

# Gamma-Glutamyl Transpeptidases: Structure and Function

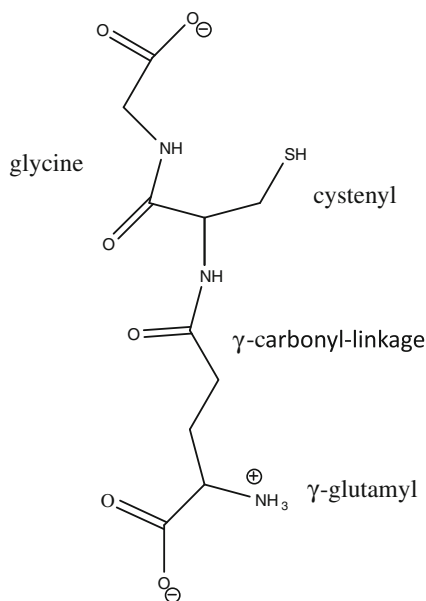
**Abstract** Gamma-glutamyl transpeptidases ( $\gamma$ -GTs) belong to the N-terminal nucleophile hydrolase superfamily, enzymes that cleave the  $\gamma$ -glutamyl amide bond of glutathione to give cysteinylglycine. The released  $\gamma$ -glutamyl group can be transferred to water (hydrolysis) or to amino acids or short peptides (transpeptidation).  $\gamma$ -GT plays a key role in the gamma-glutamyl cycle by regulating the cellular levels of the antioxidant molecule glutathione, hence it is a critical enzyme in maintaining cellular redox homeostasis.  $\gamma$ -GT is upregulated during inflammation and in several human tumors, and it is involved in many physiological disorders related to oxidative stress, such as Parkinson's disease and diabetes. Furthermore, this enzyme is used as a marker of liver disease and cancer. This book covers the current knowledge about the structure–function relationship of  $\gamma$ -GTs and gives information about  $\gamma$ -GT applications in different fields ranging from clinical biochemistry to biotechnology and biomedicine.

**Keywords** Gamma-glutamyl transpeptidase • Glutathione • Autoproteolytic activation • Gamma-glutamyl hydrolase • Biotechnological applications

## 1 Introduction

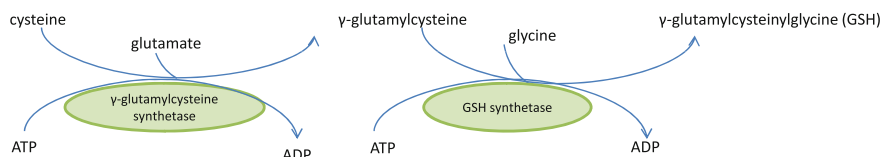
Glutathione (GSH,  $\gamma$ -L-glutamyl-L-cysteinylglycine) is a tri-peptide present in all mammalian tissues at 1–10 mM concentrations. It is the most abundant antioxidant molecule in cells and is involved in several crucial cellular functions, such as redox signaling, detoxification of xenobiotics and/or their metabolites, modulation of cell proliferation, apoptosis, and fibrogenesis. GSH is also a key determinant of nitric oxide (NO) and cysteine storage and transport, of sulfur assimilation, protection of cells against oxidative stress [1]. GSH cannot be hydrolyzed by general peptidases, because it contains an unusual peptide bond linking glutamate and cysteine through the  $\gamma$ -carboxyl group of glutamate rather than the conventional  $\alpha$ -carboxyl group (Fig. 1).

**Fig. 1** Structure of glutathione. The amino-terminal group of cysteine is linked to the  $\gamma$ -carboxyl group of glutamate

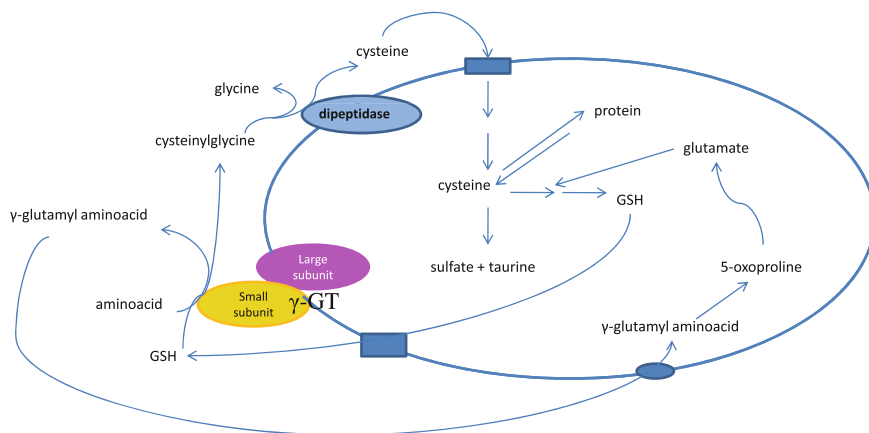


In mammalian, GSH biosynthesis [2] occurs through two ATP-dependent reactions, usually involving distinct enzymes such as glutamate-cysteine ligase, also known as  $\gamma$ -glutamylcysteine synthetase and glutathione synthetase (Fig. 2). The first step is catalyzed by  $\gamma$ -glutamylcysteine synthetase, whose structure is composed of heavy (or catalytic) and small (modifier) subunits, which are encoded by different genes in humans. This step conjugates cysteine with glutamate and generates  $\gamma$ -glutamylcysteine. In the second step, glutathione synthetase (GSH synthetase) leads to the production of GSH from  $\gamma$ -glutamylcysteine and glycine. In humans, GSH synthetase is a homodimer.

The  $\gamma$ -glutamyl cycle utilizes GSH as a continuous source of cysteine for the cell (Fig. 3). It is synthesized in the cytosol and is then translocated out of the cell. Extracellular GSH metabolism is initiated by the  $\gamma$ -Glutamyl Transpeptidase ( $\gamma$ -GT, EC 2.3.2.2), the first enzyme of the GSH degradation pathway, and then completed by membrane dipeptidases (Fig. 3).



**Fig. 2** GSH synthesis. GSH synthesis occurs via a two-step ATP-requiring enzymatic process. The first step is catalyzed by  $\gamma$ -glutamylcysteine synthetase. The second step is catalyzed by GSH synthetase. GSH exerts a negative feedback inhibition on  $\gamma$ -glutamylcysteine synthetase



**Fig. 3**  $\gamma$ -glutamyl cycle. GSH is transported out of the cell where  $\gamma$ -GT, which is constituted by a large and a small subunit, transfers the  $\gamma$ -glutamyl moiety of GSH to an amino acid (acceptor), forming  $\gamma$ -glutamyl amino acid and cysteinylglycine. The  $\gamma$ -glutamyl amino acid can then be transported back into the cell. Once inside, it can be further metabolized to release the amino acid and 5-oxoproline, which can be converted to glutamate and reincorporated into GSH. Cysteinylglycine is broken down by dipeptidases to generate glycine and cysteine, which is also transported back into the cell. Most of the cysteine taken up by the cell is reincorporated into GSH while the remaining amount is incorporated into newly synthesized proteins and/or broken down into sulfate and taurine. Figure adapted from Lu [192]

$\gamma$ -GT (E.C.2.2.3.2) is an evolutionary conserved enzyme that specifically catalyzes the cleavage of the  $\gamma$ -glutamyl bond of GSH and the transfer of the  $\gamma$ -glutamyl group to water (hydrolysis), amino acids, or peptides (transpeptidation) [3, 4].

Mammalian  $\gamma$ -GT is a glycoprotein integrated in the plasma membrane with its active site facing the extracellular space, where  $\gamma$ -glutamyl moieties of GSH are likely hydrolyzed and transferred to other amino acids, leading to the formation of  $\gamma$ -glutamyl amino acids, which are then transported into the cell. The cleavage of the  $\gamma$ -glutamyl bond of extracellular GSH enables the cell to use this antioxidant compound as a source of cysteine, which in turn serves as essential substrate for intracellular glutathione and protein synthesis. Under this concern,  $\gamma$ -GT-mediated metabolism of extracellular GSH is especially critical for protein synthesis in rapidly dividing neoplastic cells [5].

In this book, we try to connect structural and functional data on  $\gamma$ -GTs from different sources, with the aim to stimulate interest and provide an overview for future research on  $\gamma$ -GTs.

## 2 Occurrence, Genes, and Gene Expression

High levels of  $\gamma$ -GT are constitutively expressed in adult animals, and enzymatic activity is relatively higher in the kidney, intestine, and epididymis [6]. In particular, the enzyme is the most abundant in the proximal tubule of the kidney. The

expression of  $\gamma$ -GT is regulated in a tissue-specific manner and is closely related to differences in glutathione metabolism. In general, the enzyme is often expressed in tissues that are involved in the transport of different biological compounds, and the activity of the enzyme is relatively high in the luminal surface of ductal tissues. In  $\gamma$ -GT-deficient mice [7], the lack of  $\gamma$ -GT leads to the excretion of large amounts of cysteine in the urine, thus suggesting that the kidney  $\gamma$ -GT plays a critical role in the recovery of cysteine from urinary glutathione. As described above, the availability of cysteine for protein synthesis and other metabolic pathways is greatly dependent on cellular GSH levels rather than on free cysteine and cystine, thus, glutathione represents a nutrient source for the cell. Indeed, the reabsorption of the cysteine from urine occurs by the action of kidney  $\gamma$ -GT. Most cells are not able to directly uptake both reduced and oxidized GSH, thus  $\gamma$ -GT makes cysteine, contained in extracellular GSH, available to cells. For this reason,  $\gamma$ -GT seems to be associated with drug resistance in some cancer cells, in which the enzyme is up-regulated [8].

GSH is synthesized by almost all mammalian cells, but the liver is the tissue which produces the largest amount. This tissue is relatively unique in terms of the absence of  $\gamma$ -GT hydrolase activity toward glutathione. In contrast to tissues such as the kidney and intestine, the activity of  $\gamma$ -GT is low and nearly undetectable in the adult liver. However, a transient increase in  $\gamma$ -GT activity is observed in the liver at the perinatal stage during development [9]. On the other hand, the activity of the enzyme in the kidney is initially low in the neonate, then the enzyme level increases during the neonate growth and reaches a maximal level at maturity. In both rats and mice, although fetal liver hepatocytes express the enzyme at significant levels,  $\gamma$ -GT expression is suppressed after birth. Together with gene suppression, the activity becomes lower and finally decreases to undetectable levels. The different profiles of  $\gamma$ -GT activity during embryonic development vary among different species. It has been proposed that the glucocorticoid hormone plays a role in the occurrence of this increase [10]. Because this transient increase in  $\gamma$ -GT activity occurs in concomitant with the active growth of cells in the fetal liver, it was proposed that two events are associated [11].

## ***2.1 Hepatic Expression of $\gamma$ -GT***

An alteration in the activity of  $\gamma$ -GT in the liver is observed during development from the embryo to the neonate and in chemical carcinogenesis. In the development of the liver,  $\gamma$ -GT activity is transiently increased at the perinatal stage, whereas it is restricted to biliary cells in the adult liver. Thus, from fetal development to the adult stage,  $\gamma$ -GT is expressed essentially in biliary cells and immature perinatal hepatocytes [9]. The expression of  $\gamma$ -GT in liver and other organs such as the kidney is distinctly regulated. Although in the kidney a large amount of mRNA is transcribed by the most proximal promoter, in biliary cells and in immature hepatocytes the gene is transcribed by different promoters [12]. During liver development, immature hepatic cells differentiate into hepatocytic

and biliary lineages. These bipotential precursor cells alter the gene expression pattern of  $\gamma$ -GT and the use of promoters during differentiation.

## 2.2 $\gamma$ -GT Gene Structure

$\gamma$ -GTs are encoded by a single gene and are translated as a unique polypeptide chain, which then undergoes an autoproteolytic cleavage into a heavy and a light chain, usually called large and small subunit, respectively (see below). The  $\gamma$ -GT gene was found as a single copy in both rat and mouse, whereas the human counterpart was found as a multicopy gene. The rodent  $\gamma$ -GT genes contain 12 coding exons, interrupted by 11 introns. The organization of the  $\gamma$ -GT gene is well conserved in rat and mouse, and multiforms of mRNA arise from the single copy gene in a tissue-specific manner. Multipromoter and splicing variations allow transcriptions of several species of mRNA for  $\gamma$ -GT [13]. In the rat, five different promoters have been identified, and, at least six transcripts, including a splicing variant, have been characterized. The tissue-specific expression of  $\gamma$ -GT is conferred by multiple promoters, even though the different transcripts share an identical coding sequence. Promoters-I, -II, and -IV are active in the rat kidney, small intestine, and epididymis, where the highest enzyme activity is found, and seem to serve for the constitutive expression of  $\gamma$ -GT. The promoter-II contains a TATA-like sequence at the position expected from the transcription startsite. These findings have revealed that the tissue-specific expression of  $\gamma$ -GT is conferred by multiple promoters, even though all the different transcripts share an identical coding sequence.

The human multigene family coding for  $\gamma$ -GT encompasses at least seven different genes [14]. Most members of the gene family are localized on chromosome 22 [15]. Human  $\gamma$ -GT (hGT) is also transcribed under the control of multipromoters, and several mRNA species have been identified. Alternative splicing and transcription from multiple genes also contribute to the diversity of the transcripts [16]. Some transcripts do not code for an entire protein sequence but for truncated forms of the protein, in which the open reading frame comprises sequences that largely consist of either large or small subunits. Translation has been found to be regulated by the 5' untranslated region of an mRNA for  $\gamma$ -GT from a human hepatocellular carcinoma cell line, HepG2 cells, and thus, the region seems to serve as a tissue-specific active translational enhancer [17]. Such a regulating mechanism for translational efficiency together with transcriptional regulation by the multipromoter system would confer more flexible control of the expression of  $\gamma$ -GT.

## 2.3 Induction of Hepatic $\gamma$ -GT

An increase in mRNA levels and enzyme activity has been found in the liver of adult animals in response to the administration or intake of xenobiotics, carcinogens, and alcohol (see also  $\gamma$ -GT in bioclinical). Drugs cause two peaks of

$\gamma$ -GT activity. The former is reversible as  $\gamma$ -GT returns to basal levels after stopping the administration. Instead, the latter is irreversible and independent from the discontinuation of the chemical. This  $\gamma$ -GT profile seems to be consistent with the Solt-Farber's initiation/promotion model of chemical carcinogenesis [18, 19]. The multistep process of liver carcinogenesis encompasses three different steps: initiation, promotion and progression, and the proceeding reversible elevation of  $\gamma$ -GT activity corresponds to the initiation step. At this state,  $\gamma$ -GT-positive foci and hyperplastic nodules are observed in the liver, and it is thought that such histological alterations can be extinguished by redifferentiation of the cells and tissue remodeling. The late irreversible increase in enzyme activity seems to be an event associated with carcinogenesis. Besides a number of chemical carcinogens, other chemical compounds are also able to induce the hepatic expression of  $\gamma$ -GT. For example, phenobarbital, polychlorobiphenyls, and a choline-deficient/low-methionine diet promote the initiation/promotion model in the liver [18, 19].

## 2.4 $\gamma$ -GT-Related Gene Products

On the basis of primary structure similarity, an enzyme structurally related to  $\gamma$ -GT has been identified in humans. This enzyme, named as glutamyl-leukotrienase, catalyzes the hydrolysis of glutathione and a glutathione-conjugate, leukotriene. However,  $\gamma$ -glutamyl-*p*-nitroanilide, the synthetic substrate widely used in the assay of  $\gamma$ -GTs (see enzymatic assays), does not serve as a substrate for this enzyme. Glutamyl-leukotrienase from mouse seems to be very similar to its human counterpart. However, the enzymatic activity of these  $\gamma$ -GT-like proteins are different in terms of substrate specificity (e.g., the mouse enzyme is not capable of hydrolyzing GSH). Glutamyl-leukotrienase is abundant in the spleen and is involved in the inflammatory response. The discovery of structurally and functionally related  $\gamma$ -GT-enzymes has led to the suggestion that  $\gamma$ -GT is not only involved in glutathione metabolism but that it can play other roles to finely respond to different biological processes. Indeed, it has been reported that  $\gamma$ -GT, despite its enzymatic activity toward GSH, can induce osteoclast formation in bone marrow cultures [20].

## 3 Structure and Function of $\gamma$ -GTs

The first  $\gamma$ -GT was isolated from sheep kidney in 1950 [21]. Today the primary structures of  $\gamma$ -GTs from various sources, ranging from bacteria to mammals, are known. As already mentioned,  $\gamma$ -GT polypeptide chain undergoes an autoproteolytic cleavage into a large and a small subunit. Sequence alignments point to a strong conservation of structure and function. Indeed, these enzymes share >25 % identity, with the sequence of the small subunit slightly more conserved than that

of the large subunit (Figs. 4 and 5) [22]. From a multiple alignment of 501  $\gamma$ -GT sequences, about 80 residues appear highly or partially conserved. Part of these residues is responsible for the correct protein folding, others are essential for substrate recognition and needed to maintain the hydrolase activity, and other residues are conserved for unknown aspects. Among the conserved positions, some important residues have been identified: (i) a Thr (T391 in *Escherichia coli*  $\gamma$ -GT, EcGT; T381 in human GT, hGT), the nucleophile responsible for both the auto-proteolysis and the enzymatic activity and (ii) two Gly residues (G483-G484 in EcGT, G473-G474 in hGT) proposed to have a role in the stabilization of the tetrahedral transition state of the enzyme (Fig. 4) [22, 23].

