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Authors: Cinzia Calvio, Fabio Romagnuolo, Francesca Vulcano, Giovanna Speranza, Carlo F. Morelli

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Evidences on the role of the lid loop of γ-glutamyltransferases (GGT) in substrate selection

Cinzia Calvio,[1,2] Fabio Romagnuolo,[3] Francesca Vulcano,[1] Giovanna Speranza[3,4] and Carlo F. Morelli[3*]

[1] University of Pavia, Department of Biology and Biotechnology "Lazzaro Spallanzani", via Ferrata, 9 –27100 Pavia – Italy

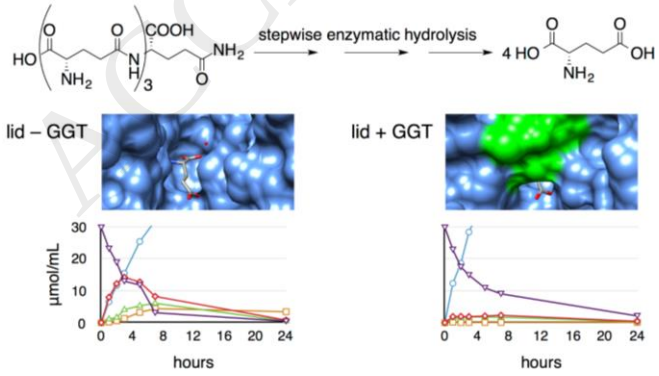
[2] Italian Interuniversity Consortium on Materials, Science and Technology (INSTM), Pavia research Unit, 27100 Pavia (I) – Italy.

[3] University of Milan, Department of Chemistry, via Golgi, 19 – 20133 Milano – Italy

[4] Institute of Molecular Science and Technologies (ISTM-CNR), via Golgi, 19 – 20133 Milano – Italy

[*]**Corresponding author**:
Carlo F. Morelli
University of Milan
Department of Chemistry
via Golgi, 19
20133 Milano – Italy
tel. +39 02 50314099
e-mail: carlo.morelli@unimi.it
Department website: www.chimica.unimi.it
ORCID: 0000-0001-6314-6902

Graphical Abstract:



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Highlights:

- A mutant GGT was obtained by inserting the lid loop of *E. coli* GGT into the structure of *B. subtilis* GGT.
- The role of the lid loop in GGTs was investigated by comparing the activities of the two wt enzymes with those of the mutant.
- The lid loop regulates the access of substrates into the active site, depending on molecular size.
- The presence of the lid loop enhances the transpeptidase activity of the mutant enzyme with respect to the wt counterparts.

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Abstract

γ-Glutamyltransferase (GGT) catalyzes the transfer of the γ-glutamyl moiety from a donor substrate such as glutathione to water (hydrolysis) or to an acceptor amino acid (transpeptidation) through the formation of a γ-glutamyl enzyme intermediate.

The vast majority of the known GGTs has a short sequence covering the glutamate binding site, called lid-loop. Although being conserved enzymes, both *B. subtilis* GGT and the related enzyme CapD from *B. anthracis* lack the lid loop and, differently from other GGTs, both accept poly-γ-glutamic acid (γ-PGA) as a substrate. Starting from this observation, in this work the activity of an engineered mutant enzyme containing the amino acid sequence of the lid loop from *E. coli* GGT inserted into the backbone of *B. subtilis* GGT was compared to that of the lid loop-deficient *B. subtilis* GGT and the lid loop-carrier *E. coli* GGT. Results indicate that the absence of the lid loop seems not to be the sole structural feature responsible for the recognition of a polymeric substrate by GGTs. Nevertheless, time course of hydrolysis reactions carried out using oligo-γ-glutamyl glutamines as substrates showed that the lid loop acts as a gating structure, allowing the preferential selection of the small glutamine with respect to the oligomeric substrates. In this respect, the mutant *B. subtilis* GGT revealed to be more similar to *E. coli* GGT than to its wild-type counterpart. In addition, the transpeptidase activity of the newly produced mutant enzyme revealed to be higher with respect to that of both *E. coli* and wild-type *B. subtilis* GGT. These findings can be helpful in selecting GGTs intended as biocatalysts for preparative purposes as well as in designing mutant enzymes with improved transpeptidase activity.

