutamyl Cγ receives two hydrogen bonds from the backbone N atoms of

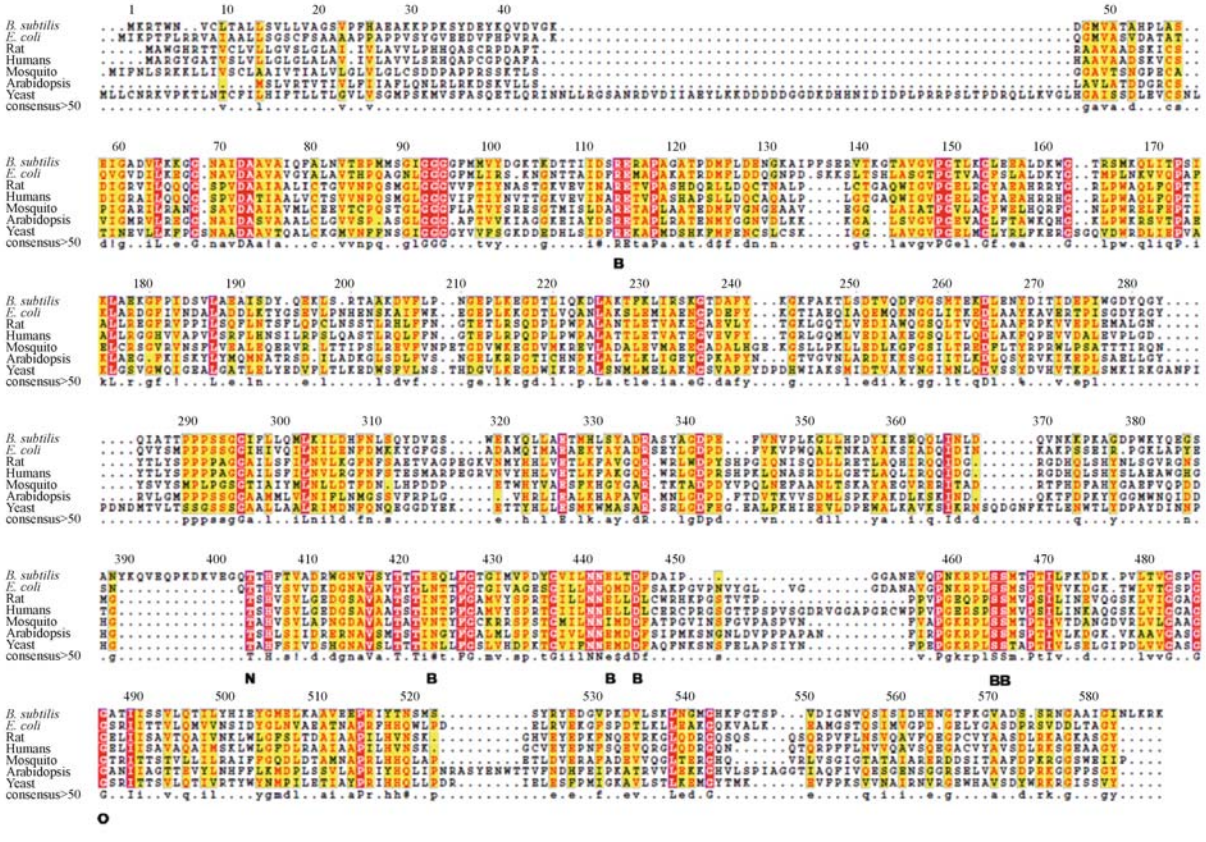

Figure 1 Multiple sequence alignment of GGT from different organisms. The sequence numbering corresponds to *B. subtilis* GGT. Identical residues are shaded, and similar residues are boxed. The residues representing catalytic nucleophile, substrate binding, and oxyanion hole are marked with the letters N, B, and O, respectively (residue identity as in Ref X). Sequences shown are for GGTs of, *B. subtilis* (P54422), *E. coli* (P18956), rat (Q9QWE9), human (P36269), *Culex* mosquito (B0XE48), *Arabidopsis thaliana* (Q39078) and yeast (Q05902). The respective UniProtKB accession number is given in the parenthesis. The figure was prepared with CLUSTALW (Thompson et al., 1994) and ESPRIPT (Gouet et al., 1999).