The molecular weights of the two chains are generally found to be within 38–72 kDa for the large subunit and within 20–66 kDa for the small subunit [24–27]. These variations in the molecular masses are due to the high glycosylation levels of the animal and plant enzymes [28–30]. Glycosylation is believed to confer protection against proteases. In mammalian, the large subunit has an intracellular N-terminal sequence, a single transmembrane domain anchored to membrane by a hydrophobic fragment of eight residues and a large extracellular domain that binds the small subunit. The extracellular component can be separated from the membrane by papain treatment [31]. The hydrophobic anchor can be removed without affecting the enzyme activity [32]. Bacterial homologs occur as soluble proteins, localized in the periplasmic space [25] by an N-terminal signal peptide or secreted in the extracellular space [33].

hGT has a variety of isoforms that differ in their carbohydrate content and structure. It contains seven N-glycosylation sites [34]: six sites in the large subunit (N95, 120, 230, 266, 297, and 344) and one in the small subunit (N511). Four N-glycosylation sites (N95, 120, 344, and 511) are highly conserved among eukaryotes, two additional ones (N230 and 297) are conserved among mammalian  $\gamma$ -GTs. The remaining N-glycosylation site (N266 in hGT) is not conserved: for example, it is not present in pig and rat. C50 and C74, and C192 and C196 most likely form disulphide bonds. The C192–C196 pair is only found in a subset of mammalian  $\gamma$ -GTs [35], whereas the C50–C74 pair is highly conserved in non-bacterial homologs. Since these residues are not involved in the catalytic mechanism, they could have a structural role and/or a regulatory function [23].

X-ray crystallography has played a crucial role in the study of  $\gamma$ -GTs, providing researchers with detailed structural models that have been essential to unveil the enzyme function and reactivity. Until now, structural investigations of  $\gamma$ -GTs have been mostly concentrated on bacterial enzymes, with just 15 structures deposited in the Protein Data Bank (PDB, Table 1), probably because of difficulty in crystallization of the heavily and heterogeneously glycosylated mammalian proteins. Okada et al. [22] published the first crystal structure of a  $\gamma$ -GT in 1996, that is, the structure of EcGT (PDB code 2DBU). EcGT crystals were grown from 15 % PEG 4,000, 0.2 M magnesium sulfate, 5 % glycerol, at pH 5.0 and at a temperature of 277 K. They were shown to diffract to a resolution of 1.95 Å. Since then, the homologous proteins from *Helicobacter pylori*, HpGT (PDB code 2NQO) [23], and from *Bacillus subtilis* GT (BsGT) (PDB code 2V36) have also been

hGT	TAHLSVVAEDGSAVSATSTINLYFGSKVSRSPVSGILFNEMDDFSS-PSITNEFGVPPSP	439
ratGT	TAHLSVVEDGSAVAATSTINLYFGSKVLSRVSGILFNEMDDFSS-PNFTNQFGVAPSP	438
<i>D. rerio</i> GT	TAHLSVIAEDGSAVAATSTINLYFGSKVMSRSTGIIFNDEMDDFSS-PYITNGFGVPPSP	359
<i>D. mel</i> aGT	TAHMNVLATNGDAVSITSTINNYFGSKVASTQTGIILNDEMDDFST-PGVINGFGVPASP	440
<i>S. cer</i> GT	TAHFSIVDSHGNAVSLTTTINLLFGSLVHDPKTVIFNNEMDDFAQ-FNKSNSFELAPSI	528
<i>Ec</i> GT	TTHTSVVDKDGNAVAVTYTLNTTFTGTGIVAGESGILLNNQMDDFSAKPGVPPNVYGLVGGD	450
<i>Hp</i> GT	TTHTSVADRWGNVSVTYTINASYGSAASIDGAGLNNEMDDFSAIKPGPNPLYGLVGGD	439
<i>Bs</i> GT	TTHTVTADRWGNVSVYTTTIEQLFGTIGIMVPDYGVILNNELTDFDA-----IPGG	452
<i>Bl</i> GT	TTHTVTVDQWGNVSVYTTTIEQLFGTGILVPGYGLFLNNELTDFDA-----IPGG	448
<i>Gth</i> GT	TTVYLAADDEGNNMVFSIQSNYMGFGSGLVVPGTGIALHNRGHNFFV-----DENH	402
<i>Dr</i> GT	TVYLAADDEGGMVSMIQSNYMGFGSGVVVPGTGIALHNRGHNFFHT-----DPAH	402
<i>Tt</i> GT	TVYLAADGE--VMVSLIQSNYQGFSGSVLVVPGTGIALQNRGLGFSL-----EEGH	394
<i>Ta</i> GT	TTYFSISDSEGRSVSIIQSNYMGFGSGVIVPKGTGFVLQNRGSYFTL-----QRDH	380
*		
hGT	ANFIQPGKQPLSSMCPTIMVQDQGQVRMVVGAAGGTQITTATALAIYINLWFGYDVKRAV	499
<i>rat</i> GT	ANFIKPGKQPLSSMCPSTIIVDKDGKVRMVVGAAGGTQITTSVALAIINSLWFGYDVKRAV	498
<i>D. rerio</i> GT	NNFIQPGKRLSSMCPSTIIFDKHNRVKMVVGASGGTKITTATALVILNSLFFNVDLKRAV	419
<i>D. mel</i> aGT	ANYIYPGKRPMSSMSCPIIVDQEGNVRLVGAAGGTRITTSVAAMVIMKYLRLKESLTAAV	500
<i>S. cer</i> GT	YNPPEPGKRLSSSTAPTIVLSELGIPDLVVGASGGSRITTSVLQTIIVRTYWNMPILETI	588
<i>Ec</i> GT	ANAVGPNKRLSSMSPTIIVVKD-GKTWLVTGSPGGSRITITVLQMVVNSIDYGMNVAEAT	509
<i>Hp</i> GT	ANAIEANKRPLSSMSPTIIVLKN-NKVFLVVGSPGGSRITITVLQVISNVIDYNNMISEAV	498
<i>Bs</i> GT	ANEVQPNKRPLSSMTPTILFKD-DKPVLTVGSPGGATIISSVLQITILYHIEYGMELKAAV	511
<i>Bl</i> GT	ANEVQPNKRPLSSMTPTIVFKD-EKPVLTVGSPGGTIIASVFQITILNYFYEGMSLQDAI	507
<i>Gth</i> GT	PNLGLAPKKPYHTIIPGFLTKGG-KPIGPFVGMGGFMQPGQHMVQIMNTVDFALNPQAAAL	461
<i>Dr</i> GT	PNALAPGKRPFYHTIIPGFLGRADGTPVGPFVGMGGFMQPGQLQVVVNTVRYGMNPQAAAL	462
<i>Tt</i> GT	PNRVGPGRKPFHTIIPGFLAREG-KPLGPFVGMGGFMQPGHVQVVVGLADFGLNPQAAAL	453
<i>Ta</i> GT	PNALMPGKRTFHTLAACMVEKEH-DLYASLGSMSGDIIQPVQMQLMEILKDNTDPQAAIL	439
**		
hGT	EEPRLHNQLLPNVTTVERN--IDQAVTAALET-----RHHHT---QIASTFIAVVQAI	548
<i>rat</i> GT	EEPRLHNQLLPNTTVEKEN--IDQVVTAGLKT-----RHHHT---EVTPDFIAVVQAVV	547
<i>D. rerio</i> GT	TEPRVHNQLNPNMTVVEQD--FEQSVLDGLEQ-----KNHVT---ELQRTPGAVVQAI	468
<i>D. mel</i> aGT	NNGRLLHQLLAPMRVSYEQE--VDSSVTDYLKQ-----VGHEMYE-EPVGSSSFAAVTAIG	551
<i>S. cer</i> GT	AYPRIHHQLLPDRIELESFPMIGKAVLSTLKE-----MGYIMK--EVFPK--SVVNPAIR	638
<i>Ec</i> GT	NAPRFHHQWLPPDELRLVEK--GFSPDTLKLLEA-----KGQKVALKEAMGSTQSIMVGPD	561
<i>Hp</i> GT	SAPRFHMQLWLPDELRLIEKF-GMPADVKNLTK-----MGYQIVTKPMVGDVNAIQVLKP	551
<i>Bs</i> GT	EEPRIYTNMSSSYRYEDG---VPKDVLSKLN-----MGHKFGT-SPVDIGNVQSISID	561
<i>Bl</i> GT	EEPRIYTNLSLTSYRYESG---MPEDVRRKLND-----FGHKFGS-NPVDIGNVQSIFID	557
<i>Gth</i> GT	DAPRWQWMEGKTVLVEPH---FPRHIAEALAR-----KGHDICVALDGGPFGRGQTIWR	512
<i>Dr</i> GT	DAPRWQWLQGRTEVEPEA---LGDQLARALVA-----RGHDVRVQLDPGSGFRGQMIRR	513
<i>Tt</i> GT	DRPRWQVVPDGEVLLPEPG---IPQATALFLKD-----LGRHVRVYEAEGYGLFGRGQVFR	504
<i>Ta</i> GT	DKPRWTETP--YTYEAPG---AVYVESEELYRNVSKQISGRKVLRDVSQEFGTQAQITTL	494
hGT	RTAGGWAAASD-SRKGGEPAGY-----	569
<i>rat</i> GT	RTSGGWAAASD-SRKGGEPAG-----	568
<i>D. rerio</i> GT	RQGDKLCAECD-PRKGGYPAGY-----	489
<i>D. mel</i> aGT	-ALEQPEPFYD-RRRIGSALTAKTNKMQH	579
<i>S. cer</i> GT	NVRGEHVAHSVYWRKRGISSVY-----	660
<i>Ec</i> GT	GE---LYGASDPRSVDDLTAAGY-----	580
<i>Hp</i> GT	TKGSVFYGSTDP--KEF-----	567
<i>Bs</i> GT	HENGTFKGVADSSRNAAIGINLKRK----	587
<i>Bl</i> GT	RENKTFMGVADSSNGTAVGVNNKTSAE--	585
<i>Gth</i> GT	DPDPTGLAAGTEPRTDGAVA--	534
<i>Dr</i> GT	DPDPTGLVLEGDESRTDGHIALW-----	535
<i>Tt</i> GT	--LGEALVAGSDPRAEGLALAW-----	524
<i>Ta</i> GT	-IRGDVVVGAAADPRGDGIAIPYS-----	516

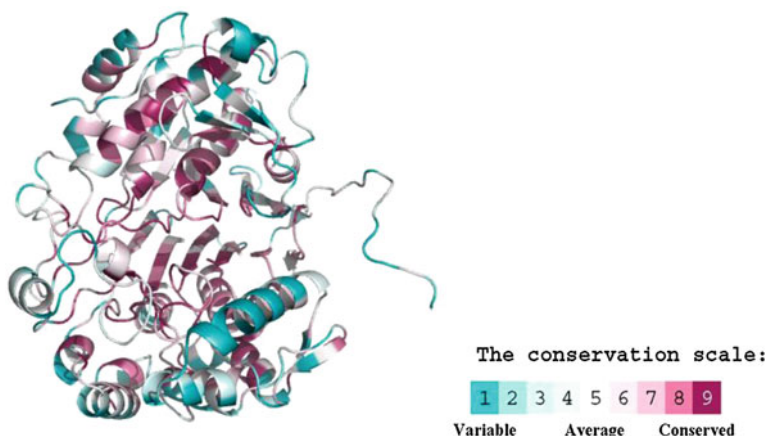
structurally characterized. The structures of the putative  $\gamma$ -GT from *Bacillus halodurans* (BhGT, PDB code 2NLZ, annotated as cephalosporin acylase) and from the thermoacidophilic archaeon *Thermoplasma acidophilum* (TaGT, PDB code 2I3O) have been deposited in the PDB without a primary citation. Furthermore, it has been recently solved the structure of CapD from *Bacillus anthracis*, a protein considered related to  $\gamma$ -GTs, but not able to hydrolyze GSH (PDB code 3G9K) [36]. Crystallization conditions of these proteins are reported in Table 2.



◀ **Fig. 4** Multiple alignment of amino acid sequences of the small subunits of some  $\gamma$ -GTs. The amino acid sequences of  $\gamma$ -GT from *H. sapiens* (Swiss-Prot P19440), *R. norvegicus* (Swiss-Prot P07314), *D. rerio* (Swiss-Prot Q7T2A1), *D. melanogaster* (Swiss-Prot Q9VWT3), *S. cerevisiae* (Swiss-Prot Q05902), *E. coli* (Swiss-Prot P18956), *H. pylori* (Swiss-Prot O25743), *B. subtilis* (Swiss-Prot P54422), *B. licheniformis* (Swiss-Prot Q62WE3), *G. thermodenitrificans* (YP001127364.1), *D. radiodurans* (Gene ID: 1798291), *T. thermophilum* (Gene ID: 3168743) and *T. acidophilum* (NP394454.1) are included. The alignment was performed using the ClustalW method (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). The conserved threonine responsible for autoprocessing of the enzyme and the two strictly conserved glycines involved in binding of the  $\gamma$ -glutamyl moiety (T391, G483, and G484 in EcGT) are indicated by an asterisk. The lid loop extending toward the active site (spanning from P438 to G449 of EcGT) and absent in some  $\gamma$ -GTs is underlined

All these  $\gamma$ -GTs exhibit a similar structure: they belong to the superfamily of the N-terminal nucleophilic (Ntn) hydrolase [37, 38]. The members of this superfamily share a common active site architecture, tertiary and quaternary fold, although they can show pairwise sequence identities as low as 15 %. The predominant elements of secondary structure in Ntn-hydrolases are a long four-layer  $\alpha\beta\beta\alpha$ -structure, with two antiparallel  $\beta$ -sheets packed against each other and sandwiched between  $\alpha$ -helical layers (Fig. 6a, b). In the central  $\beta$ -sheet sandwich, both large and small subunits provide strands. They are arranged in an antiparallel fashion: one of the two central sheets is essentially flat, whereas the other may be twisted at the end (Fig. 6b).

In the studied  $\gamma$ -GTs, the small and the large subunits are highly intertwined throughout the structure. The inter-subunit interface is stabilized by both hydrogen bonds and hydrophobic contacts. The crystal structure of EcGT has been solved



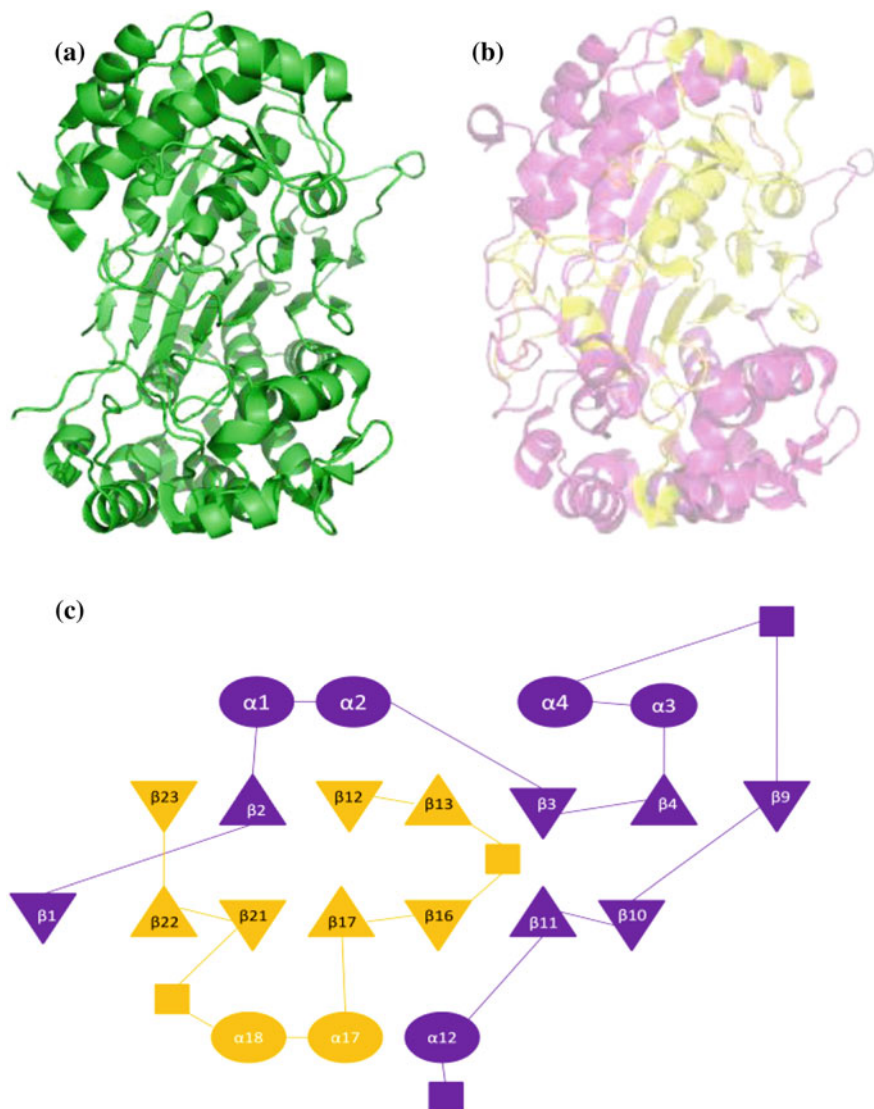
**Fig. 5** Ribbon representation of one subunit of the precursor structure of the  $\gamma$ -GT from *B. halodurans* (PDB code 2NLZ) colored by residue conservation. Residues were colored according to their evolutionary conservation score using the color-coding bar on the right, as implemented in the *CONSURF* web server (<http://consurf.tau.ac.il/>)