Keywords: γ-glutamyltranspeptidase; lid-loop; poly-γ-glutamic acid; hydrolysis reaction; transpeptidation reaction

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1. Introduction

γ-Glutamyltransferase (GGT, E.C. 2.3.2.2, known also as γ-glutamyltranspeptidase) is a widely distributed enzyme that catalyzes the breakdown of the γ-glutamyl bond in γ-glutamyl derivatives such as glutathione [1] through the formation of a covalently bound γ-glutamyl enzyme intermediate [2,3]. The γ-glutamyl moiety is then transferred from the enzyme to a water molecule in a hydrolysis reaction, or to an amino acid or a short peptide in a transpeptidation reaction. Mammalian GGTs are membrane-bound, glycosylated enzymes, whereas bacterial GGTs are non-glycosylated and found as soluble proteins in the periplasmic space [4] or extracellularly excreted [5]. Despite these differences in posttranslational modification and localization, GGTs are evolutionarily well conserved. GGT is a heterodimeric enzyme consisting of a small and a large subunit, belonging to the *N*-terminal nucleophile (Ntn) superfamily [6]. The two subunits originate from a single polypeptide chain after an autocatalytic post-translational cleavage [7,8]. The *N*-terminal threonine of the newly formed small subunit is the catalytically active residue involved in both enzyme maturation [8,9] and catalysis [10]. The glutamate binding site of GGTs from different sources is highly conserved, as demonstrated by a number of x-ray crystallographic studies of GGT apoenzymes or GGTs complexed with glutamic acid or inhibitors [9,11-18]. Although GGT is structurally conserved in evolutionary terms, some functional differences can be highlighted among the enzymes from different organisms. Differently from mammalian GGTs, microbial ones accept glutamine as the donor substrate [19,20]. In addition, mammalian GGTs as well as the *E. coli* enzyme show strict stereospecificity for L- acceptor amino acids [21,4] while *B. subtilis* GGT seems to have a broader substrate stereospecificity [22]. Conversely, all GGTs show relaxed stereospecificity towards the configuration of the γ-glutamyl donor compounds, accepting both L- and D-γ-glutamyl derivatives.

GGTs from Eukaryotes and some microorganisms carry a short sequence consisting of 12-16 residues that covers the glutamate binding site, called lid loop [11]. A conserved aromatic residue in

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a central position within the lid loop shields the enzyme-bound glutamate residue [11,16,23]. The nature of this aromatic residue seems to be evolutionary divergent, in that animals have a phenylalanine (Phe433 in *H. sapiens*), whereas bacterial lid loop-containing GGTs have a tyrosine in the same position (Tyr444 in *E. coli*). Indeed, some of the functional differences in the enzymatic activities of mammalian and bacterial GGTs have been ascribed to the different nature of such an aromatic residue [24]. Analysis of the structure of bacterial enzymes revealed that the phenolic hydroxyl group of the tyrosine residue inside the lid loop is hydrogen-bonded to the side chain amide function of a conserved asparagine residue (Asn411 in *E. coli*; Asn400 in *H. pylori*), keeping the lid loop in place but preventing the aromatic ring from occluding the entrance of the active site [23]. The faster transpeptidation reaction observed with mammalian enzymes with respect to bacterial ones has been partially ascribed to the lack of such a hydrogen bond, which allows a higher mobility of the lid loop thus favoring an easier product release [25]. The high mobility of the lid loop in mammalian GGTs was confirmed by recent crystallographic studies carried out on human GGT1 [17].

Even if the lid loop seems to be important for enzyme activity [23], its exact role has not been fully understood. The discussion is even more intriguing and complicated by the fact that the lid loop is ostensibly missing in the GGTs from some microorganisms, which are yet structurally and functionally very similar to lid loop-containing version of the enzyme [26]. GGT from *B. subtilis* [5,14], *B. licheniformis* [27], *Geobacillus thermodenitrificans* [28], *Deinococcus radiodurans*, *Thermus thermophilus* [29] and *Pseudomonas syringae* [30] are among the enzymes lacking the lid loop that have been characterized. Also the GGT-related enzyme CapD from *B. anthracis* is devoid of the lid loop [31]. In *B. anthracis*, the causative agent of anthrax disease, long poly-γ-D-glutamate chains (γ-D-PGA) form a capsule surrounding the bacterial cell constituting a fundamental virulence factor [32]. Such γ-D-PGA capsule allows the bacterium to evade the immune system and represents a physical barrier to antibiotic drugs and phages infection [33]. CapD is involved in the

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formation of the covalent linkage between the γ-D-PGA chains and the meso-diaminopimelic acid residues of the peptidoglycan through a transpeptidation reaction [34,35].