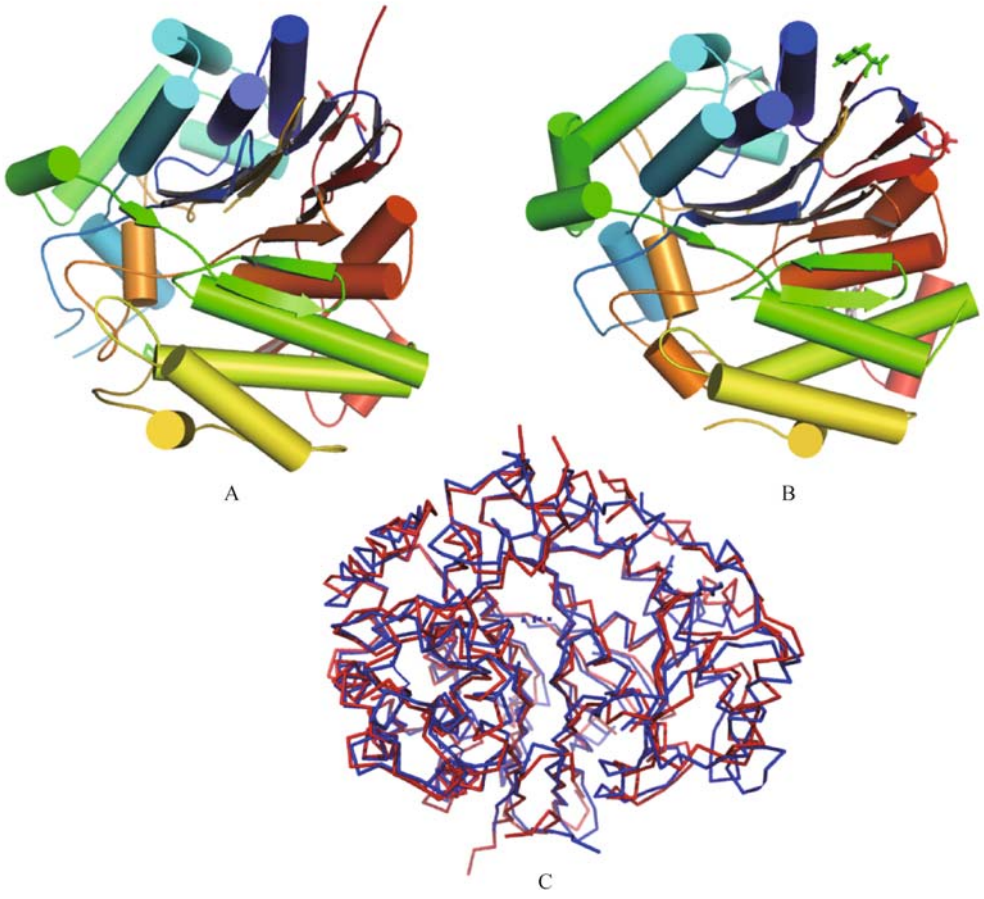

Figure 2 Structures of GGTs from (A) *B. subtilis* (PDB Code 2v36) and (B) humans (PDB Code 4GDX) α-helices and β-strands are represented as cylinders and arrows respectively. (C) Superposition of Cα trace of GGTs from *B. subtilis* (red) and humans (blue). The figures were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.6 Schrödinger, LLC).

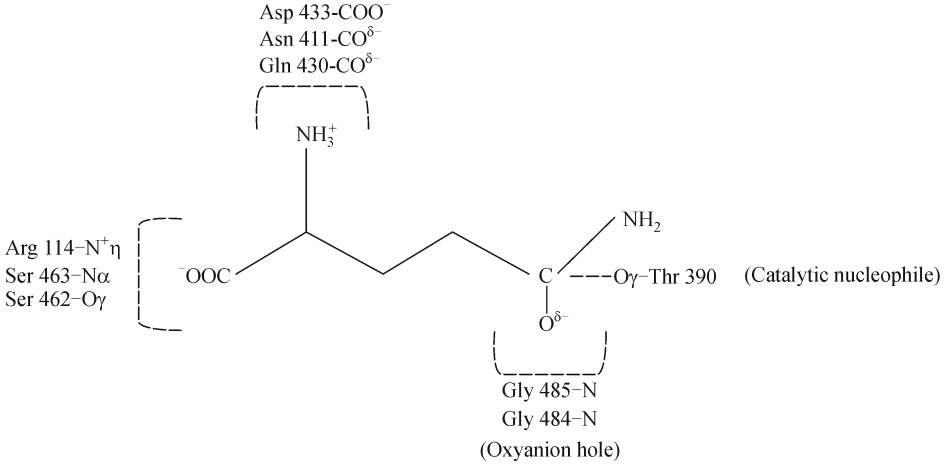

Figure 3 Diagrammatic representation of interactions that bind the γ-glutamyl moiety of the substrate to the active site. Interactions with the catalytic nucleophile and stabilization of the tetrahedral intermediate by oxyanion hole are also shown.

two tandem Gly residues. These interactions form the oxyanion hole that stabilizes the negative charge formed on the γ-carbonyl oxygen in the tetrahedral transition state. In both *E. coli* and *H. pylori* GGTs, the mouth of the active site is partially closed by a Tyr residue that occurs at the apex of the so called lid loop. The conformation of this loop is similar in both native and complex structures. The lid loop is relatively shorter with a terminal Phe residue in humans and other

mammalian GGTs; it is completely absent in *B. subtilis* GGT. These differences in the lid loop increases the exposure of the active site to the solvent. It is hypothesized that the increased exposure facilitates the entry of the Acyl-Acceptors and therefore forms the basis for the relatively higher levels of transferase activity of mammalian and *B. subtilis* GGT (Mathew et al., 2013).

GGTs are Ntn hydrolases

GGTs belong to a super-family of enzymes 'N-terminal nucleophile (Ntn) hydrolases'(Brannigan et al., 1995). The diagnostic characters of these enzymes are:

1) The core structure of the enzyme is composed of a 'αββα' tetralamellar fold.

2) They are produced as inactive precursor/zymogen, which then matures into the active form after an autocatalytic chain cleavage. The cleavage results in the formation of a new N-terminal residue.

3) The newly formed N-terminal residue functions as the catalytic nucleophile. The three possible nucleophilic residues are Thr, Ser or Cys. The type of the residue is fixed for a given member. Thr serves as the catalytic nucleophile in GGTs.

4) Their catalytic machinery comprises of a 'single residue nucleophile' system. The general base catalyst required for deprotonation of the side chain-OH or-SH moiety is provided by the free α-amino group of the nucleophilic residue itself. The nucleophilic residue occurs at the N terminus because of this need for a free α-amino group

5) Ntn hydrolases catalyze amidolytic reactions.

The other members of the family are Bile Salt Hydrolases (Kumar et al., 2006), Cephalosporin Acylases (Kim et al., 2000), Glucosamine-6-Phosphate Synthase (Isupov et al., 1996), Glutamine-PPRP-Amidotransferase (Kim et al., 1996), Glycosylasparaginase (Guo et al., 1998), Heat Shock Locus V (Bochtler et al., 1997), L-aminopeptidase-D-Ala-esterase/amidase (Bompard-Gilles et al., 2000), Isoaspartyl Aminopeptidase (Michalska et al., 2005), MTH1020 (Saridakis et al., 2002), Ornithine Acetyltransferase (Elkins et al., 2005), Penicillin G Acylases (Duggleby et al., 1995), Penicillin V Acylases (Suresh et al., 1999), β subunit of 20 S Proteasome (Löwe et al., 1995), U34 Peptidase (Pei and Grishin, 2003).