**Table 1** Crystal symmetry and unit cell dimensions for  $\gamma$ -GT structures

Enzyme	Structure description	PDB code	Resolution (Å)	Space group	Unit cell dimension a,b,c (Å) $\alpha, \beta, \gamma$ (°)
<i>EcGT</i>	Ligand free form	2DBU	1.95	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	78.7, 126.9, 128.8 90, 90, 90
<i>EcGT</i>	Complex with hydrolyzed GSH	2DG5	1.60	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	78.1, 126.6, 129.2 90, 90, 90
<i>EcGT</i>	Complex with Acivicin	2Z8K	1.65	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	77.7, 126.5, 129.4 90, 90, 90
<i>EcGT</i>	Complexed with L-Glu	2DBX	1.70	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	77.7, 126.5, 129.2 90, 90, 90
<i>EcGT</i>	Complex with Azaserine	2Z8I	1.65	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	77.5, 126.2, 129.2 90, 90, 90
<i>EcGT</i>	Acyl-Enzyme Intermediate	2DBW	1.80	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	78.8, 126.7, 128.9 90, 90, 90
<i>EcGT</i>	Monoclinic form	2E0X	1.95	P2 <sub>1</sub>	65.2, 127.9, 75.1 90, 94.8, 90
<i>EcGT</i>	Sm derivative	2E0Y	2.02	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	78.9, 125.9, 128.9 90, 90, 90
<i>EcGT</i>	Complex with Azaserine in the dark	2Z8J	2.05	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	76.3, 126.8, 128.1 90, 90, 90
<i>EcGT</i>	T391A mutant	2E0W	2.55	P4 <sub>3</sub> 2 <sub>1</sub> 2	134.6, 134.6, 118.4 90, 90, 90
<i>HpGT</i>	Ligand free form	2NQO	1.90	P2 <sub>1</sub>	54.3, 105.2, 91.1 90, 92.0, 90
<i>HpGT</i>	Complex with Glu	2QM6	1.60	P2 <sub>1</sub>	55.0, 104.8, 91.9 90, 91.8, 90
<i>HpGT</i>	Complex with Acivicin	3FNM	1.70	P2 <sub>1</sub>	54.7, 105.4, 91.9 90, 91.7, 90
<i>HpGT</i>	Mature T380A with S(nitrobenzyl)GSH	2QMC	1.55	P2 <sub>1</sub>	57.1, 106.7, 87.2 90, 105.0, 90
<i>BsGT</i>	Ligand free form	2V36	1.85	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	72.3, 108.8, 161.3 90, 90, 90
<i>BsGT</i>	Complex with glutamate	3A75	1.95	P2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	49.4, 98.9, 227.2 90, 90, 90
<i>TaGT</i>	Ligand free form	2I3O	2.03	P2 <sub>1</sub>	116.1, 95.5, 119.0 90, 109.8, 90
<i>BhGT</i>	Ligand free form	2NLZ	2.70	P3 <sub>1</sub> 2	105.7, 105.7, 385.1 90, 90, 120
CapD <i>B. anthracis</i>		3G9K	1.99	P2 <sub>1</sub>	52.7, 120.6, 77.4 90, 90.9, 90

**Table 2** Crystallization conditions of  $\gamma$ -GTs

Protein	Technique	pH	Buffer/Salt	Precipitant	Additive/Cryoprotectant	Temperature (K)
EcGT	Vapor diffusion	5	0.2 M Mg sulfate,	15 % PEG 4,000	5 % glycerol	277
	Hanging drop	8.5	0.2 M CaCl <sub>2</sub> , 0.1 M Tris-HCl	20 % PEG 4,000		
HpGT	Vapor diffusion	7.5	200 mM HEPES	25 % MPEG2000		291
	Sitting drop					
BsGT	Vapor diffusion	7	100 mM MES	20 % PEG 4,000, 600 mM NaCl	5 % Jeffamine M-600	293
TaGT	Hanging drop					
	Vapor diffusion	5.5	Mg Formate			293
BhGT	Hanging drop					
	Vapor diffusion	7.5	200 mM Na Formate	20 % PEG 3,350	20 % glycerol	294
	Sitting drop					



**Fig. 6** Ribbon representations of the structure of (a) precursor (green) and (b) mature form of EcGT. In the latter, small and large subunits are shown in yellow and purple, respectively. (c) A topology diagram of EcGT showing the disposition of secondary structure elements in the protein. In this diagram, circle, triangle, and square indicate  $\alpha$ -helix,  $\beta$ -strand and not conserved insertions, respectively

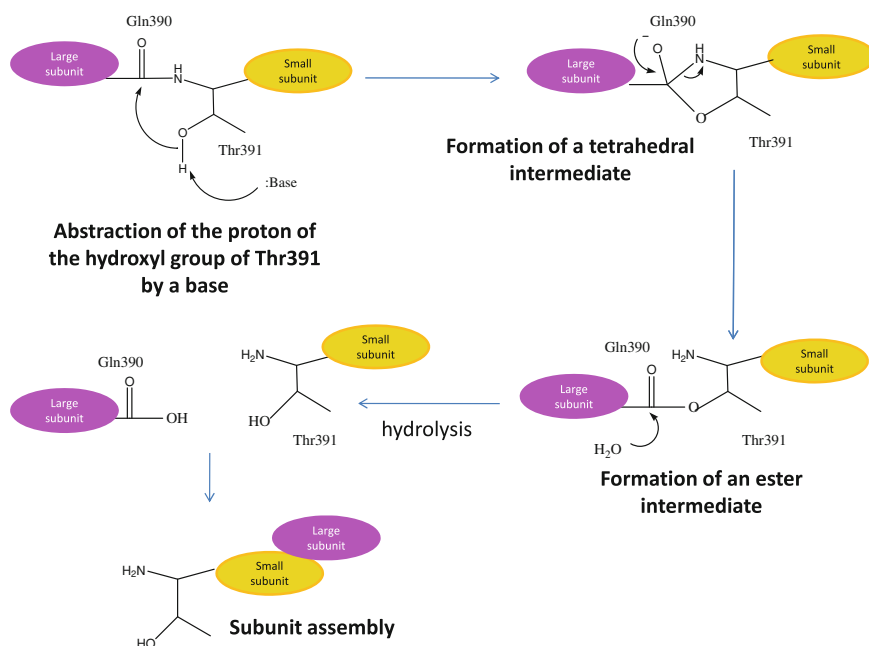
both for the unprocessed T391A mutant (PDB code 2E0W) [39] and for the mature enzyme (PDB code 2DBU) [22]. A detailed comparison of these structures is reported below.

An important contribution to the understanding of  $\gamma$ -GT function has been the elucidation of X-ray structures of EcGT and BsGT complexes with L-glutamate, which acts as substrate/product analog (PDB codes 2DBX and 3A75, respectively [22, 40]). Structures of  $\gamma$ -GTs in complex with ligands include those of HpGT with Acivicin (PDB code 3FNM) [41] and of EcGT with Azaserine (PDB code 2Z8I) or Acivicin (PDB code 2Z8K), two potent inhibitors of the protein (see later).

## 4 Autoprocessing and Reaction Mechanism of $\gamma$ -GTs

### 4.1 Autoprocessing

Members of the Ntn-hydrolase superfamily are produced as inactive proenzymes (also called precursors or zymogens) that undergo a post-translational modification to form catalytically competent enzymes. This process is a proteolytic activation that releases a catalytic serine, threonine, or cysteine at the N-terminal position [37, 38, 42] (Fig. 7).



**Fig. 7** Proposed mechanism for intramolecular autocatalytic processing of  $\gamma$ -GT from *E. coli*. A base activates Thr OG atom thereby enabling it to attack the preceding residue on C; the resultant tetrahedral transition state collapses into an ester intermediate; hydrolysis of the ester separates the two chains. Adapted from Castellano and Merlino [102]

In detail, in  $\gamma$ -GTs, the hydroxyl group of the strictly conserved threonine (T391 in EcGT, T381 in hGT) of the proenzyme serves as nucleophile for the cleavage. Thr attacks the carbonyl group of the preceding residue, which is often a glutamine (Gln390 in EcGT) or a glycine, forming a tetrahedral intermediate. The cleavage of the C–N bond through protonation of the amino group of the Thr yields an ester intermediate (N–O acyl shift), which is then hydrolyzed by a water molecule to form a large and a small subunit with the Thr as the new N-terminal residue. A crucial role in this process could be played by a water molecule, which could enhance the nucleophilicity of the hydroxyl group of the Thr [39, 43, 44]. In hGT, glycosylation is required to process the proenzyme, even though nothing is known about the role of glycosylation in the autoprocessing [34, 45].

The precursor and the mature (and active)  $\gamma$ -GTs have distinct structural features in different organisms. In mammals, as well as in many bacteria like *E. coli*, the proenzyme is a dimer of two identical monomers  $(\alpha\beta)_2$ , whereas the activated  $\gamma$ -GT is a heterodimer of the small ( $\alpha$ -) and large ( $\beta$ -) subunits (Fig. 6b) [22]. In this notation, the term  $\alpha\beta$  refers to the single unprocessed chain, whereas  $\alpha$ - and  $\beta$ - are the two chains after processing. In the case of the thermophilic enzyme from *Geobacillus thermodenitrificans* (GthGT), the inactive precursor exhibits a homotetrameric  $(\alpha\beta)_4$  structure, whereas the mature enzyme behaves as a heterotetramer  $(\alpha_2\beta_2)$  [46]. Moreover, crystal structures of both EcGT and HpGT revealed the presence of two heterodimeric molecules, that is, a heterotetramer, in the asymmetric unit [22, 23]. Interestingly, gel filtration and dynamic light scattering data on HpGT [47] also indicate the existence of heterotetrameric assemblies. The effect of oligomerization of  $\gamma$ -GTs is unknown, and its role in catalytic function and structural stabilization needs further studies. In HpGT, EcGT, and BIGT [39, 43, 44], the substitution of the conserved Thr with an Ala results in a homodimeric protein unable to undergo the autocatalytic maturation, whereas in GthGT [46] the same replacement leads to a homotetrameric proenzyme, which keeps a reduced but significant hydrolase activity. It has been suggested that this small catalytic activity might be due to a water molecule that mimics the side chain hydroxyl group of the catalytic threonine [46]. In HpGT, removal of the  $\text{CH}_3$  group of the Thr, that is, substitution of the catalytic Thr with Ser, results in a nearly 80-fold reduction in the rate of processing when compared to the wild-type enzyme. In this case, the protein needs more than 30 days for complete maturation [23, 47]. In EcGT, when Thr391 is replaced by Ser or Cys, the derived precursors are processed, albeit slowly [48].

The role of the residue preceding the Thr in the autocatalytic processing of  $\gamma$ -GTs is controversial. It was investigated by site-directed mutagenesis in the case of EcGT [49, 50] and BIGT [51]. This residue, which in BIGT is a glutamate, was substituted by either A, D, R, or Q. Analysis of SDS Polyacrylamide Gel Electrophoresis showed that the  $\text{E} \rightarrow \text{A}$ ,  $\text{E} \rightarrow \text{D}$ , and  $\text{E} \rightarrow \text{R}$  substitutions yield mutants unable to undergo cleavage. However, when glutamate was replaced by a glutamine, the mutant was able to process itself into a large and a small subunit, thus acquiring a catalytic activity comparable to that of the native enzyme [51]. On the contrary, studies on EcGT showed that Gln390 has no role in autoprocessing since its substitution with A

does not produce any maturational defect [49, 50]. Other controversial results were obtained when a His (His393 of EcGT) rather well conserved in  $\gamma$ -GTs was replaced: the H  $\rightarrow$  G substitution yields to the formation of unprocessed precursors in the case of EcGT [52], whereas the corresponding hGT mutant (H383A) forms a mature protein [53]. This residue is a Y in GthGT, BsGT, TtGT and TaGT (Fig. 4).

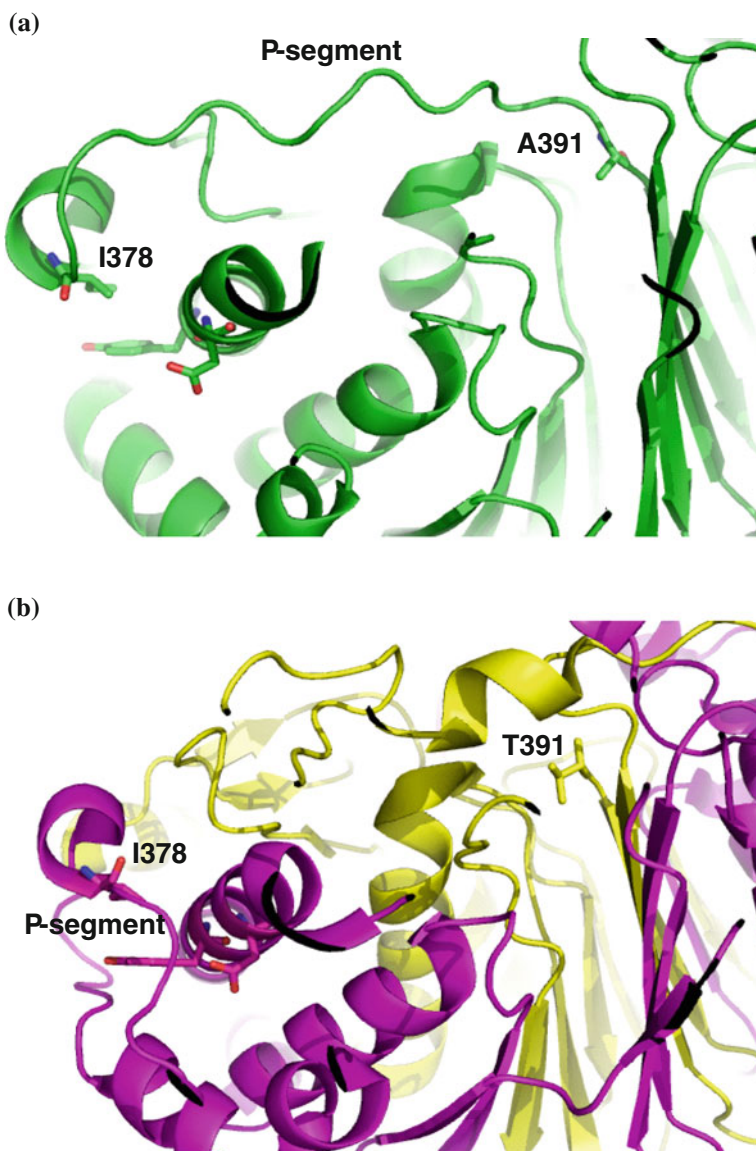
The C-terminal region of  $\gamma$ -GTs has also been demonstrated to have a critical role in their autocatalytic capability [43]. In particular, site directed mutagenesis and deletion studies on BIGT and HpGT have revealed that the C-terminus participates in an undefined way to the autocatalytic process and that the deletion of nine residues significantly affects the processing. In BIGT, V576 (P566 in hGT and T577 in EcGT) is located at the boundary [43].