Post-translational maturation

The mechanism of processing has been analyzed in *E. coli* GGT by developing maturationally blocked mutants (Suzuki and Kumagai, 2002) and from the crystal structure of the precursor (Okada et al., 2007). Maturational processing transforms the single chain precursor into the active heterodimer. The precursor differs from the mature enzyme mostly in the region corresponding to the active site. The C terminus of the heavy chain, called as P-segment, occupies the future active site. During processing, the peptide bond preceding the catalytic Thr (Thr-391 in *E. coli* GGT) is cleaved. Thereafter, the P segment rotates about Ile-378 – which occurs at the base of the segment– (ψ changes from −45° to127°) thus displacing the segment from the catalytic Thr by a distance of ~34Å. Finally, the space emptied by the retrieval of the P-segment is occupied by the lid loop. Also, the nascent active site contracts due to the inward translocation of the residues between 411 and 416, thus defining a complementary γ-glutamyl binding site. The maturational proteolysis is mediated by the side chain of the catalytic Thr; substitution of this residue with Ala completely blocks the processing. In addition, mutational substitution of the catalytic Thr with Cys sensitizes the maturation process to Cys modifiers like DTNB, PCMB, iodoacetamide. These reagents do not affect the processing of the wild type or the Ser substituted mutant (Lee et al., 2000). Furthermore, the maturation process is intra-molecular as incubation of maturationally blocked Thr-391→Ala mutant with the active enzyme does not induce processing. In contrast, the C-terminal residue, Gln in *E. coli*, appears to be unimportant as its substitution with Ala does not produce any maturational defects. The unimportance of this residue is possibly the basis for its poor conservation (Fig. 1).

In the mature enzyme, catalytic property is imparted to the side chain –OH of the N-terminal Thr by a general base catalyzed deprotonation. The free α-amino group of the catalytic residue itself functions as the general base. This moiety is not available in the precursor as it is engaged in the peptide bond. In the crystal structure of the precursor, a water molecule appears to be in a competent position to mediate the process. The involvement of a water molecule has also been noticed in the processing of cephalosporin acylases (Lee et al., 2000) and β subunit of yeast 20S proteasome (Arendt and Hochstrasser, 1999). The peptide bond cleavage is believed to proceed by N-O acyl shift mechanism as there are evidences for formation of a critical ester bond during the process. These results have allowed elucidation of the processing mechanism: a water molecule activates Thr-391 Oγ thereby enabling it to attack Gln-390 C; the resultant tetrahedral transition state collapses, thus transferring the amide link between Gln-390 and Thr-391 into an ester link between Gln-390 C and Thr-391 Oγ (N-O acyl shift); hydrolysis of the ester intermediate separates Gln-390 and Thr-391.

Distribution and expression

Cellular location

Mammalian GGTs occur on the surface of the epithelial cells mostly in tissues involved in secretion or absorption such as kidneys, bile duct, intestine, pancreas and epididymis (Ikeda and Taniguchi, 2005). The enzyme is located on the cell membrane even in the plant cells (Storozhenko et al., 2002).

On the contrary, the homolog in yeast is localized on the inner face of the vacuolar membrane (Mehdi et al., 2001). The enzyme occurs in the periplasm of gram negative bacteria like *Escherichia coli, Proteus vulgaris* and *Helicobacter pylori* (Nakayama et al., 1984; Suzuki et al., 1986; Chevalier et al., 1999). In contrast, the homolog in *Neisseria meningitidis* is located in the cytoplasm on the inner face of the inner membrane (Takahashi and Watanabe, 2004). The enzyme in gram positive bacteria like *B. subtilis* is secreted into the extracellular medium (Xu and Strauch, 1996).

Expression in mammals

In humans, GGT is represented by at least 7 different genes, located mostly on chromosome 22. In contrast, a single *ggt* gene occurs in rat and mouse. In both cases, the presence of multiple promoters and splicing variations result in a variety of transcripts (Chikhi et al., 1999). The diversity in rodents, which arises due to tissue specific expression, is confined to the 5′ untranslated region (5′ UTR) with the coding region being essentially same. However, the human transcripts extend the variation even to the coding region. Some of the transcripts are truncated and may contain sequence only for the heavy or the light subunits. It is not known if these incomplete transcripts are eventually translated. Catalytic activity requires the participation of both the subunits. These potentially truncated proteins may therefore serve a non-enzymatic function. Interestingly, the human GGT has been shown to induce osteoclasts by a process that is independent of its catalytic activity (Niida et al., 2004). The human GGT does not form isozymes and the apparent electrophoretic heterogeneity arises due to tissue specific variation in glycosylation (Nemesánszky and Lott, 1985).

Comparison of prokaryotic and eukaryotic GGTs

Prokaryotic and eukaryotic GGTs differ in both structural and functional aspects. While the prokaryotic GGTs occur as soluble protein either in the periplasmic or the extracellular space, the eukaryotic homologs are type II transmembrane proteins. A typical eukaryotic GGT has a large extracellular domain anchored to the membrane by a transmembrane hydrophobic anchor (~8 residues) and a short cytoplasmic tail (containing 4 polar residues). The large extracellular domain, where the catalytic function is located, can be separated from the membrane by papain treatment (Tate and Meister, 1985). The procedure does not affect the enzyme function and is therefore employed in the purification of the enzyme from animal tissues (Ikeda et al., 1995). Furthermore, eukaryotic GGTs have N and O linked glycans whose composition varies in tissue specific manner. The sugars have no catalytic function as their removal by enzymatic deglycosylation does not affect the activity (Smith and Meister, 1994). The sugars appear to confer protection against proteases. Bacterial and eukaryotic GGTs show marked differences in the nature of kinetics. Eukaryotic GGTs are catalytically more powerful than their bacterial homologs. Specific activity of the rat kidney GGT is nearly 100 fold higher than that of the *E. coli* GGT (Ikeda et al., 1995). Further differences occur in the nature of transpeptidation. The breakdown of the acyl-donor by the eukaryotic GGTs is increased by up-to 100 fold in the presence of the acyl-acceptor. In contrast, the acyl-acceptor has smaller effect on the prokaryotic GGT catalyzed breakdown of the γ-glutamyl-substrates (Suzuki et al., 1986). The increase in the rate of acyl-donor breakdown is marginal in the case of the GGTs from gram negative bacteria; the increase in the rate of acyl-donor breakdown is ~2 fold in the *E. coli* GGT and almost none in the *H. pylori* GGT (Boanca et al., 2006). The transpeptidation activity of the GGTs from gram-positive bacteria like *B. subtilis* is relatively better as the acyl-acceptor can increase the breakdown of the acyl-donor by up-to 11-fold (Ogawa et al., 1991).

Other γ-glutamyl hydrolases

Earlier, GGT was thought to be the only enzyme capable of hydrolysing the γ-glutamyl bond. This has been the basis for indiscriminate association of the enzyme with every process that involves break and/or transfer of a γ-glutamyl moiety. GGT was considered as a participant in the biosynthesis of leuckotrienes (LTs). LTs are arachidonate derivatives that function as active mediators of inflammatory and allergic reactions. Biosynthesis of LTs occurs mostly in basophils, eosinophils, mast cells and macropahges. The synthesis occurs in multiple steps, one of which involves deglutamylation of the intermediate LTC4 to produce LTD4, supposedly by GGT (Anderson et al., 1982). The putative importance of GGT was further supported by the occurrence of higher levels of LTC4 in the urine of individuals with genetically deficient GGT (Mayatepek et al., 2004). However, contrasting results were observed in case of *ggt* knockout mutants in mice. The mutant mice are stunted, grow slowly, fail to mature sexually, develop cataract and die prematurely by ~12 weeks of age (Lieberman et al., 1996). But no serious impairment was observed in the cellular conversion of LTC4 to LTD4, thus indicating the presence of an alternate enzyme for the process (Carter et al., 1997). Recently, an enzyme capable of supporting LTC4→LTD4 conversion was cloned and characterized (Carter et al., 1998). The primary structure of this enzyme, named as γ-glutamyl leuckotrinase (GGL), is identical to the mouse GGT by nearly 41%. However, it fails to act on γ-glutamyl-p-nitroaniline, the chromogenic substrate used in GGT assay.

An enzyme (called GGT-rel) related to but distinct from human GGT has been cloned from the human placental

cDNA library (Heisterkamp et al., 1991). GGT-rel shows 39.5% sequence identity to human GGT, occurs on the cell membrane, acts on glutathione and converts LTC4 to LTD4. However, it is inactive on the chromogenic assay substrate. A variant of GGT that expresses exclusively in the rat brain has been isolated and characterized. It is 33% identical and 73% similar to the form expressed in the liver. The variant is inactive on γ-glutamyl-4-nitroaniline, the assay substrate, but active on glutathione (Yamaguchi et al., 2000).

In addition to GGT, *B. subtilis* produces another γ-poly-glutamate hydrolase (Ywr D) that cleaves the chain between D-glutamates (Minami et al., 2004). The amino acid sequences of Ywr and GGT from *B. subtilis* are identical by 27%. The former enzyme is considered to be cytoplasmic as there is no evidence for a signal peptide in its gene.

ΦNIT1 – a bacteriophage that infects *B. subtilis* – produces a γ-poly-glutamate hydrolase that lacks stereospecificity (Kimura and Itoh, 2003). It breaks both α and γ linked polyglutamates into tri, tetra and pentamers. The enzyme serves to perforate the bacilli's capsule during infection. Other enzymes capable of breaking the γ-glutamyl amide bond includes PGA hydrolases from *Flavobacterium* (Volcani and Margalith, 1957) and *Myrothecium* (Tanaka et al., 1993), carboxypeptidase G (3.4.17.11) (Sherwood et al., 1985), γ-glutamyl hydrolase (GGH) from animal tissues (EC 3.4.19.9) (Yao et al., 1996) and glutamate caboxypeptidase II (EC 3.4.17.21) (Galivan et al., 2000). GGH is a thiol enzyme that hydrolyses the γ-glutamyl tail of folyl- γ-PGA. Glutamate caboxypeptidase II is a metalloenzyme that uses Zn^{2+} for endopeptidic lysis of folyl-γ-PGA. Carboxypeptidase G is also a zinc metalloenzyme that releases C-terminal glutamate residues from γ-glutamyl peptides and folyl- γ -PGA.

The cell wall of *Bacillus anthracis,* the etiologic agent of anthrax, is lined by a mucilaginous immunoprotective capsule that is made of γ-poly-glutamic acid (PGA). Most of the PGAs are racemic mixtures of D and L enantiomers. However, the polymer from *B. anthracis* is exclusively made of D-form. The capsule constitutes the principal virulence factor along with the anthrax toxin. Its removal either by genetic mutation or heat treatment reduces the virulence. The capsule is covalently linked to the peptidoglycan of the cell wall by an enzyme called Cap-D. The covalent anchorage is vital for the anti-immunoproperty as mutants lacking Cap-D have reduced virulence (Candela and Fouet, 2006). The immobilized nature of the PGA is unique to *B. anthracis* as most other bacillary PGAs are released into the medium. Cap-D is considered to be a member of the GGT family because of its sequence identity (Candela and Fouet, 2006). The enzyme is synthesized as an inactive precursor which then undergoes autocatalytic maturation process. The active enzyme is a heterodimer with Thr at the N terminus of the light chain. Despite the similarities, Cap-D is unable to unable to hydrolyze γ-glutamyl-p-nitroaniline, the chromogenic assay substrate.

Catalysis

Measurement of GGT activity

A number of substrates have been reported for assaying GGT activity. Of these, γ-glutamyl-4-nitroaniline is the popular substrate (Szasz, 1969). GGT breaks the molecule into glutamic acid and 4-nitroaniline.The bright yellow color of 4-nitroaniline (ε_{410} = 8800M^{-1}) allows quantification by spectrophotometry. For conditions requiring higher concentrations, the limited solubility of the anilide is overcome by using γ-glutamyl-(3-carboxyl)-4-nitroaniline. Glycylglycine is used as the standard Acyl-Acceptor for analyzing the kinetics of transpeptidation. In clinical diagnostics, the reaction between γ-glutamyl-4-nitroaniline and glycylglycine is used for assaying serum GGT based on the recommendation of the International Federation of Clinical Chemistry (IFCC) (Shaw et al., 1983). The transferase activity is preferred as it produces relatively higher activity than the hydrolytic counterpart. Accurate measurement, which is crucial in diagnostics, is impeded by autotranspeptidation (Whitfield, 2001). The aberrant reaction becomes significant at higher substrate concentrations and results in downward curling of the saturation curve. Thus, the substrate is employed at a concentration well below the Km for a clinical assay.

Mechanism of catalysis

Active site

Specificity studies support the classification of the active site into three sub-sites. The γ-glutamyl-moiety and the leaving group of the acyl-donor bind to sub-site 1 and sub-site 2 respectively. Nature of the acyl-acceptor binding site (sub-site 3) is ambiguous and will be discussed later.