Structural information about the inactive precursor has also been obtained by exploring the mutagenized native polypeptides with a prevented or delayed activation site. Comparison of the overall structure of the unprocessed T391A mutant of EcGT (PDB accession code: 2E0W) with that of the mature enzyme shows no major differences between the backbone atom positions, especially in the core regions [39]. Large changes are found near the active site: in fact, in the precursor analog, the segment corresponding to the C-terminal region of the large subunit occupies the site where a loop (residues 438–449 in EcGT, hereafter denoted as lid loop) forms the lid of the  $\gamma$ -glutamyl group-binding pocket in the mature  $\gamma$ -GT (Fig. 8). This feature suggests that, upon cleavage of the N-terminal peptide bond of T391, the newly produced C-terminus of the large subunit (residues 375–390 in EcGT), hereafter denoted as P-segment, flips out, allowing the formation of the  $\gamma$ -glutamyl group-binding pocket. The conformational variation of the P-segment can be described as a rotation about residue Ile378, which changes its  $\psi$  angle from  $45^\circ$  to  $127^\circ$ , thus allowing the displacement of the segment from the catalytic Thr (Fig. 9a, b). The rearrangement of the mobile P-segment upon the autoprocessing is favored by the formation of electrostatic interactions. The P-segment conformational changes could not be a general property of  $\gamma$ -GTs, since the position of

**Fig. 8** Superimposed structures of inactive precursor (*green*) and mature form of EcGT. In the mature enzyme, the large subunit is shown in *violet* and the small subunit in *yellow*.  $\gamma$ -glutamyl binding site, catalytic Thr, P-segment, and lid loop have been evidenced. Adapted from Castellano and Merlino [102]







**Fig. 9** Structures of inactive precursor (a) and mature form of EcGT and (b). In the mature enzyme, the large subunit is shown in *violet* and the small subunit in *yellow*. The position of catalytic important Thr (Thr391), Ile378, P-segment, and lid loop have been evidenced. In the precursor, the Thr is replaced by an Ala. To emphasize the similar orientation of the structures in the two panels, the side chains of Tyr332 and Asp336 are also shown

this segment in the large subunit of the protein from *Bacillus subtilis* is not significantly affected by the autocatalytic processing, being located close to the N-terminal region of the small subunit [40].



## 4.2 Reaction Mechanism

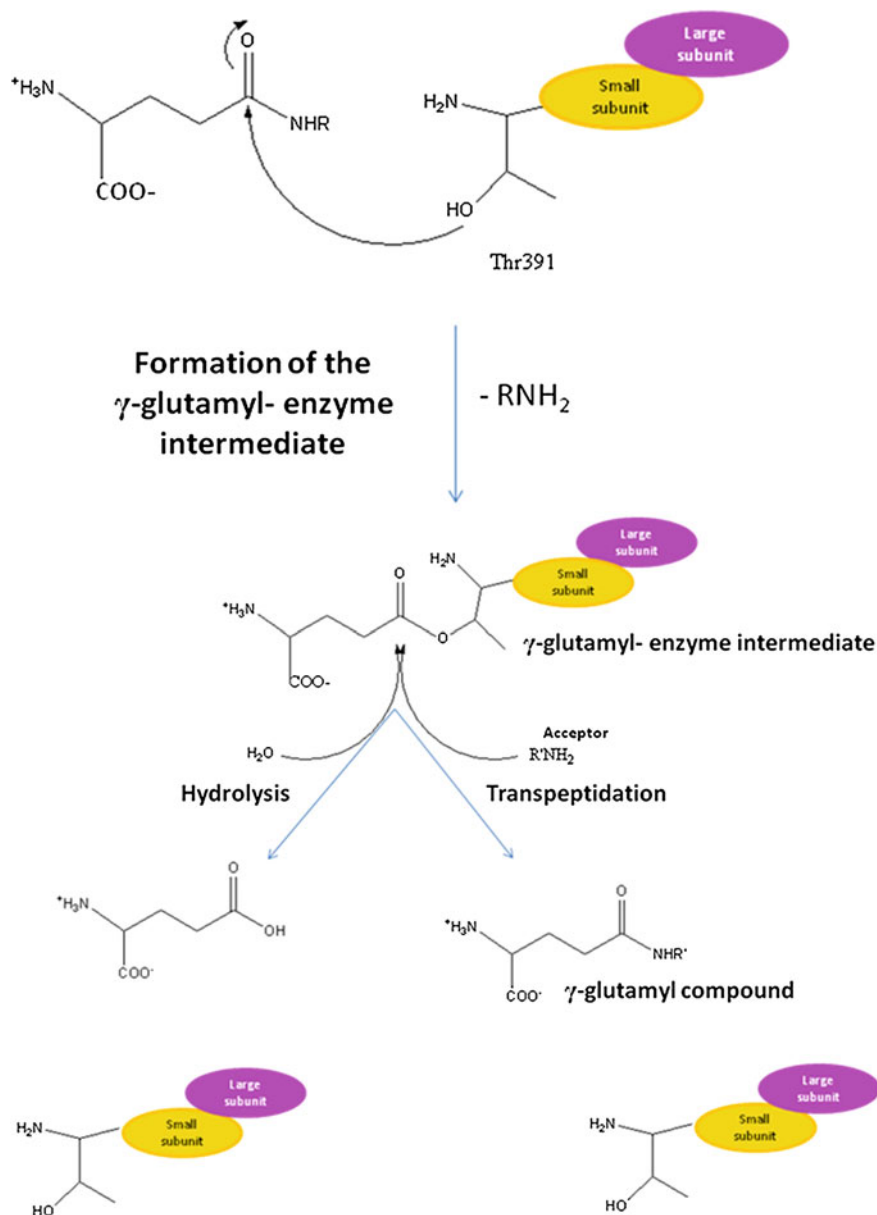
$\gamma$ -GTs catalyze the cleavage of the  $\gamma$ -glutamyl linkage of  $\gamma$ -glutamyl compounds, such as GSH and its S-conjugated, including leukotriene C4, S-nitroso-glutathione (GSNO), and GSH adducts of xenobiotics formed by the action of glutathione-S-transferases [8]. In a second step, most  $\gamma$ -GTs catalyze the transfer of the  $\gamma$ -glutamyl moiety to other aminoacids or short peptides [54].

In the most widely accepted reaction mechanism, the highly conserved Thr, which is also responsible for autoprocessing, attacks the C=O group of the  $\gamma$ -glutamyl-compound to form a  $\gamma$ -glutamyl-enzyme intermediate. The intermediate then reacts with water, to release glutamate in a hydrolysis reaction, or with an acceptor, to give a transpeptidation reaction forming new  $\gamma$ -glutamyl compounds (Fig. 10). The simplest acceptor substrates include single amino acids and glycine dipeptides (Gly-Ala or Z-Gly, where Z is W, F, Y, H, A, Q, C, M, S, L, K, A, or G).

The attack is likely promoted by one or more residues adjacent to the catalytic Thr, which can form hydrogen bonds with the catalytic Thr, increasing the reactivity of its hydroxyl group that then attacks the  $\gamma$ -glutamyl peptide [23]. Barycki and coworkers have proposed that the hydroxyl group of a second Thr, which follows the catalytic one in the sequence of the small subunit and is well conserved in the  $\gamma$ -GT family, forms two hydrogen bonds with the catalytic Thr [47]. This Thr–Thr dyad is critical for efficient cleavage of the  $\gamma$ -glutamyl peptide bond of GSH. Recombinant DNA techniques have been used to produce BIGT and HpGT variants in which either the catalytic Thr or the second Thr of the dyad are replaced with an alternative residue. These studies have shown that the former Thr is essential for the catalytic activity, since most of unprocessed proteins, in which this residue was mutated in Ala, were found almost completely inactive [39, 47], while the T  $\rightarrow$  S substitution results in a protein with catalytic activity significantly altered when compared to the native enzymes. Notably, the replacement of the latter Thr of the dyad with lysine can impair the autoprocessing [55]. To establish whether the lack of catalytic activity by the T  $\rightarrow$  A mutants is due to the unprocessing of the enzyme or to the absence of the hydroxyl group of the Thr, a co-expression system in which the processing of the enzyme was uncoupled from the catalytic activity was studied. Co-expression of the small and large subunits results in a catalytically competent enzyme [47].

## 4.3 $\gamma$ -Glutamyl Binding Site

As described above, the reaction catalyzed by  $\gamma$ -GTs proceeds via the formation of a  $\gamma$ -glutamyl-enzyme intermediate followed by nucleophilic substitution by water, amino acids, or peptides. Formation of the intermediate was demonstrated by a number of experimental evidences acquired by chemical, kinetic, and crystallographic studies. A clear picture of the  $\gamma$ -glutamyl-enzyme intermediate was



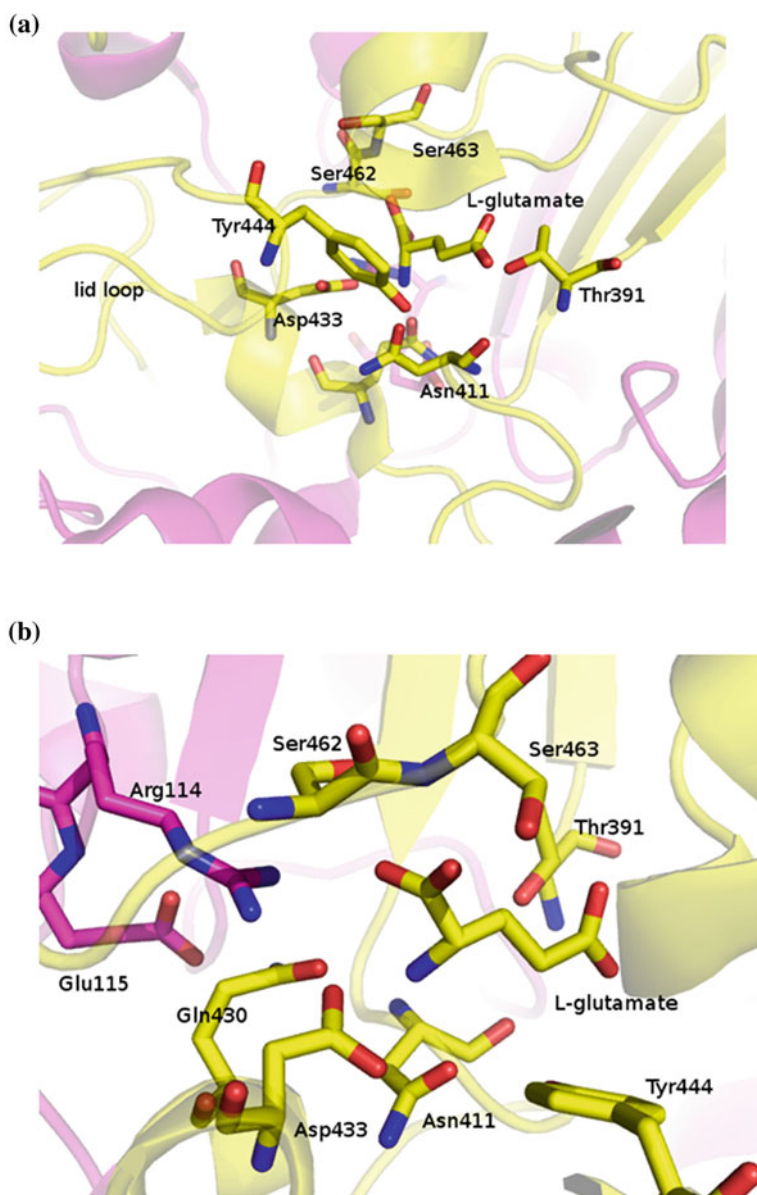
**Fig. 10** Proposed mechanism for catalysis of  $\gamma$ -glutamyl peptide cleavage by  $\gamma$ -GTs. In mature enzyme, the hydroxyl group of the N-terminal Thr residue of the small subunit, conserved in all homologous  $\gamma$ -GTs, attacks the  $\gamma$ -glutamyl peptide bond of GSH and leads to the formation of a tetrahedral transition state, whose collapse leads to the formation of a tetrahedral intermediate ( $\gamma$ -glutamyl enzyme complex). The intermediate is stabilized through interactions with two conserved glycines, and concomitant expulsion of the leaving group. A second Thr in the sequence of the small subunit can increase the reactivity of catalytic Thr. Adapted from Castellano and Merlino [102]

obtained solving the structures of EcGT in complex with GSH ( $\gamma$ -glutamyl-enzyme intermediate, PDB code 2DG5) and L-glutamate (PDB code 2DBX) (Fig. 11). The substrate-binding pocket is located at the bottom of a deep groove, close to the catalytic Thr ( $\gamma$ -glutamyl binding site) (Fig. 11). This site exhibits a scarce specificity, since it is able to accept both L- and D isomers of glutamic acid, and a number of different substrates.

When bound within  $\gamma$ -glutamyl binding site, the carbonyl group of the  $\gamma$ -glutamyl moiety is covalently linked to the OG atom of the catalytic Thr. The  $\gamma$ -glutamyl moiety is held in position by an extensive network of hydrogen bonds and salt bridges with several residues (Fig. 11). In particular, in the complex of EcGT with L-glutamate, the carboxyl group of the ligand is bonded with R114, S462, and S463, whereas the amino group interacts with N411, Q430, and D433 (Fig. 11b). The  $\gamma$ -glutamyl carbonyl oxygen is hydrogen bonded with the main-chain atoms of G483 and G484. Other known active site residues are L461, P482, and I487. Except for R114, all residues involved in  $\gamma$ -glutamyl group recognition belong to the small subunit. Mutational studies of hGT have supported the involvement of conserved Asp and Ser residues (D423, S451, and S452 in hGT) in the  $\gamma$ -glutamyl binding site formation [56, 57].

It has been proposed that when the  $\gamma$ -glutamyl binding site is occupied by a substrate (or inhibitor) the lid loop has a well-defined position and shields the catalytic pocket from the solvent, otherwise, when the pocket is empty, the lid loop is disordered. Thus, the lid loop, which is conserved in all eukaryotic  $\gamma$ -GTs, should regulate the access of the substrate to the active site or the binding of the substrate to the active site cleft. Multiple sequence alignments showed that several microbial enzymes including GthGT and those from *Bacillus subtilis*, *Bacillus halodurans*, *Thermoplasma acidophilum*, *Deinococcus radiodurans*, *Picrophilus torridus*, and *Thermus thermophilus* lack the sequences corresponding to this loop [46, 58]. In EcGT, Y444, located at the middle of this loop, is hydrogen-bonded with N411, thus forming the wall of the substrate binding pocket. Y444 acts as a gating residue. Molecular dynamics studies have revealed that in hGT the lid loop (residues 423–438) exhibits large fluctuations in both the apo and the holo state, hence also in the presence of the  $\gamma$ -glutamyl moiety [59]. These results undermine previous hypothesis on the reduction of mobility of the loop in EcGT and HpGT upon the formation of the covalent adduct. In hGT, the gating residue is replaced by a Phe, which lacking the side chain OH group, cannot form the above described stabilizing interaction. As a result, the lid loop of hGT in the substrate-bound complex should exhibit larger fluctuations than those of the corresponding region of EcGT and HpGT. The higher conformational variability of hGT lid loop, that is, the ability to change its structure from an open to a closed conformation, could explain higher transpeptidase activity (faster product release) of hGT when compared to the other members of the family.

Interestingly, the structure of the L-glutamate-bound BsGT revealed that neither the lid loop nor alternative ordered segments cover the active site, which remains exposed to the solvent [40].  $\gamma$ -GTs with solvent exposed active sites could accept other potential substrates.



#### 4.4 Acceptor Binding Site

Structural data aimed at identifying residues involved in the recognition of the acceptor and forming the acceptor binding site are still missing. Some results indicate that the acceptor binds to the site occupied by the leaving group [60, 61]. This hypothesis would suggest that the catalytic mechanism proceeds in a sequential manner, that is, the enzyme binds the  $\gamma$ -glutamyl compound and the acceptor in the same site in a sequential way. However, at the moment, this hypothesis is not conclusive. Further attempts to unveil the molecular bases of acceptor recognition are based on docking studies performed using structural models of glycyl-glycine and *Picrophilus torridus*  $\gamma$ -GT (PtGT) or its Y327N mutant [62] and on site directed mutagenesis and computational studies performed on hGT and on CapD from *B. anthracis*, a protein related to  $\gamma$ -GTs but missing the ability to hydrolyze GSH [59]. A computational study performed on the PtGT Y327N mutant has suggested that the glycyl-glycine docks perfectly in a binding pocket where five residues (L87, E90, Y305, N327, and S348) can form hydrogen bonds with the ligand [62]. In the same binding pocket, in the case of wild-type PtGT, only four residues (S348, F350, T351, and Y425) interact with the acceptor. This result is strengthened by comparative intrinsic fluorescence emission spectra. In fact, a shift of maximum emission wavelength from 330 to 345 nm is observed when the acceptor-free mutant Y327N is compared to the same protein in the presence of glycyl-glycine. In the case of the wild type, addition of the acceptor causes only a fluorescence quenching.

The computational studies performed on hGT have suggested that the putative acceptor binding site is located in the region along the groove of the donor binding site. The binding pocket in human enzyme should be formed by a number of polar residues such as D46, N79, H81, S82, Y403, Q476, and K562. Site-directed mutagenesis studies of hGT and CapD identify residue K562 in hGT (R520 in CapD), N373, and R432 in CapD (R in EcGT) as involved in the formation of the acceptor site. It is worth noting that two small loops of hGT at the acceptor site, formed by residues 459–464 and residues 549–553 were found to exhibit large motions in molecular dynamics studies [59].

Since the mechanism of transpeptidation has not been clarified yet, further studies are needed to verify the speculative aspects of these models on the basis of new structural insights. These studies should also explain why the absence of any single glycosylation in mammalian enzymes affects both the transpeptidation and hydrolysis reactions of hGT [34].

#### 4.5 Transpeptidation Versus Hydrolysis

$\gamma$ -GTs catalyze both hydrolysis and transpeptidation reactions. In vivo, the primary reaction is the hydrolysis [34]. The two reactions have different pH-rate profiles.