The γ-glutamyl sub-site exhibits broader optical specificity by accepting both L and D isomers of glutamic acid. Both α-ammonium and α-carboxylate groups of the γ-glutamyl moiety are involved in binding to sub-site 1. However, α-ammonium group appears to be critical as substitution of its nitrogen atom significantly diminishes the affinity. On the contrary, α-carboxylate group tolerates derivatization into uncharged, isosteric or bulkier (e.g., methyl, t-butyl) forms (Keillor et al., 2005; Cook et al., 1987).

The presence of a discrete site for binding the acyl-acceptor (sub-site 3) is still inconclusive. Some results tend to indicate that the acyl-acceptor binds to the site occupied by the leaving group (Taniguchi and Ikeda, 1998; Thompson and Meister, 1977). GGT is considered to employ ping-pong mechanism for its catalysis because of two lines of evidences. In ping-pong mechanism, the first substrate binds to the native enzyme (E) and a part of it is transferred to the active site to form a modified enzyme (E*) while the other part is expunged. The second substrate then reacts with the modified

enzyme to regenerate the native enzyme. First set of evidences come from kinetic studies with human and rat kidney GGTs. Here, parallel Lineweaver-Burk plots that are diagnostic of ping-pong catalytic mechanism have been observed when one of the substrate is held at a fixed concentration (Tate and Meister, 1974; Elce and Broxmeyer, 1976; Karkowsky et al., 1976; Stromme and Theodorsen, 1976; Huseby, 1977; Shaw et al., 1977, 1978). The second set involves a number of experiments that show the presence of a modified enzyme in the form of an acyl-enzyme complex.

Acyl-enzyme intermediate

The formation of the γ-glutamyl-enzyme complex has been demonstrated by chemical, kinetic and crystallographic methods. Treatment with glutamine analogs like 6-diazo-5-oxo-L-norleucine (DON) (Tate and Meister, 1977) and *O*-diazoacetyl-L-serine (azaserine) (Smith et al., 1995) inactivates the enzyme by covalent and stoichiometric binding to the γ-glutamyl site. Thus the reaction is assumed to proceed by nucleophilic attack on the amide bond resulting in a tetrahedral transition state. The collapse of the tetrahedron leads to the formation of the γ-glutamyl-enzyme complex concomitant with the expulsion of the leaving group. The free enzyme is regenerated by the reverse reaction involving a non-enzymic group as the nucleophile. Catalysis thus proceeds in two steps, with an initial 'acylation of the enzyme' followed by it's 'deacylation'. The mechanism of hydrolysis is schematically represented below:

$$E + \text{Glu-R} \rightleftharpoons \text{E.Glu-R} \rightarrow \text{E-Glu} + R \rightarrow E + \text{Glu}$$

Formation of an intermediate is additionally supported by stop flow studies (Keillor et al., 2004). Under pre-steady-state conditions, the activity follows a biphasic pattern that has been interpreted to represent a faster acylation step followed by a rate limiting deacylation step. The putative intermediate has been confirmed by the crystallographic studies on *E. coli* GGT, wherein γ-glutamylation of the active site was observed in glutathione soaked crystals (Okada et al., 2006).

Catalytic nucleophile

Treatment with labeled DON results in the localization of the radioactivity in the light chain, thus mapping the site of covalent attachment (Tate and Meister, 1977). Furthermore, the attachment was found to be through O-ether bond indicating the involvement of the side chain hydroxyl group of either a Ser or Thr residue. Participation of a critical hydroxyl group is in agreement with the inhibitory effect of serine-borate complex (Tate and Meister, 1978). In borate buffer, the affinity of L-serine for the γ-glutamyl binding site is greatly enhanced and results in competitive inhibition. Borates are known to form reversible complexes with vicinal hydroxyl groups. Therefore, the affinity of L-serine is believed to be due to a borate-bridge between its side chain and a hydroxyl group in the active site. The nucleophilic residue was finally identified by trapping it with a mechanism based inhibitor *viz.,* 2-amino-4-fluorophosphono-butanoic acid (Inoue et al., 2000). The treatment results in phosphonylation of a Thr residue that occurs at the N terminus of the light chain. This residue is conserved in all the GGTs. The candidacy of the N-terminal Thr is in agreement with the co-crystal structure of the *E. coli* GGT where a covalent link was observed between the Oγ of the N-terminal Thr and the γ-glutamyl moiety (Okada et al., 2006).

Catalytic mechanism

Insights provided by chemical, kinetic and crystallographic studies have led to the elucidation of the mechanism by which GGT catalyzes hydrolysis and transpeptidation. The activated site chain of the catalytic Thr ($-O^-$) attacks the carbonyl carbon of the scissile bond, thus forming an unstable anionic tetrahedral transition state. The back bone nitrogen atoms of two tandem Gly residues donate hydrogen bonds to offset the negative charge developed on the carbonyl oxygen. The two Gly residues thus form an oxyanion hole. C-N bond cleavage occurs with the collapse of the tetrahedron simultaneous with the expulsion of the leaving group. The bond breakage occurs concomitantly with the protonation of the amide nitrogen by general acid catalysis (Ménard et al., 2001). The additional proton satisfies the valency of the nitrogen atom and thus stimulates it to withdraw from the amide bond. At the end of the acylation step, the γ-glutamyl moiety is retained on the enzyme by an ester link with the Thr nucleophile. The free enzyme is regenerated from the esterified nucleophile in a reverse process catalyzed by a non-enzymic nucleophile. The deacylating function is provided by a water molecule or the free amino group of the acyl-acceptor in case of hydrolysis and transpeptidation respectively. The deacylating agent is motivated to attack the ester intermediate by a general base catalyzed deprotonation (Castonguay et al., 2003). The catalytic mechanism is schematically represented in Fig. 4.

Catalytic modulators

Both inhibitors and activators have been reported for GGT. The well known inhibitors are DON (Tate and Meister, 1977), serine-borate complex (Tate and Meister, 1978), L-azaserine (*O*-diazo-acetyl-L-serine) (Keillor et al., 2004), acivicin (L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid)(Gardell and Tate, 1980), L-methionine sulphoxide (S_cS_s) (Lherbet and Keillor, 2004) and γ-phosphono diester analogs of glutamate(Han et al., 2006). These compounds have been useful in the elucidation of the catalytic mechanism. The mode of inhibition by DON and serine-borate complex was described earlier. Ser and borate associate through a reversible link to form the inhibitory complex. It was believed that a permanent link might improve the affinity of the inhibitor and thus a boronate derivative of glutamine was designed and synthesized (London and Gabel,

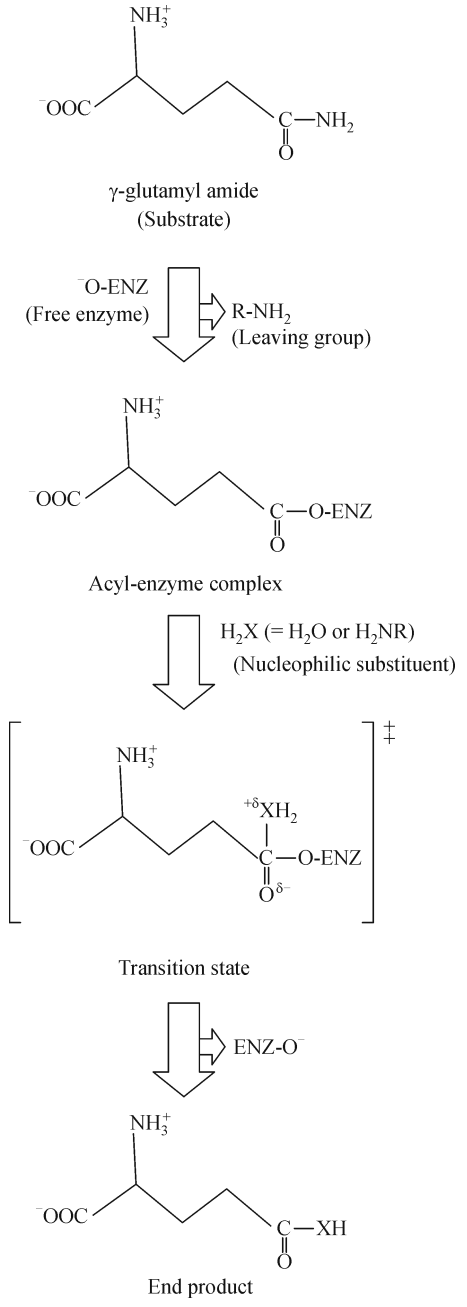

Figure 4 Schematic representation of the mechanism of GGT catalyzed hydrolysis of a γ-glutamyl amide.

2001). As predicted, the affinity of the derivative was improved from 20μM to 17nM. Like DON, γ-phosphono diester derivatives of glutamate inactivate the enzyme by forming a complex with the Thr nucleophile. The sulphoxide moiety of methionine sulphoxide mimics the gamma carbonyl of the substrate and thus interacts with the oxyanion hole to form a reversible complex (Lherbet and Keillor, 2004). High-through-put screening of a small molecule library against the human GGT resulted in the discovery of N-(5-(4-methoxybenzyl)-1, 3, 4-thiadiazol-2 yl)benzenesulfonamide (King et al., 2009) . Unlike the foresaid examples, this molecule inhibits the acyl-acceptor site rather than the acyl-donor binding site. Also, this molecule is 150 times less toxic than the GGT inhibitor acivicin on dividing cells. It is interesting to note that the inhibitory effect of the benzenesulfobamide is species-specific as it is weakly effective against rat, mouse, pig and monkey GGTs. The species-specificity indicates that the chemistry of the Acyl-Acceptor binding site is weakly conserved.

Kinetics of the rat GGT catalyzed hydrolysis and transpeptidation is affected by free bile acids and their glycine/taurine conjugates (Gardell and Tate, 1983). Both reactions are stimulated by free bile acids (cholate, chenodeoxycholate and deoxycholate), supposedly by inducing conformational change upon binding to an allosteric site. In contrast, conjugated bile acids, like maleate and hippurate, stimulate hydrolysis but inhibit transpeptidation. It is believed that the site at which these inhibitors bind partially overlaps with the substrate binding site. Free bile acids and conjugated bile salts predominantly occur in bile duct and intestine, where significant amounts of GGT also occur. The differential effect of maleate on the hydrolytic and the transfer activities appears to originate from the increased availability of water for deacylation of the γ-glutamyl-intermediate (Tate and Meister, 1974). The improvement of the hydrolytic reaction apparently inhibits the transpeptidation reaction. However, the effect is slightly different when glutamine, which is a poorer substrate than glutathione, is used in the reaction. Maleate stimulates the hydrolysis of glutamine by > 10 fold while its transpeptidation to hydroxylamine is enhanced by ~5 fold. The increased activity on glutamine is believed to reflect the facilitated binding of an otherwise unfavorable substrate. This explains the maleate induced increase in the inactivation rate of γ-glutamyl analogs like DON. Hippurate and its derivatives are also capable of uncoupling the hydrolytic and the transpeptidation reactions (Thompson and Meister, 1980).

Physiology and pathology

GGT deficiency in humans and mice

GGT deficiency is a rare autosomal recessive disease and has been documented in 5 patients (Zhang et al., 2005). The affected patients show increased concentration of glutathione in blood (glutathionemia) and urine (glutathionuria) and also mental retardation. GGT knockout mice have been developed by embryonic stem cell technique (Lieberman et al., 1996). The mutant mice are normal at birth but grow slowly, develop cataracts, are half the weight of the wild type, produce gray instead of agouti fur and fail to develop sexually. In the deficient mice, glutathione level in plasma and urine were increased by 6-fold and 2500-fold respectively while the plasma Cys level was just 20% of the wild type.

Physiological functions

Many lines of evidences point to glutathione as the likely physiologic substrate of mammalian GGTs. Glutathione (γ-Glu-Cys-Gly) is a thiol peptide occurring ubiquitously in eukaryotic cells at levels as high as 0.5–10 mM (Mesiter and Anderson, 1973). The nucleophilic character of the cysteinyl thiol enables glutathione (GSH) to react with metabolically produced oxidants and electrophiles. GSH removes oxidants like hydrogen peroxide by reacting with them to form diglutathione. The latter molecule is formed by the cross-linking of cysteinyl residue of two glutathione peptides by a disulphide bond. The cysteinyl thiol of GSH also reacts with electrophilic compounds to form their respective complexes called glutathione-S-conjugates (Zhang et al., 2005):