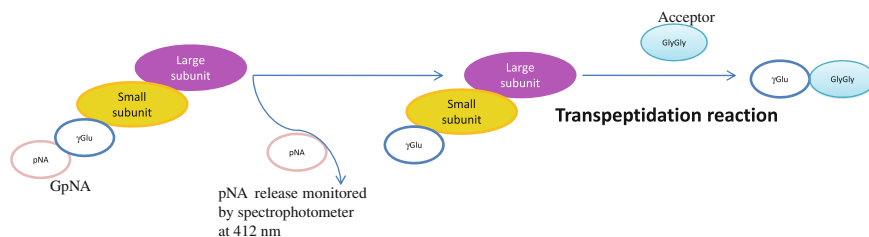
Their behavior can also be different when homologous enzymes are compared. For example, transpeptidation shows a bell-shaped pH-rate profile for hGT, but only an ascending limb in EcGT [25]. The hydrolysis reaction yields a relatively flat pH-rate profile. These differences make a  $\gamma$ -GT able to catalyze one of the two reactions selectively, by adjusting the pH of the reaction mixture. Using rat  $\gamma$ -GT it has been shown that the formation of acyl-enzyme intermediate is the rate-limiting step [54, 63, 64].

Interestingly, some  $\gamma$ -GTs from extremophilic organisms are specialized in hydrolase activity [46, 58]. Comparative sequence and structural analysis between extremophilic  $\gamma$ -GTs and mesophilic counterparts with high sequence identity suggested that the transpeptidation is mediated by residues S462 and N411 (numbering scheme of *E. coli*), which are located in  $\gamma$ -glutamyl binding site (Fig. 11). In site-directed mutagenesis studies, in which these residues were introduced in the sequence of the hydrolase specialized  $\gamma$ -GT from *Picrophilus torridus*, the enzyme acquired significant transpeptidase activity [62].

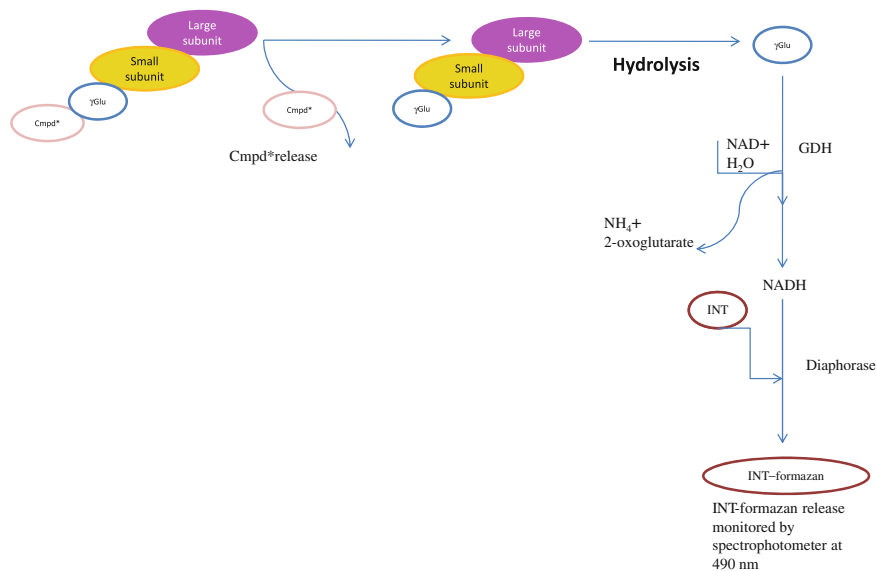
## 4.6 Enzymatic Assays

$\gamma$ -GT activity has been assessed by using a number of different substrates. Determination of the hydrolytic activity has been generally performed using  $\gamma$ -glutamyl-*p*-nitroanalide (GpNA) [31, 65]. The release of *p*-nitroaniline (pNA) was monitored by spectrophotometer at 410–412 nm ( $\epsilon = 8800 \text{ M}^{-1}$ ). When the activity of the recombinant proteins is too low to allow continuous monitoring, the release of pNA is assessed by end point assay after 5–10 min of incubation with the substrate. When high concentrations are required, the limited GpNA solubility is overcome by using  $\gamma$ -glutamyl-(*m*-carboxyl)-*p*-nitroanilide. The transfer of the  $\gamma$ -glutamyl group to a dipeptide acceptor, which occurs by a modified Ping-Pong mechanism, has been generally followed using GpNA and high concentrations of the acceptor glycyl-glycine (GlyGly), at pH 8.0 or higher (Fig. 12).

Recently, Hanigan and coworkers have developed a new quantitative assay to measure hydrolytic activity of  $\gamma$ -GTs [66]. According to this new method, the



**Fig. 12** Standard  $\gamma$ -GT enzymatic assay



**Fig. 13** Schematic representation of the new quantitative  $\gamma$ -GT enzymatic assay developed by Hanigan and coworkers [66]

hydrolysis of  $\gamma$ -glutamyl bonds is detected by a coupled assay in which the glutamate released by the hydrolysis of the  $\gamma$ -glutamyl bond is quantified by a modified version of an assay developed by Beutler [67]. In particular, the glutamate is oxidized by glutamate dehydrogenase (GDH), reducing  $\text{NAD}^+$  to  $\text{NADH}$ .  $\text{NADH}$  in the presence of diaphorase reduces idonitrotetrazolium (INT) to INT-formazan, a colored product, which can be detected at 490 nm. The production of glutamate in the second reaction can be monitored continuously. In the third reactions, INT-formazan is produced in stoichiometric ratio with the amount of L-glutamate (Fig. 13).

#### 4.7 Biochemical Properties of $\gamma$ -GTs

Despite the considerable sequence identity among members of the  $\gamma$ -GT family, a direct comparison of the catalytic activity of enzymes from different source is difficult, because of the different experimental conditions and substrate molecules used to study each enzyme. However, a rough comparison indicates that significant catalytic differences exist among  $\gamma$ -GTs. In general, plant  $\gamma$ -GTs are similar to the mammalian enzymes in their biochemical characteristics [68], whereas bacterial  $\gamma$ -GTs are generally catalytically less powerful than their eukaryotic homologs.

For example, the activity of rat kidney  $\gamma$ -GT is about 100-fold higher than that of EcGT [56]. hGT catalyzes transpeptidation 180-fold faster than hydrolysis, while HpGT shows only a modest 2-fold increase [47]. Large differences in catalytic activities also exist when bacterial homologs are compared. BsGT is 33-fold more active than EcGT [55]; furthermore, as already mentioned, extremophilic bacterial  $\gamma$ -GTs display reduced hydrolase activities, when compared to the other bacterial and eukaryal counterparts, and do not display any ability to transfer the  $\gamma$ -glutamyl group to acceptors [46, 58]. Further differences occur for the catalytic rate. Eukaryotic  $\gamma$ -GTs can be stimulated up to 100-fold by acceptors, whereas prokaryotic enzymes only exhibit marginal changes: about 2-fold in EcGT and about 10-fold in BsGT [25, 26, 56].

For the reactions catalyzed by  $\gamma$ -GTs, the optimal pH is generally between 8 and 9, with the exceptions of the enzymes from *Bacillus subtilis* SK 11.004 and *Pseudomonas nitroreducens* which have an optimum pH of the transfer reaction of 10 and 10.5, respectively [69, 70]. The optimal temperatures for  $\gamma$ -GT activities range from 37 to 60 °C. Some enzymes are highly stable below 50 °C [70], whereas others are highly sensitive to thermal inactivation [26, 71]. For example, the enzyme from *Bacillus pumilus* KS is highly thermostable, retaining 50 % of the original activity at 70 °C [72], whereas GthGT and BIGT show approximately 80 and 40 % of the original catalytic activity at 45 °C [44]. Altogether, these features could reflect different mechanisms of adaptation of the enzyme to colonize different niches. Otherwise, they could remark different evolutionary relationships in  $\gamma$ -GT family, with extremophilic  $\gamma$ -GTs as the ancient progenitors [58].

Since  $\gamma$ -GT has been considered a suitable enzyme in industrial processes that require high-salt conditions, such as the manufacture of fermented foods where >18 % NaCl is generally used, it is important to find halotolerant  $\gamma$ -GTs that retain complete transpeptidase activity in the presence of such high concentration of NaCl. At the moment, very few halotolerant  $\gamma$ -GTs are known; the majority of the members of the family are inactivated in the presence of salt. Examples of halotolerant  $\gamma$ -GTs include BsGT, BIGT, GthGT, and a monomeric 30 kDa  $\gamma$ -GT purified from *B. licheniformis*, probably generated by proteolytic digestion of the mature BIGT by subtilisin [33, 73–76]. Hypotheses on the structural basis for the salt tolerance of BsGT and GthGT have been reported [76]. The peculiar feature of halotolerant  $\gamma$ -GTs to be stable in the presence of large amount of salts is related to the existence of a higher proportion of acid residues on their surface with respect to their non-halotolerant counterparts. These acid patches on the surface may allow proteins to remain in the hydrated state and avoid self-aggregation even under high-salt conditions [40]. In this respect, GthGT can be a prototype. In view of its thermophilic origin and halophilic behavior, GthGT possesses a larger ratio of negatively charged (D + E) residues to positively charged (R + K) ones and a larger percentage of exposed negatively charged side-chains with respect to mesophilic counterparts. These side chains are globally distributed on GthGT molecular surface. This finding is in line with the results of several independent



**Table 3** Effects of mutations on enzymatic activity of hGT

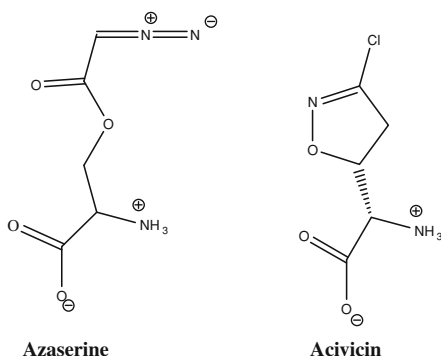
Human GT	Mutated residue	Effect on catalytic activity
	K100 $\rightarrow$ N:	No effect on activity
	E102 $\rightarrow$ Q	No effect on activity
	R107 $\rightarrow$ K	Reduces enzyme activity by 99 %
	R107 $\rightarrow$ Q	Abolishes enzyme activity
	R107 $\rightarrow$ H	Abolishes enzyme activity
	E108 $\rightarrow$ Q	Reduces enzyme activity by 98 %
	R112 $\rightarrow$ Q	No effect on activity
	R139 $\rightarrow$ Q	No effect on activity
	R147 $\rightarrow$ Q	No effect on activity
	R150 $\rightarrow$ Q:	No effect on activity
	H383 $\rightarrow$ A	Reduces enzyme activity by 66 %
	S385 $\rightarrow$ A	No effect on activity
	S413 $\rightarrow$ A	No effect on activity
	D422 $\rightarrow$ A	Reduces enzyme activity by 90 %
	D423 $\rightarrow$ A	Abolishes enzyme activity. Increases KM by over 1000-fold
	S425 $\rightarrow$ A	No effect on activity
	S451 $\rightarrow$ A	Reduces enzyme activity by 99 %. Abolishes activity when associated with S452A
	S452 $\rightarrow$ A:	Reduces enzyme activity by 99 %. Abolishes activity when associated with S451A
	C454 $\rightarrow$ A	No effect on activity
	H505 $\rightarrow$ A	Reduces enzyme activity by 90 %

studies suggesting that halophilic enzymes present higher proportion of acid residues on the surface than their non-halophilic homologs [77, 78] and with a recent comparative structural analysis of residue distribution in a database comprising 15 pairs of halophilic/non-halophilic homologs [76]. On the bases of these results,  $\gamma$ -GT mutants with increased resistance versus high salt environment could be designed.

Several molecules are able to uncouple hydrolysis and transpeptidase activity of  $\gamma$ -GTs. Activators of the hydrolase activity include maleate, free bile acids cholate, chenodeoxycholate, and deoxycholate, and some metal ions. Maleate stimulates the hydrolase activity of rat kidney  $\gamma$ -GT [79], but inhibits its transpeptidase activity. Hippurate and its derivatives are also capable of uncoupling hydrolysis and transpeptidase activity [80], whereas free bile acids affect the kinetics of both hydrolysis and transpeptidation [81]. Regarding metal ions as activators,  $\text{Li}^+$ ,  $\text{Rb}^+$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Cs}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Co}^{2+}$ , and  $\text{Mn}^{2+}$  activate EcGT [82], whereas the enzymatic activity of BsGT is enhanced in the presence of  $\text{Al}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$  [70].  $\text{Mg}^{2+}$ ,  $\text{K}^+$ , and  $\text{Na}^+$  also enhance catalytic activity of BIGT [44].

Site directed mutagenesis has been used to reveal which residues are important for the catalytic activity of  $\gamma$ -GTs. Mutations investigated in the human enzyme are reported in Table 3.

**Fig. 14** Structures of glutamine analogs that inhibit  $\gamma$ -GTs: Azaserine and Acivicin



## 4.8 Quest for Inhibitors

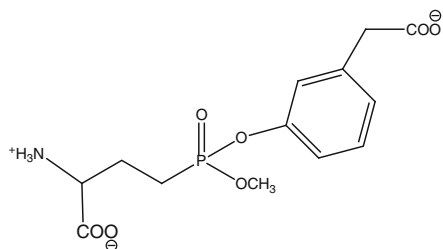
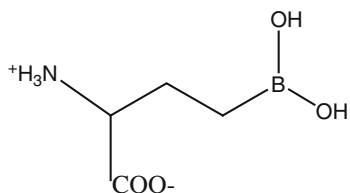
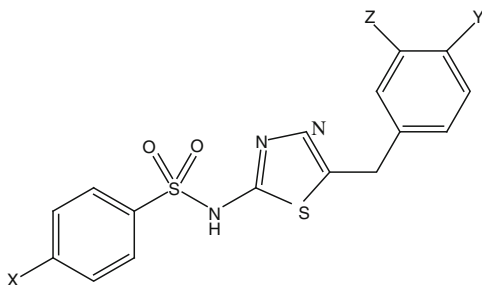
Rational design of  $\gamma$ -GT inhibitors has encountered a lot of difficulties, due to uncertainty about  $\gamma$ -GT reaction mechanisms. Compounds which are known to inhibit  $\gamma$ -GT include the glutamine analogs Acivicin (L-( $\alpha$ S,5S)- $\alpha$ -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid), 6-diazo-5-oxo-L-norleucine (DON), and Azaserine (O-diazoacetyl-L-serine) [83] (Fig. 14).

These inhibitors have been widely used for the in vitro and in vivo experiments on  $\gamma$ -GTs. Acivicin, the most potent inhibitor that has been tested clinically, has a protective effect on cisplatin-induced nephrotoxicity [84] and suppresses  $\gamma$ -GT-dependent oxidative damage in ischemic rat kidney [85]. However, this molecule also inhibits various glutamine amidotransferases, including imidazole glycerol phosphate synthase and guanine monophosphate synthetase, and inactivates a number of biosynthetic enzymes for purine and pyrimidine, amino acids, and amino sugars, resulting in a potent cytotoxicity [86–88]. Both Acivicin and Azaserine cause bone marrow suppression.

Design of  $\gamma$ -GT inhibitors based on studies of active site has led to the identification of additional  $\gamma$ -glutamyl analogs. Lherbet and Keillor have designed sulfur derivatives of L-glutamic acid that inhibit  $\gamma$ -GTs [89] and are less toxic than the glutamine analogs. Han and co-workers have synthesized and tested a series of  $\gamma$ -(monophenyl)phosphono glutamate analogs which also function as inhibitors of  $\gamma$ -GTs [90]. Among these molecules, 2-amino-4-(3-(carboxymethyl)phenyl(methyl)phosphono)butanoic acid (GGsTOP, Fig. 15) seems one of the most promising.

GGsTop is an electrophilic phosphonate phenyl ester, it is non-toxic but not stable enough to be used for in vivo studies [91]. It does not inhibit the asparagine synthetase and shows 100-fold higher inhibitory activity toward hGT than that of Acivicin [90, 91].

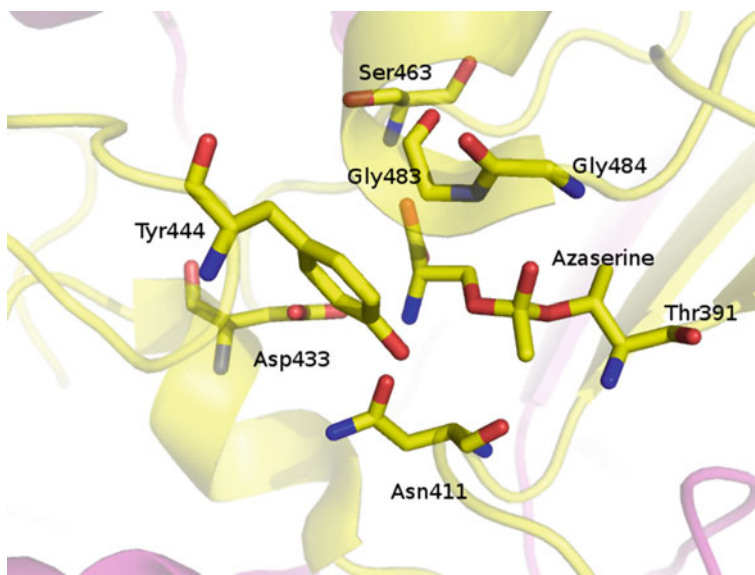
Another potent inhibitor is the  $\gamma$ -boronic acid analog of L-glutamic acid, 3-amino-3-carboxypropaneboronic acid ( $\gamma$ -boroGlu, Fig. 16) [92]. Weak inhibitors are alkylboronic acids [93], *m*-formylphenylboronate [93], and the serineboric acid complex [94].