$$2GSH + H_2O_2 \rightarrow GS = SG + 2H_2O$$

$$GSH + \text{electrophile} \rightarrow \text{GS-conjugate}$$

The physiologic availability of Cys, the critical residue in the glutathione peptide appears to be limited (Meister, 1973). Mice fed with protein deficient diet have lower levels of glutathione but higher levels of GGT activity. The deficit can be remedied by nutritional supplementation with methionine. These findings demonstrate the dietary importance of sulfur containing amino acids in maintaining the homeostatic levels of glutathione and the role of GGT in the process. However, the mechanism by which GGT contributes to glutathione homeostasis is not clear. GGT was proposed to mediate the transmembrane transfer of amino acids by participating in the 'γ-glutamyl cycle' (Fig. 5) (Meister, 1973). The cycle begins with the transfer of the γ-L-glutamyl moiety from glutathione to any amino acid (other than proline) to form γ-L-glutamyl-amino acid. It was assumed that γ-glutamylation somehow facilitates the transport of the amino acid into the cytoplasm. In this view, glutathione serves as a donor of the vital glutamyl residue. While in the cytoplasm, the glutamylated amino acid forms 5-oxoproline with concomitant release of the amino acid. Glutamic acid is then reformed from 5-oxoproline (= pyroglutamic acid) for reuse in the synthesis of glutathione. The predominant localization of GGT in the epithelia that are active in the absorptive function apparently supports the putative role in the amino acid absorption.

GGT also appears to be involved in the formation of mercapturic acids (N-acetyl S-substituted cysteine derivative) from glutathione (Hinchman et al., 1991). Mercapturic acid derivative improves the solubility of xenobiotics, thus enabling their removal by the excretory system. Biosynthesis of mercapturic acids begins in the cytoplasm of the livers cells by conjugation with glutathione, a reaction catalyzed by glutathione *S*-transferase. The conjugates are then transported into the extracellular space where they are converted into cysteine *S*-conjugates by membrane bound GGT and dipeptidases. These trimmed conjugates are then returned to the cytoplasm for acetylation by N-acetytransferases.

The role of GGT in plant tissues is still unclear as there is no evidence for the presence of the γ-glutamyl cycle in plant tissues (Leustek et al., 2000). Furthermore, some plant GGTs have lower affinity for glutathione under in vitro conditions. The enzyme is speculated to participate in the biosynthesis of γ-glutamyl dipeptides that are formed during fruit ripening and accumulate in storage tissues such seeds and bulbs in certain plants (Ishikawa et al., 1967; Kean and Hare, 1980; Kasai et al., 1982; Kawasaki et al., 1982). There are indications that GGT in onion catalyzes the last step in the formation of the precursors of the volatile compounds (Martin and Slovin, 2000). Studies with suspension cultures of tobacco cells indicate the potential participation of GGT in glutathione catabolism (Storozhenko et al., 2002). Likewise, little is known about the physiologic role of bacterial GGTs. In *E. coli,* inactivation of *ggt* gene impairs the cells from utilizing glutathione as a nitrogen source (Suzuki et al., 1993). *B. subtilis* GGT plays a role in deriving nitrogenous nutrition during limiting conditions. The bacterium produces mucila-

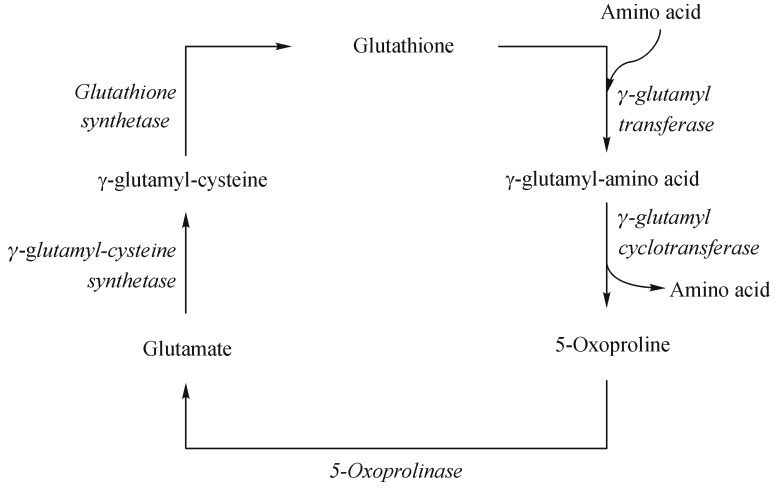

Figure 5 Schematic representation of γ-glutamyl cycle.

ginous poly-γ-glutamic acid during the log phase and releases it into the external medium. The polymer is consumed as a source of nitrogen during the late stationary phase, at which time expression of the GGT is also at its peak. Knock out of the GGT gene incapacitates the bacterium from consuming poly-gamma-glutamic acid (Kimura et al., 2004).

Involvement in diseases

GGT has been implicated in many physiologic disorders like neurodegenerative diseases (Sian et al., 1994), inflammation (Singh et al., 1986), diabetes (Lee et al., 2003) and cardiovascular diseases (Hashimoto et al., 2001). Over-expression of GGT is often observed in human tumors. There are indications that overexpression of the enzyme plays a role in tumor progression (Hanigan, 1995) and in expression of malignant phenotypes of cancer cells, such as drug resistance (Pompella et al., 2006) and metastasis (Obrador et al., 2002; Ortega et al., 2003; Benlloch et al., 2005).

Brugia malayi, a lymphatic filarial parasite, causes tropical pulmonary eosinophilia (TPE), a disease characterized by the inflammation of the respiratory epithelium (Gounni et al., 2001). The filarial GGT functions as an allergen to induce humoral response which subsequently cross-reacts with the homolog present on the pulmonary epithelium of the host. The molecular mimicry between the filarial and the human homologs forms the basis for the autoimmune response that occurs during the filarial infection.

GGT appears to be involved both positively and negatively in the physiology of oxidative stress (Whitfield, 2001). The enzyme protects the cells against oxidative effects by its involvement in the synthesis of glutathione. Induction of oxidative stress results in upregulated expression of GGT. Surprisingly, GGT also appears to generate prooxidant effects. The thiol of Cys-Gly (leaving group) is more reactive than that in the parent molecule. The enhanced reactivity promotes the reduction of ferric ion Fe (III) to ferrous ion (II), thus starting a redox-cycling process that could ultimately lead to the production of reactive oxygen species (ROS) and thiyl ($-S^{\bullet}$) radicals.

Helicobacter pylori, a gram negative spiral bacterium, causes gastritis, ulcers and gastric cancer in humans. GGT is expressed constitutively by *H. pylori* cells and is used as a marker for its identification. The bacterium survives by reprogramming the host immune system. GGT along with vacuolating cytotoxin were found to be the virulence factors as isogenic mutants lacking these two proteins fail to colonise mice and have lost the ability to modulate the host immunity (Oertli et al., 2013). The infection introduces double strand breaks in the genomic DNA. Chronic infection saturates the DNA repair system and leads to imprecise repair and thus the mutagenic and carcinogenic properties of *H. pylori* (Toller et al., 2011). Subsequent studies with other mice strains and piglets indicate that GGT may not be primarily necessary for colonization, but when present enhances the extent of colonisation. *Niesseria meningitidis* is a gram negative bacterium that colonises the nasopharynx of humans. It sometimes spreads into the bloodstream and reaches the cerebrospinal fluid, where it induces meningitis. The GGT produced by this bacterium appears to promote its growth by deriving cysteine from the extracellular γ-glutamyl-cysteinyl compounds (Takahashi et al., 2004).

GGT as a diagnostic marker

GGT level in the serum is measured in clinical laboratories as a marker for liver function (Whitfield et al., 1972). The diagnostic assay is based on empirical studies and has been in practise for over four decades. Though precise physiological function of the enzyme is still nebulous, large amounts of data are available on the factors influencing its activity in the serum. GGT assay is a sensitive test but lacks specificity. Many conditions like hepatitis C infection, cholestasis, pancreatitis, diabetes, obesity, excessive-alcohol intake and enzyme-inducing drugs can elevate serum GGT. GGT is particularly sensitive to alcoholic liver diseases and is elevated in a large proportion of alcoholics. The levels are lower in 'moderate' and occasional drinkers than in those consuming potentially hazardous volume. Due to this correlation, GGT is useful as a marker for monitoring alcohol deaddiction (Scouller et al., 2000). Many attempts have been made to correlate abnormal serum GGT with drink-drivers (drivers with non-permissible levels of blood alcohol potentially leading to debilitating driving skills due to neurological impairment) with the objective of restricting the issue of licenses to safe drivers. A significant correlation has been noticed in studies conducted in Germany, Scotland and Norway (Dunbar et al., 1985; Gjerde et al., 1986; Niederau et al., 1990). The lack of specificity restricts the use of GGT test for decision on individuals. The mechanism leading to the rise in serum GGT is not well understood. The rise in case of obstructive jaundice is mostly due to solubilization of the membranes by bile salts, while it is due to enhanced expression in the case of alcoholism (Gardell and Tate, 1980).

Relationship between GGT and cephalosporin acylase

Cephalosporin acylases (CA) are Ntn hydrolases that are employed in the pharmaceutical industry to convert the naturally produced cephalosporin-C into 7-amino-cephalsoporanic acid (7-ACA). The latter product is used as the parent molecule in the manufacture of clinically used semi-synthetic cephalosporin antibiotics. Cephalosporin acylases are classified into five categories. Acylases of class I, II and III employ Ser as the catalytic nucleophile while Class IV acylases uses Thr as in GGTs. Class I-IV acylases are heterodimers while the class V acylase is a single chain protein. Homology

Table 1 Physiological role of some GGT members

Organism	Function	Reference
Mammals	Homeostasis of glutathione	Mesiter and Anderson, 1973
	Renal reabsorption of cysteine	Meister, 1973
	Detoxification of xenobiotics	Hinchman et al., 1991
Bacillus subtilis	Depolymeration of polyglutamic acid into glutamic acid for nutritional need	Kimura et al., 2004
Helicobacter pylori	Colonization of gastro-intestinal mucosa	Oertli et al., 2013
Neisseria meningitidis	Colonization of brain	Takahashi et al., 2004
Allium sepa (Onion)	Production of volatile compounds	Martin and Slovin, 2000

modeling studies indicates that the class V enzyme assumes the α/β fold and not the αββα fold, that is diagnsotic of Ntn hydrolases (Boanca et al., 2007). Class IV acylases and GGTs have similarities in the amino acid sequence and the catalytic function. The amino acid sequence of class IV CAs and bacterial GGTs are identical by about 30% and the enzymes are active on both CA and GGT substrates. In *E. coli* GGT, substitution of Asp-433 (which bind the α-amino group of the substrate) with Asn abolishes the transferase activity and diminishes the hydrolytic function by 13% but imparts cephalosporin acylase activity which was hitherto absent (Suzuki et al., 2004). It is believed that class IV acylases are primarily GGTs with an adventitious CA activity as the affinity for the GGT substrate is usually higher than that for the CA substrate (Li et al., 1999).

Conclusions

Despite extensive interest in GGTs for over three decades, not much is known about the basis for its conservation across the living system. Extensive conservation of an enzyme is possible only if the cognate function is likewise conserved. Comparative analysis by multiple sequence alignment indicates extensive similarities between bacterial, yeast, mammalian and plant GGTs (Fig. 1). However, there are marked differences in the chemistry of the reactions catalyzed by the homologs. The transferase activity catalyzed by mammalian GGTs is nearly 100 times stronger than the corresponding hydrolytic activity. Available evidences tend to indicate that mammalian homologs utilize the transferase function to maintain homeostatic levels of glutathione. In contrast, the transferase activity catalyzed by bacterial homologs is relatively weak. Their Km for Acyl-Acceptors is usually high.

Table 1 lists GGTs whose physiologic function is known. The potential physiological role of bacterial GGTs is still nebulous. Glutathione occurs in gram negative bacteria but is completely absent in gram positive bacteria like *B. subtilis*. Therefore, glutathione is not the universal physiologic substrate for GGTs. The homolog from *B. subtilis* and *N. meningitidis* are involved in deriving amino acids under conditions of nutritional limitation. *B. subtilis* GGT appears to accept large polymers like polyglutamic acid as a substrate. Then there are the aberrant GGTs like cap-D and GGT rel, which show extensive sequence similarity to the GGTs but do not act on the standard assay substrate. Furthermore, class IV cephalosporin acylases are active on both glutamyl and glutaryl (cephalosporin) substrates. The differences in the physiological role and the variations in the substrate specificity compel the assumption that the GGT family is structurally homogenous but functionally heterogeneous.

Compliance with ethics guidelines

Sharath Balakrishna and Asmita Prabhune declare that they have no conflict of interest.

This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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