**Fig. 15** Structure of GGsTOP**Fig. 16** Structure of  $\gamma$ -boroGlu**Fig. 17** Structure of analogs of OU749. X, Y, Z may be, for example, H, Cl, F, Br, I, OH, an alkoxy, or NO<sub>2</sub> [95]

Recently, a novel class of  $\gamma$ -GTs uncompetitive inhibitors that are structurally distinct from and less toxic than the glutamine analogs has been also developed. These compounds, which are derived from N-[5-(4-methoxybenzyl)-1,3,4-thiadiazol-2-yl] benzenesulfonamide, called as analogs of OU749 (Fig. 17) [95], should not occupy the  $\gamma$ -glutamyl binding site, but the acceptor binding one (see below). These inhibitors are species-specific since they inhibit hGT, but not mouse, rat, and pig enzymes [96].

## 4.9 Inhibitor Mechanisms

Inhibition mechanisms of Acivicin and Azaserine have been recently elucidated by X-ray crystallographic studies [97]. CA atom superposition of the Acivicin and Azaserine complexes (PDB accession codes: 2Z8I and 2Z8K) with the ligand-free enzyme structure (PDB accession code: 2DBU) yields a RMSD  $<0.2$  Å, indicating that binding of these inhibitors does not significantly affect the overall structure of

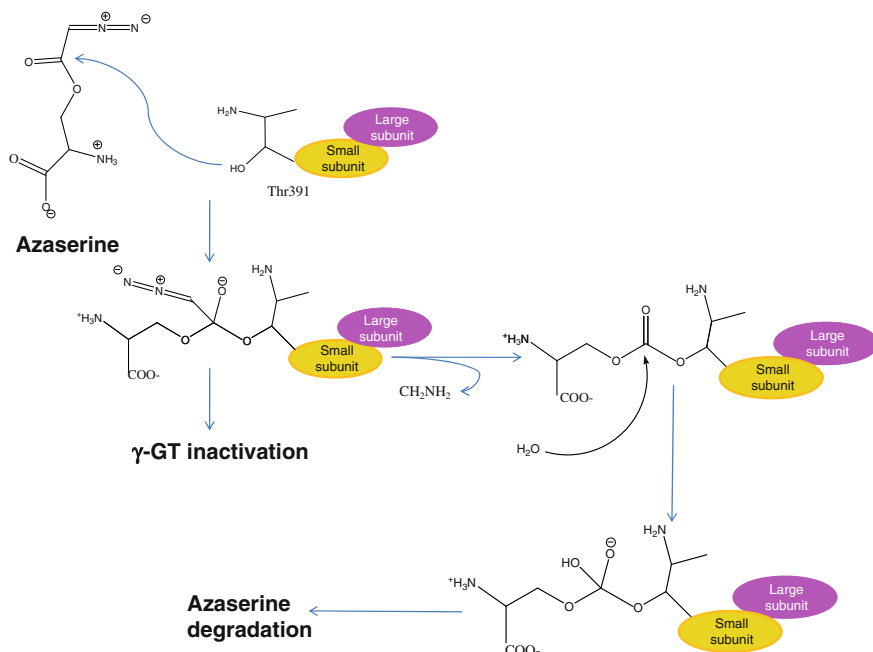


**Fig. 18** Binding mode of Azaserine. Location and orientation of the molecules are very similar to those of the  $\gamma$ -glutamyl enzyme intermediate

the (mature) protein. X-ray structures of the complexes EcGT-Acivicin and EcGT-Azaserine have disclosed that the tight protein-inhibitor association is largely due to the formation of an adduct with  $\gamma$ -GTs, which is structurally analogous to transition state that forms during the reaction pathway for catalysis (Fig. 18). In particular, these inhibitors form a covalent bond with the OG atom of the catalytic threonine. However, structure and hybridization of these inhibitors are unusual. In EcGT-Azaserine complex, the CH atom of Azaserine (Fig. 18) involved in the formation of the covalent bond with the Thr is hybridized  $sp^3$  and adopts a tetrahedral geometry (Figs. 18, 19). This is not the final inactivation product: it rearranges to form a more stable adduct that irreversibly inhibits the protein. A proposed inhibition mechanism of Azaserine is reported in Fig. 19. In the case of EcGT-Acivicin complex, the imino carbon of the dihydroiaoxazole ring is linked to the catalytic Thr. Interestingly, the inspection of the electron density maps of this adduct reveals that Cl atoms are removed from the inhibitor upon the binding (Fig. 20). The proposed inhibition mechanism of Acivicin is reported in Fig. 21.

The inhibition mechanism of the other known  $\gamma$ -GT inhibitors has not been confirmed by experimentally determined structures of adducts, but have been supposed on the bases of biochemical data. The inhibition mechanism supposed for GGsTOP is reported in Fig. 22. Also in this case, the inhibitor should link to the catalytic Thr in the enzyme active site.

$\gamma$ -boroGlu (Fig. 23) [94] and DON [98] should form a covalent, tetrahedral adduct with the protein as well.

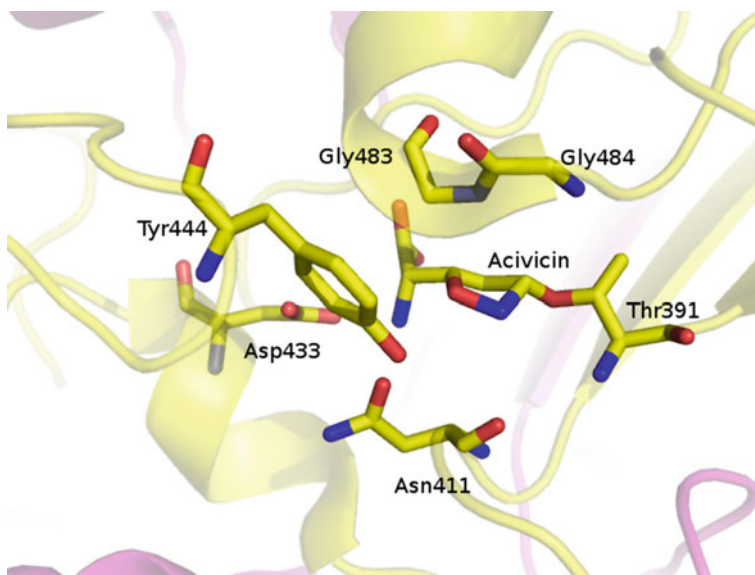


**Fig. 19** Proposed inhibition mechanism for Azaserine. The OG atom of catalytic Thr attacks the carbonyl carbon of Azaserine leading to the formation of a tetrahedral adduct. The activated diazoketone may inactivate the enzyme by alkylation or eliminate diazomethane to form an *O*-carboxyseryl enzyme intermediate. The addition of a water molecule to the carbonyl forms a tetrahedral adduct that may lead to the overall degradation of Azaserine by the enzyme

On the contrary, OU749 mechanism of action seems different. The predicted binding model of OU749 with hGT showed that this molecule is located in the acceptor binding site with its phenylcarboxylate group that can form a hydrogen bond with Lys562 [59]. The discovery of OU749 is promising for the design of non-toxic, species-selective inhibitors of  $\gamma$ -GTs directed to the acceptor binding site.

#### 4.10 Conformational Stability of $\gamma$ -GTs

Understanding the structure-stability-function relationship of an enzyme is crucial for its applicative aspects. Conformational studies may provide insight into the molecular basis of the enzyme stability, which in turn can be used to design protein variants with special properties for biotechnological applications. Few reports deal with biophysical characterization of precursors and mature forms of  $\gamma$ -GTs. This is in part due to the difficulty to purify  $\gamma$ -GTs at homogeneity, since a mixture of the



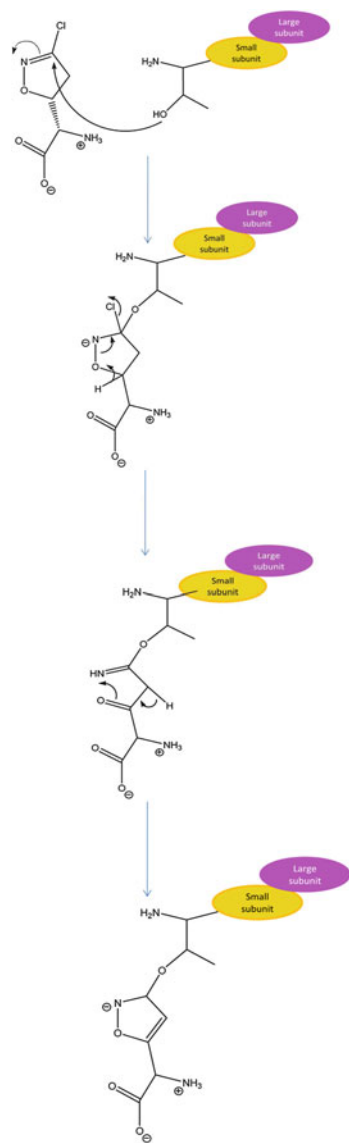
**Fig. 20** Binding mode of Acivicin. Location and orientation of the molecule is very similar to that of the  $\gamma$ -glutamyl enzyme intermediate

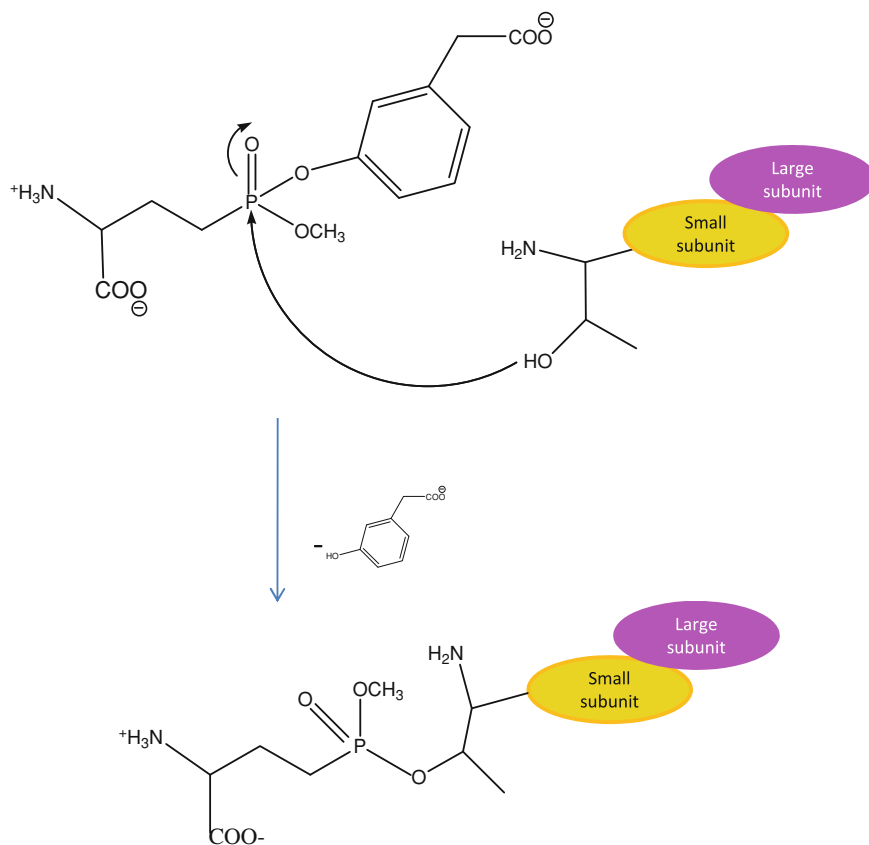
unprocessed and mature protein is often obtained [46, 58, 72]. Unfolding analyses using circular dichroism and tryptophan emission fluorescence have revealed that members of the  $\gamma$ -GT family display different sensitivity toward temperature and guanidinium hydrochloride induced-denaturation [75, 99, 100]. Thermal denaturation of EcGT and BIGT follows a simple irreversible two-state mechanism ( $N \rightarrow U$ ) [75], whereas that of GthGT was described using a three-state model involving the formation of a stable intermediate [100]. Among the characterized enzymes, GthGT is the most thermostable, in line with its thermophilic origin, whereas BIGT is the most resistant to chemical denaturation with guanidinium hydrochloride. Interestingly, it has been recently shown that heat-inactivated EcGT left at 4 °C temperature recovers its activity, whereas heat-treated BsGT does not. These results have been explained with the hypothesis that heat-treated EcGT dissociates into large and small subunits, which reassemble to form a catalytically competent enzyme at low temperature [101].

#### 4.11 Search for Thermostable $\gamma$ -GTs

In recent years, demands for thermostable enzymes have increased in industrial processes. Biocatalyst thermostability allows a higher operation temperature, which

**Fig. 21** Proposed inhibition mechanism for Acivicin. Catalytic Thr attacks the C3 imino carbon in the dihydroisoxazole ring of Acivicin. This results in the formation of anaza-anion. The O–N bond is then cleaved with concomitant loss of the hydrogen in C5 and of the Cl atom to give a  $\beta$ -oxoimidoyl group. Finally, the imidoyl carbon attached to the OG atom of Thr becomes  $sp^3$  hybridized by elimination of the H in C4 with concomitant ring closure and formation of a double bond between C4 and C5



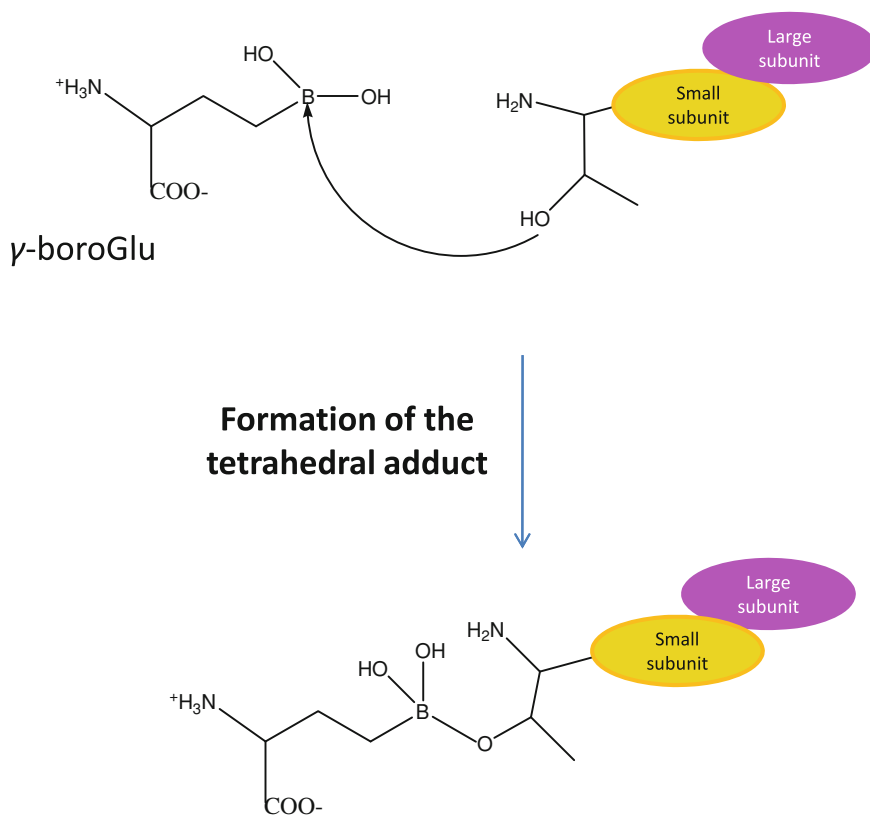


**Fig. 22** Proposed inhibition mechanism for GGsTOP

is clearly advantageous because of a higher reactivity (higher reaction rate, lower diffusion), higher process yield (increased solubility of substrates and products and favorable equilibrium displacement in endothermic reactions), lower viscosity and fewer contamination problems. For this reason, the purification and characterization of thermostable  $\gamma$ -GTs is required. Thermophiles represent an obvious source of thermostable enzymes. Recent progress in genomics has provided a wealth of genome sequences, and several sensitive homology search programs have discovered  $\gamma$ -GT homologs in many thermophilic microorganisms [62].

Cloning, expression, and purification of thermostable  $\gamma$ -GT from *G. thermodenitrificans* was reported in 2010 [46]. GthGT was the first thermostable  $\gamma$ -GT to be characterized. One year later, overexpression of the gene encoding the  $\gamma$ -GT from *T. thermophilus* and *D. radiodurans* was reported [102]. More recently, the protein from *P. torridus*, an acido-thermophilic archaeon, has been characterized [62].





**Fig. 23** Proposed inhibition mechanism for  $\gamma$ -boroGlu [92]

## 5 $\gamma$ -GT in Bioclinical

hGT levels in blood are routinely measured in clinical laboratories. Indeed,  $\gamma$ -GT serum is mainly derived from liver, thus the enzyme is used as marker of liver or biliary tract-associated diseases [103, 104]. The higher the  $\gamma$ -GT level the greater the “insult” to the liver. Serum levels of  $\gamma$ -GT are affected by many factors: alcohol intake [13], body fat content, plasma lipid/lipoproteins, and glucose levels.

Increased serum  $\gamma$ -GT levels have also been associated with pancreatitis, type II diabetes [105], cardiovascular disease [106, 107], stroke, and hypertension [108]. However, the reasons of these correlations are not always known. In patients with hypertension,  $\gamma$ -GT is positively associated with inflammation markers such as fibrinogen, C-reactive protein, and F2-isoprostanes<sup>109</sup> and is expressed in human atherosclerotic lesions, thus contributing to the progression of atherosclerosis [109]. Finally,  $\gamma$ -GT plays a key role in the production of free radical species through its interaction with iron [110]. Therefore, elevated  $\gamma$ -GT levels could be a

marker of inflammation condition and oxidative stress, typical features of hypertension. An increase in serum levels of  $\gamma$ -GT has been also suggested to be predictive of atherosclerosis [111], but the mechanism of this association has not been elucidated. One hypothesis is that  $\gamma$ -GT acts as proatherogenic factor [112]. Otherwise, the increase of  $\gamma$ -GT could be a marker of the presence of the metabolic syndrome [113]. Indeed, evaluation of  $\gamma$ -GT activity in obesity is associated with insulin resistance and consequent metabolic syndrome [114].

The reference range for  $\gamma$ -GT is 0–30 IU/L. Levels of  $\gamma$ -GT are higher in men than in women (ca 25 %) and, in women, increase with age [115]. Infants levels overcome 6–7 times the upper limit of the adult reference range, which declines to adult levels around age 7 months [116]. Elevated  $\gamma$ -GT levels have also been associated with use of many drugs, including barbiturates, antibiotics, histamine receptor blockers, antifungal agents, antidepressants, and testosterone. Smoking can also increase  $\gamma$ -GT levels. Clofibrate and oral contraceptives can decrease  $\gamma$ -GT levels.

$\gamma$ -GT has been implicated in many physiological disorders, such as neurodegenerative diseases [117, 118]. Moreover,  $\gamma$ -GT has also been reported as a virulence factor associated with the colonization of the gastric mucosa by *H. pylori*, the pathogen responsible for gastritis, ulcer and gastric cancer [27].

$\gamma$ -GT deficiency, a rare autosomal recessive disease documented in five patients [114] has been linked to disruptive glutathione homeostasis, DNA damage, reproductive defects, mental retardation, and cataract [119]. Genetic  $\gamma$ -GT deficiency in mouse impairs glutathione metabolism and transport and results in systemic glutathionemia, glutathionuria, and oxidant stress in the kidney [120].

## 5.1 Pathways of $\gamma$ -GT Induction

The appearance of  $\gamma$ -GT-positive foci in animals exposed to chemical carcinogens and the increased expression of  $\gamma$ -GT in proliferating pre-neoplastic foci in the liver have suggested that  $\gamma$ -GT can be considered as an early marker of neoplastic transformation [5, 8, 13, 121, 122]. It has been shown that  $\gamma$ -GT is up-regulated in different cell types after acute exposure to oxidative stress [123] also involving the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways. Indeed,  $\gamma$ -GT expression and Ras-MAPK pathways activation have been correlated in colon cancer cells following  $\gamma$ -irradiation [124], as well as exposure to oxidative stress [125]. Reactive oxygen species (ROS) have been implicated in the process of carcinogenesis, and, the redox regulation of many genes in response to ROS/electrophiles seems to modulate  $\gamma$ -GT expression.

Interestingly,  $\gamma$ -GT mRNA was shown to be induced also by cytokines, including tumor necrosis factor alpha (TNF-alpha) [126], and interferon (IFN)-alpha and -beta [127, 128]. These results suggest that the increased  $\gamma$ -GT expression could reflect in some conditions a defensive mechanism against

oxidative stress or in other conditions a regulatory mechanism, involving metabolism of leukotrienes and GSNO.

## 5.2 $\gamma$ -GT and Cancer

Increased levels of  $\gamma$ -GT have been observed in cancer of ovary, colon, liver, astrocytic glioma, soft tissue sarcoma, melanoma, and leukemias [129]. Elevated  $\gamma$ -GT activity has also been found in melanoma cells with an increased invasive growth, and  $\gamma$ -GT expression with unfavorable prognostic signs has been found in human breast cancer. In contrast, in prostatic, colorectal and breast cancer any correlation between expression and standard clinical pathological parameters have been found. These differences can be explained by considering the high variability of cancer cells and the effect of other factors, such as the environment, drugs and diet, which may alter the phenotype of neoplastic lesions [129].

Several studies have addressed the relationships of  $\gamma$ -GT activity with the malignant phenotype [13]. The involvement of  $\gamma$ -GT in cellular supply of extracellular GSH and the increased resistance to pro-oxidant drugs observed in several  $\gamma$ -GT-expressing cell lines have suggested that  $\gamma$ -GT plays a role in the cellular defense mechanisms. On the other hand, recent findings indicate that, under particular conditions, the metabolism of GSH by  $\gamma$ -GT can exert pro-oxidant effects [8, 122, 123].

GSH is synthesized inside cells and transported in the extracellular milieu through plasma-membrane transporters [130], with a concentration gradient (millimolar vs. micromolar). Extracellular metabolism of GSH by  $\gamma$ -GT, together with cell surface dipeptidases, promotes the release of constituent amino acids, the glutamic acid and the essential cysteine as well as their recovery by cells. Indeed,  $\gamma$ -GT overexpressing cells are able to utilize extracellular GSH as a source of cysteine more efficiently than normal cells [7], with a consequent selective growth advantage both at physiological and at limiting cysteine concentrations [131]. Thus,  $\gamma$ -GT can act as a source of essential amino acids, both for protein synthesis and for the maintenance of intracellular levels of GSH. Adequate levels of GSH play a key role in cellular resistance against several electrophilic/alkylating compounds. Indeed,  $\gamma$ -GT-overexpressing cells were shown to be more resistant to hydrogen peroxide, and chemotherapies such as cisplatin [132]. In melanoma cells, GSH depletion and  $\gamma$ -GT inhibition significantly increased cytotoxicity of oxidative stress conditions [133]. Interestingly, the same treatments were also shown to induce  $\gamma$ -GT expression, likely as a protective adaptation induced by oxidative stress itself. As such,  $\gamma$ -GT expression would perfectly fit with the so-called 'resistance phenotype', that is a common defense mechanism of transformed, pre-neoplastic cells, against injury by oxidants and xenobiotics [8]. However, conflicting results are reported on the supposed roles of GSH and  $\gamma$ -GT in protection against cell injury. Indeed,  $\gamma$ -GT could function in different ways, leading to

extracellular detoxication of platinum-based drugs, but also to pro-oxidant effects catalyzed by metal ions in the extracellular space.

### 5.3 $\gamma$ -GT and Cisplatin Resistance

Sulfur amino acids, in particular cysteine, and other small peptides containing cysteine, such as cysteinyl-glycine and GSH, are able to form adducts with cisplatin, and such complexes are poorly transported across plasma membrane. The effects are a decreased intracellular accumulation and a reduced toxicity of cisplatin towards treated cells [134]. Interestingly, cisplatin adducts with cysteinyl-glycine are formed ten times faster than those with GSH; such adducts are present in the extracellular medium of  $\gamma$ -GT overexpressing cells treated with cisplatin. The pKa of cysteinyl glycine thiol is significantly lower than that of GSH (6.4 vs. 8.6, respectively), thus causing its more rapid dissociation at physiological pH and its more efficient interaction with cisplatin.  $\gamma$ -GT transforms poorly reactive GSH into highly reactive cysteinyl-glycine, thus triggering the formation of cisplatin/thiol complexes in the extracellular space [135]. This results in lower cellular accumulation of cisplatin, reduced DNA platination, and reduced cytotoxicity [136]. Thus, the protective effect of  $\gamma$ -GT expression against cisplatin cytotoxicity seems to be dependent on the extracellular detoxication of cisplatin, rather than the higher intracellular GSH levels. However, it is likely that the rate of detoxification mediated by  $\gamma$ -GT may depend on the specific biological context, in which other resistance mechanisms can coexist. Indeed, cellular resistance to toxic agents acts as a multifactorial phenomenon, involving not only defense mechanisms, but also cellular response to genotoxic stress (DNA repair efficiency, DNA damage tolerance, stress response and susceptibility to apoptosis) [8].

Finally, dosage of cisplatin *in vivo* is limited by its nephrotoxicity, but the mechanism by which cisplatin kills proximal tubule cells remains unclear. However, both animal studies and clinical trials demonstrated that pre-treatment with exogenous GSH reduced nephrotoxicity induced by cisplatin without reducing its antitumor activity [129].

### 5.4 $\gamma$ -GT and Tumor Progression

$\gamma$ -GT can exert pro-oxidant effects at the membrane surface level and in the extracellular microenvironment [137]. This phenomenon was explained with the high reactivity of the  $\gamma$ -GT product, cysteinyl-glycine. Indeed, because of its lower pKa, the cysteinyl-glycine dissociates more quickly at physiological pH, reducing extracellular transition metal cations (in particular,  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$ ) more efficiently than GSH itself. Iron reduction by GSH, in fact, might be limited by the chelating properties of the alpha-carboxyl group of the glutamate residue, affecting steric

and redox interactions of the cysteine thiol. The removal of glutamic acid by  $\gamma$ -GT causes a decrease of the cysteine thiol pKa and makes it free to interact with iron. The ‘redox cycling’ starting after iron reduction was shown to produce ROS and thiyl radicals, that is, reactive species promoting several intracellular and extracellular molecular effects. The damage induced by  $\gamma$ -GT could play an active role in the progress of preneoplastic foci to malignancy. Indeed, the pro-oxidant activity of  $\gamma$ -GT can promote the iron-dependent oxidative damage of DNA in transfected melanoma cells, thus contributing to genomic instability and increased mutation risk in cancer cells [138].

Moreover,  $\gamma$ -GT activity is able to promote the release of free iron from transferrin, thus promoting the uptake of iron by cancer cells [139]. This effect may play an additional role in supplying iron to malignant cells. Physiological levels of pro-oxidants can exert regulatory roles by acting on targets sensitive to the redox state of the cell [140]. A major role in such regulation is played by cysteine thiols, which can undergo different redox modifications [141, 142]. Such reactive cysteines play key role in proteins involved in crucial processes, such as cell proliferation, apoptosis, cell adhesion, and thus in progression of cancer and other diseases. It has been reported that  $\gamma$ -GT activity can promote the oxidation of thiol groups in cell surface proteins, through the involvement of hydrogen peroxide and the formation of mixed disulfides (‘protein S-thiolation’) [141]. As hydrogen peroxide freely diffuses across the plasma membrane, pro-oxidant reactions dependent by  $\gamma$ -GT activity can also involve crucial intracellular targets, for example they can induce the binding of NF- $\kappa$ B and AP-1 to DNA [143, 144] and modulate the balance between protein kinase/phosphatase [145]. Indeed, it is well known that redox processes can play modulatory roles in the transduction of proliferative/apoptotic signals, due to interactions with growth factor receptors, protein kinases, and transcription factors.

The modulatory effects of pro-oxidant reactions could contribute to the resistance phenotype of  $\gamma$ -GT-expressing cancer cells, by regulating both signal transduction pathways involved in proliferation/apoptosis balance, as well as by inducing protective adaptations in the pool of intracellular antioxidants. For example,  $\gamma$ -GT/GSH-dependent pro-oxidant reactions were also shown to increase intracellular levels of vitamin C [146].

### 5.5 $\gamma$ -GT as a Target for Anticancer Treatments

Because high intracellular levels of GSH are involved in the resistance phenotype of cancer cells,  $\gamma$ -GT has been considered a potential target of inhibition associated with chemotherapeutics. As described above, most of  $\gamma$ -GT inhibitors, such as Acivicin, Azaserine, boronate derivatives, L-glutamic acid derivatives; gamma-(monophenyl) phosphonoglutamate analogs [90] are toxic and cannot be used in humans. Recently, a novel class of uncompetitive inhibitors of  $\gamma$ -GT, less toxic than glutamine analogs, have been described [96]. However, the development of

$\gamma$ -GT inhibitors with low toxicity remains an interesting perspective for pharmacological research, and could have a great impact on cancer therapy.

$\gamma$ -GT expression and activity are also relevant in the pathophysiology of cellular processes involving NO and related compounds, such as GSNO. Indeed, treatments of human cancer cells with NO and NO mimetics can restore the sensitivity of resistant cell populations to the cytotoxic effects of chemotherapeutics [147]. Thus, NO acts as a ‘chemosensitizing agent’, likely by modulating processes associated with prevention or inhibition of cellular drug resistance mechanisms. Reactivation of NO signaling might in some way counteract the effects produced by hypoxia in solid tumors [148]. However, the mechanisms by which NO restores sensitivity to anticancer agents are not clearly understood. This critical action might be played by vascular changes (promotion of blood perfusion and tumor oxygenation), radical scavenging, down-regulation of the GSH detoxification, inhibition of key transcription factors, as well as inhibition of drug efflux transporters and DNA repair enzymes. Growth inhibition and chemosensitization to carboplatin treatments were observed after exposure of glioma cells to NONOates [149], while significant chemosensitization to cisplatin cytotoxicity was observed in cells transfected with inducible NO synthase (iNOS) gene [150]. S-Nitrosothiols, GSNO especially, are considered physiologic NO metabolites, able to transport NO in blood and tissues.  $\gamma$ -GT selectively metabolizes GSNO, thus promoting the release of NO [8, 151]. This fact may well be exploited in order to selectively target NO to  $\gamma$ -GT-expressing cancer cells (see medical applications).

## ***5.6 $\gamma$ -GT Complexes: Novel Biomarkers for Cancer and Other Pathologies***

Serum  $\gamma$ -GT, considered as released exclusively from the liver, is widely used as a biomarker of liver dysfunction and excessive alcohol use [13]. On the other hand, several studies have revealed that  $\gamma$ -GT serum levels are positively associated with the risk of cardiovascular events [112], hypertension, type II diabetes and metabolic syndrome [113, 152], renal failure and cancer, even unrelated to hepatic involvement [153]. Therefore, diseased tissues other than the liver might contribute to serum  $\gamma$ -GT activity, thus explaining its broad predictive value. The release of  $\gamma$ -GT from cancer cells was described in several types of neoplasia, but the mechanisms by which cellular  $\gamma$ -GT is released in blood are still poorly characterized. Several papers investigated the possible specificity of serum  $\gamma$ -GT complex for certain tumors, in particular hepatocellular carcinoma, focusing on parameters such as  $\gamma$ -GT post-translational modifications and lipoprotein association. Specific  $\gamma$ -GT macroforms with clinical significance have been reported in patients with primary hepatocellular carcinoma, but the origin or structures of these complexes were not established [154, 155]. Recently, an in vitro study on

melanoma and prostate cancer cells has shown the release of a  $\gamma$ -GT containing soluble complex with a MW >2000 kDa [156]. The component molecules of this complex are still to be identified. Variations in  $\gamma$ -GT glycosylation have been described when comparing the enzyme from malignant and normal tissues. These changes appear, however, to vary with the type of tumor [157], and the amount of tumor-derived  $\gamma$ -GT forms in serum may be affected by a rapid clearance rate [158]. Other studies are needed to better understand the properties of serum  $\gamma$ -GT fractions and the way they are released from cancer cells, in view of a clinical utilization of  $\gamma$ -GT as a biomarker of disease. In a retrospective study, total serum  $\gamma$ -GT was significantly increased in patients with metastatic renal cell carcinoma, but was normal in those with localized primary growths [159]. Similar results were obtained in other works, where both alkaline phosphatase and  $\gamma$ -GT activities were normal in a majority of patients with localized renal cell carcinoma, but increased in most of the patients with metastatic disease involving liver and/or bones. In both cases,  $\gamma$ -GT appeared to be a sensitive marker of metastatic renal cell carcinoma, even though not specific for the site of metastasis.

Significantly higher serum  $\gamma$ -GT levels were also found in hepatocellular carcinoma patients with poorly differentiated tumors. Nevertheless, serum  $\gamma$ -GT levels seem to be at least partly independent of  $\gamma$ -GT expression in tumors [123, 143], and their specificity as marker of cancer has been questioned [157]. On the other hand, epidemiologic studies have suggested that increased  $\gamma$ -GT levels can predict morbidity and mortality independently from liver diseases. Elevated  $\gamma$ -GT significantly increased cancer risk and, in site-specific cancer models,  $\gamma$ -GT was significantly associated with malignant neoplasms [158].

Finally, it has been recently found that soluble  $\gamma$ -GT may play a cytokine-like function. Indeed, the structure of  $\gamma$ -GT includes the chemokine-like CX<sub>3</sub>C motif [20], which likely enables  $\gamma$ -GT to modulate bone resorption independently of its catalytic activity [160]. Moreover, urinary excretion of  $\gamma$ -GT is related to changes of biochemical markers of bone resorption [160]. Therefore, the overexpression and release of  $\gamma$ -GT by human tumors may have a role in establishment and development of bone metastasis.

## 6 $\gamma$ -GTs: Biotechnological and Biomedical Applications

The biochemical ability of  $\gamma$ -GTs to cleave the unusual peptide bond of GSH and to transfer the  $\gamma$ -glutamyl moiety to some acceptors for producing  $\gamma$ -glutamyl compounds can be exploited in different ways for pharmaceutical and biotechnological applications. Since the  $\gamma$ -glutamyl linkage cannot be hydrolyzed by serum peptidases, the half lives of  $\gamma$ -glutamyl compounds are longer than those of non modified drugs [82]. Thus, the addition of a  $\gamma$ -glutamyl group at a specific site makes the drug inactive until the  $\gamma$ -glutamyl group is cleaved off by  $\gamma$ -GTs. Bacterial  $\gamma$ -GTs are a valuable group of enzymes which display a lot of advantageous industrial properties: their functional characteristics allow a great

versatility of possible applications; they can be easily genetically manipulated; they are soluble (periplasmic or extracellular), non-glycosylated, and present a widely diversified substrate specificity, which can be used to employ different acceptors and synthesize various  $\gamma$ -glutamyl compounds. These proteins have been already successfully used as catalysts for the synthesis of pharmaceutically important peptides thanks to the transpeptidation reaction [82, 161]. Examples of the use of  $\gamma$ -GTs in pharmaceutical and biotechnological processes are reported below.

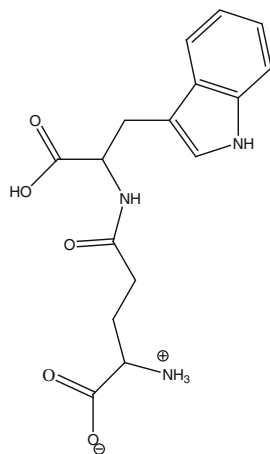
### 6.1 $\gamma$ -GTs in the Production of SCV-07 and of Glutamyl Phenyl Hydrazine Analogs

$\gamma$ -D-Glutamyl-L-tryptophan (SCV-07) is a potential drug for the treatment of tuberculosis, since it has been shown to possess a broad spectrum of immunostimulatory activities against murine tuberculosis [162]. This molecule is also a possible therapeutic for recurrent genital herpes simplex virus type 2 (Fig. 24) [163].

Because SCV-07 has several reactive groups and consists of both D- and L-amino acids, which are connected through a  $\gamma$ -glutamyl linkage, its chemical synthesis is complicated. An efficient enzymatic method to synthesize SCV-07 using not expensive reagents and employing bacterial  $\gamma$ -GTs has been developed [82, 161, 164]. The optimum reaction conditions are 50 mM D-glutamine, 50 mM L-tryptophan, and 0.2 U ml<sup>-1</sup>  $\gamma$ -GT, pH 9–9.5, and incubation at 37 °C for 5 h. In these conditions, the reaction produces 33 mM  $\gamma$ -D-glutamyl-L-tryptophan, with a conversion rate of 66 %. The product can be purified by Dowex 1  $\times$  8 column.

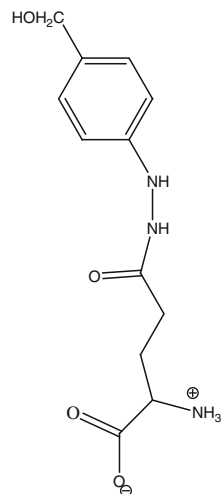
Another group of pharmacologically relevant molecules that can be synthesized using  $\gamma$ -GTs are the glutamyl phenyl hydrazine (GPH) derivatives. These

**Fig. 24** Structure of SCV-07





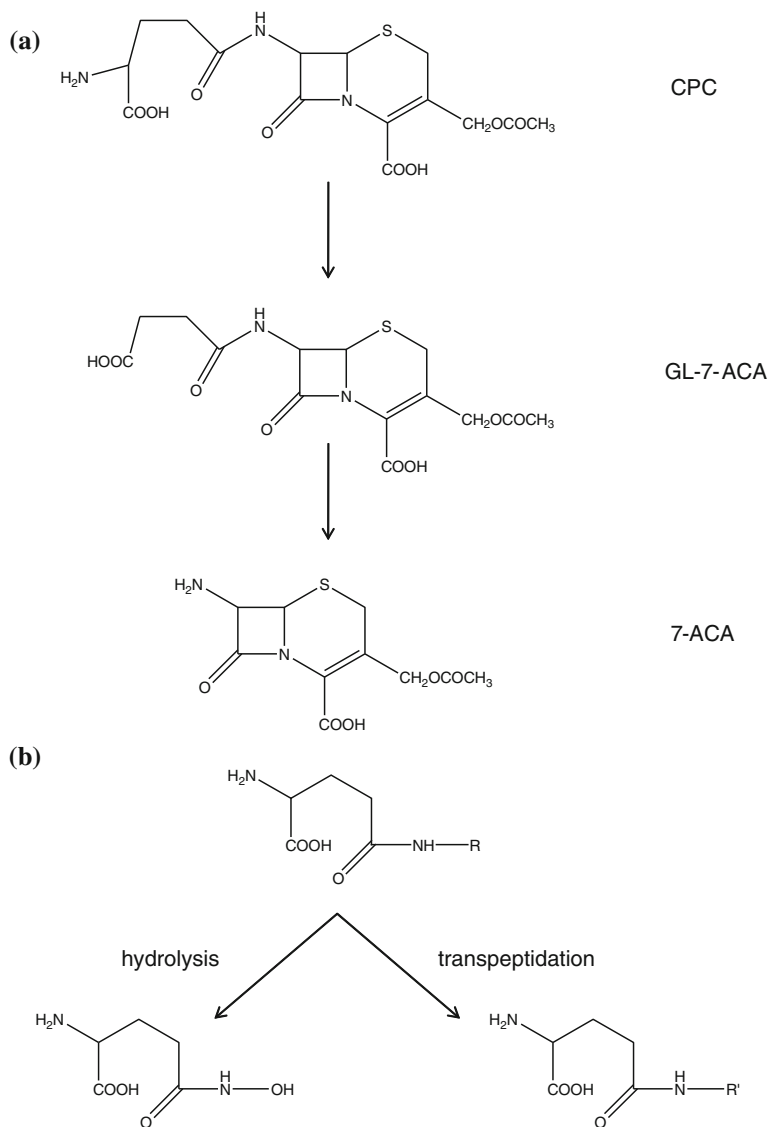
**Fig. 25** Structure of Agaritine



molecules, which exhibit antioxidant, immune-stimulatory, antiviral and anticancer effects, are generally obtained via chemical synthesis or by isolation from natural sources. Since  $\gamma$ -GTs are also involved in the synthesis of Agaritine (Fig. 25) occurring in *Agaricus bisporus* [165], it has been supposed that the GPH derivatives could be synthesized using phenyl hydrazine and L-glutamine through a transpeptidation reaction catalyzed by  $\gamma$ -GTs, and indeed this hypothesis has been proved for EcGT [166].  $\beta$ -N-( $\gamma$ -l(+)-glutamyl)-4-carboxyphenylhydrazine, a precursor of Agaritine, has been similarly prepared from L-glutamine and 4-hydrazinobenzoic acid using EcGT [167].

## 6.2 $\gamma$ -GTs as Cephalosporin Acylases

Cephalosporins, the best-selling antibiotics worldwide, are derived from 7-aminocephalosporanic acid (7-ACA). Currently, in the pharmaceutical industry, 7-ACA is produced from cephalosporin C (CPC) by sequential application of D-amino acid oxidase and cephalosporin acylase (Fig. 26). In this two-step enzymatic process, D-amino acid oxidase catalyzes the oxidative deamination of CPC to 7- $\gamma$ -(5-carboxy-5-oxypentanamide)-cephalosporanic acid, which then autoconvert to glutaryl-7-ACA (GL-7-ACA) [168]. Cephalosporin acylase (CA) then deacylates GL-7-ACA to 7-ACA (Fig. 26). A simpler and more environmentally sound conversion of CPC to 7-ACA by a single enzymatic reaction would be of great interest, but no enzyme catalyzes the direct bioconversion of CPC to 7-ACA at an acceptable rate. CAs are found in various *Pseudomonas* species and are classified into five classes on the basis of their sequence and



**Fig. 26** **a** Enzymatic production of 7-ACA: oxidative deamination reaction catalyzed by D-amino acid oxidase and autoconversion to GL-7-ACA; deacylation reaction catalyzed by class IV CA; **b** Reactions catalyzed by  $\gamma$ -GTs: hydrolysis and transpeptidation

substrate specificity [164]. The primary structure of class IV CAs and  $\gamma$ -GTs is identical by about 30 % and some of these CAs show  $\gamma$ -GT activities. Thus, it has been speculated that Class IV CAs are  $\gamma$ -GTs with adventitious secondary CA activity [169].

Some  $\gamma$ -GTs present GL-7-ACA activity and other members of this enzyme family have been mutagenized to acquire this capability (Fig. 26). In particular,  $\gamma$ -GT from *Bacillus subtilis* possesses an inherent glutaryl-7-aminocephalosporanic acid acylase [40] activity with a  $k_{\text{cat}}$  value of  $0.0485 \text{ s}^{-1}$  and has been used for 7-ACA production. Site-directed and random mutagenesis has further increased its activity. In particular, it has been shown that  $k_{\text{cat}}/K_m$  value increases to  $3.41 \text{ s}^{-1} \text{ mM}^{-1}$  and the  $k_{\text{cat}}$  value increases to  $0.508 \text{ s}^{-1}$  for the D445G and the E423Y/E442Q/D445N triple mutant, respectively. Consequently, the catalytic efficiency and the turnover rate have been improved up to about 1,000-fold and 10-fold, compared with the wildtype BsGT [170].

In EcGT, substitution of D433 abolishes transpeptidase activity, significantly reduces hydrolase activity, but imparts CA activity, which is absent in the wild-type enzyme [166]. In this frame, extremophilic  $\gamma$ -GTs represent natural mutants, lacking D433 and transpeptidase activity [46, 58].

### 6.3 $\gamma$ -GTs as Glutaminases

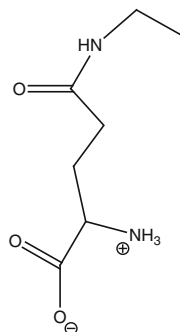
The delicious taste of soy sauce, a traditional Japanese seasoning, mainly depends on the amount of L-glutamic acid, which is an important flavor-enhancing component. During soy sauce fermentation and in the manufacture of bread, proteins are digested into peptides via degradation by enzymes contained in the raw materials and among them, proteases. The peptides are then cleaved into amino-acids by specific peptidases. The glutamine liberated is hydrolyzed to glutamic acid by glutaminases, but when these enzymes are insufficient or inhibited, as often happens in the soy sauce fermentation, the glutamines are converted spontaneously to tasteless pyroglutamic acid.

$\gamma$ -GTs can act as glutaminases, when they act as hydrolase, but lack transpeptidase activity, which could lead to the production of undesired by-products [33, 73–75]. In this respect,  $\gamma$ -GT resistant to high temperatures and high salt concentrations used during these processes are desirable [58, 76]. Minami, Suzuki, and Kumagai have produced and characterized a mutant of BsGT specialized in hydrolase activity, which could be used as a glutaminase in food industry [171]. GthGT also can function as glutaminase, since it is salt-tolerant [76], thermostable [100] and its transpeptidase activity is trascurable [46].

### 6.4 $\gamma$ -GTs in the Production of Theanine

$\gamma$ -L-glutamyl-ethylamide (5-N-ethyl-glutamine or theanine) is one of the major constituents of Japanese green and black teas (Fig. 27), known since 1949 [172] as

**Fig. 27** Structure of theanine



responsible of their taste. Recently, theanine [173] has drawn an increasing interest, especially for its potential medical applications, because it is able to decrease high blood pressure [174] and enhance the memory ability [175], to inhibit the excitation stimulated by caffeine [176] and improve attention [175], to enhance the effects of antitumor agents [177], to reduce stress [178], and to inhibit convulsive action [179]. Theanine is synthesized by theanine synthetase (EC 6.3.1.6) in plants. Since it is expensive and not readily available, several methods have been developed for its production [180]. One of the most effective methods is theanine isolation from dry tea or fresh tea plant leaves, which have high theanine content. However, this procedure is difficult, time-consuming, and expensive. Other methods such as tea callus cultivation [181] and fermentation of the mushroom *Xerocomus badius* [182] have also been reported, but the production efficiency of these procedures is low. Chemical synthesis of theanine has been performed for the first time in 1942 [183] with a yield of  $90 \text{ g kg}^{-1}$  by treating pyrrolidone-5-carboxylic acid with aqueous ethylamine for 20 days at  $37^\circ\text{C}$ . Since then, many other chemical approaches have been carried out, but all have limitations including low yield and purity, and requirement for protection and unblocking of reactive groups [184, 185]. Consequently, there is a demand for other methods of production of theanine. Enzymatic theanine synthesis using  $\gamma$ -GT from *E. coli* and glutamine as substrate have been reported [25, 42, 186]. Similar experiments have proved that theanine can be synthesized also using  $\gamma$ -GT from *P. nitroreducens* [69] or  $\gamma$ -GT from *Bacillus subtilis* SK11.004 [70]. An alternative approach uses glutamic acid  $\gamma$ -methyl ester and immobilized *E. coli* cells with  $\gamma$ -GT activity as catalyst. The results show that activity is about 1.2-fold higher with glutamic acid  $\gamma$ -methyl ester than that with glutamine as substrate. Reaction conditions have been optimized at pH 10 and  $50^\circ\text{C}$ . Under these conditions, the immobilized cells are continuously used 10 times, yielding an average glutamic acid  $\gamma$ -methyl ester to theanine conversion rate of 69.3 %.



## 7 Conclusions

Structural and functional studies on  $\gamma$ -GTs have provided important information about the autocatalysis and enzymatic reaction mechanisms of these enzymes. However, despite considerable progresses have been made in the understanding of their structure–function relationships, there are still many unanswered questions. Indeed, most of the literature on  $\gamma$ -GTs was focused on understanding how the enzymes were autoprocessed and on establishing details of their catalytic mechanism, including the identification of residues that form the  $\gamma$ -glutamyl binding site. However, further kinetic and structural studies on these enzymes are needed to determine details of the mechanisms and relative importance of the transpeptidation and hydrolysis reactions. In this respect, it is important to remark that mechanism of transpeptidation still remains unclear and that residues involved in recognition of acceptor and in formation of the acceptor binding site have not been elucidated. Unraveling the reaction mechanisms of  $\gamma$ -GTs remains one of the major challenges for the rationale design of specific and potent drugs/inhibitors for this class of enzymes. Moreover, the physiological role of the hydrolysis reaction seems to be universally accepted, while that of transpeptidation remains controversial. In mammals,  $\gamma$ -GT catalyze the  $\gamma$ -glutamylation of the amino acids produced upon the hydrolysis of extracellular GSH, and this transfer reaction seems to be a strategy to favor their re-uptake in the cell. In unicellular organisms, the transpeptidase reaction is poor or absent and could play a marginal role. These speculations need further studies to be confirmed.

Virtually nothing is known about the structures of the mammalian membrane-embedded  $\gamma$ -GTs. There are no studies that establish if oligomerization of the precursor and/or hetero-oligomerization of the mature forms are important in controlling enzyme activity and cellular localization. Moreover, the possible presence of sites for ‘allosteric’ regulation has been not still determined. The interplay between the small and the large subunits needs also to be addressed and further investigations on the possibility that these enzymes could be also involved in different metabolic pathways are needed.

Despite recent advancements in predicting the catalytic function of  $\gamma$ -GT mutants, there are big limitations in gain of function studies, probably because of our lack of understanding the molecular mechanisms that define protein behavior in space and time, that is, as flexible and dynamic macromolecules. In this respect, it should be noted that dynamic behavior of  $\gamma$ -GTs has received scarce attention from investigators. New experiments should be performed to elucidate the dynamics of  $\gamma$ -GTs and then assess the contributions of conformational changes and concerted residue motions to enzyme catalysis.

Finally, thermodynamic data on the folding/unfolding pathways of the precursor, as well as on those of the mature protein, could be interesting, since they will provide extremely useful information on the energetic forces that drive the folding and the assembly of the subunits upon the autoprocessing.

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<http://www.springer.com/978-3-0348-0681-7>

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Castellano, I.; Merlino, A.

2013, VIII, 57 p. 28 illus., 18 illus. in color., Softcover

ISBN: 978-3-0348-0681-7