Other microorganisms are able to produce long poly-γ-glutamate chains; *B. subtilis*, *B. licheniformis*, synthetize and excrete γ-DL-PGA, i.e. γ-PGA containing variable amounts of L- and D-glutamic acid residues, depending on environmental conditions [36,37]. GGT from *B. subtilis*, lacking the lid loop, is able to act as an exo-hydrolase towards γ-DL-PGA, removing a glutamic acid residue at a time from the *N*-terminal end of the polymeric chain, irrespectively from the stereochemical configuration of the cleaved glutamate moiety [38]. Conversely, long chains of γ-PGA are not accepted as a substrate by mammalian GGTs or by GGT from *E. coli* and other microorganisms like [39]. The correlation between the lack of the lid loop in both *B. subtilis* GGT and *B. anthracis* CapD and their ability to act on polymeric substrates, although with different reaction specificities (hydrolysis for *B. subtilis* GGT and transpeptidation for *B. anthracis* CapD) has led some authors to propose a role for the lid-loop in determining the maximal size of the substrate entering the catalytic cleft [31]. In our ongoing investigations of the functional features of *B. subtilis* GGT, in view of its possible use as biocatalyst for preparative purposes [20,22], we turned our attention to the possible role of the lid loop in the GGT-catalyzed reactions. We compared the activities of GGTs from *E. coli* (GGT_{Ec}), containing the lid loop, *B. subtilis* (GGT_{Bs}), lacking the lid loop, with a mutant GGT_{Bs} version, on purpose produced by inserting the sequence of the lid loop from *E. coli* GGT into the structure of GGT_{Bs}. In the present study we experimentally verified whether the presence of the lid loop is responsible for the enzyme inability to process γ-PGA. In addition, in order to gain further insights on the possible functional role of the lid loop in enzyme activities, we describe the behavior of the different enzymes towards some oligo-γ-glutamyl glutamines used as model compounds.

2. Material and Methods

2.1 General

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All reagents were from Sigma Aldrich (Darmstadt, Germany) and were used as received. Enzymes and other molecular biology reagents were from NEB (Ipswich, MA, USA). Poly-γ-glutamic acid (γ-PGA) in its salt form (mainly sodium) was from Natto Bioscience Co. (Japan) and was composed of L- and D-glutamic acid residues in a ca 43:57 ratio. HPLC solvents were from Aldrich and were used as received. Oligo γ-glutamylglutamines **1-3** were synthetized as reported in [40].

UV measurements were carried out with a Jasco V-360 spectrophotometer.

HPLC analyses were carried out with a Waters 600 instrument (Millipore) equipped with a HP Series 1050 diode array detector (Palo Alto, CA, USA) using a Gemini RP C18 250×4.60 column (Phenomenex, Torrance, CA, USA). Linear gradients of two eluents were used for elutions. Eluent A was 0.1% trifluoroacetic acid; solvent B was a 80 : 20 mixture of acetonitrile and eluent A. For analysis of compounds derivatized with 1-fluoro-2,3-dinitrobenzene (Sanger's reagent) the gradient was as follows: eluent A : eluent B 80 : 20 isocratic elution for 10 min; gradient to eluent A : eluent B 60 : 40 from 10 to 30 min; isocratic elution for 10 min and gradient to eluent A : eluent B 80 : 20 within 20 min. Flow rate was 1.5 mL/min and detection was at 356 nm. For analysis of compounds derivatized as dabsyl derivatives the gradient was as follows: eluent A : eluent B 80 : 20 isocratic elution for 10 min; gradient to eluent A : eluent B 60 : 40 from 10 to 30 min; isocratic elution for 5 min and gradient to eluent A : eluent B 80 : 20 within 20 min. Flow rate was 1.0 mL/min and detection was at 436 nm.

2.2 Enzymes production and purification

Cloning of the wild type GGT from *B. subtilis* 168 (GGT_{Bs}) was described in [22]. The wild type *E. coli ggt* gene was amplified from strain BL21(DE3) genome (accession number CP001509) using primers FGGTEcX (5'-AAAACTCGAGGCCGCGCCTCCTGCGCCGCCC-3') and RGGTEcB (5'- GGGGATCCTTAGTACCCCGCCGTTAAATCATC-3'), carrying the *Xho*I and *Bam*HI restriction sites (underlined), respectively. The PCR product was restricted and cloned in the corresponding *Xho*I and *Bam*HI sites of a pET16b expression plasmid to give plasmid $pETGGT_{Ec}$. To insert the *E. coli* lid loop in *B. subtilis* GGT three different PCR products were joined in two

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steps. Firstly, the N-terminal part of *B. subtilis ggt* gene was amplified from 168 genome, using primers ggt4X (5'-CTGCTCGAGGCTAAAAAACCGCCCAAAAGC-3') and MTR1ggtBs (5'-AAggtctcCcgttaattcattgtttaaaataacaccg-3') carrying the *Xho*I and *Bsa*I restriction sites, respectively, while the region encompassing the lid loop in *E. coli* GGT was separately amplified from BL21(DE3) genome, using primers MTFintEc (5'-AAggtctcAaacgGATTTCTCCGCCAAACCGGGCGTAC-3') and MTRintEc (5'-AAggtctcAgggtCATCGACGACAGCGGGCGTTTGTTC-3'). The two PCR products were both restricted with *Bsa*I, ligated together and re-amplified with the external primers ggt4X and MTRintEc, forming a fragment which sequence corresponds to the *N*-ter *B. subtilis* GGT fused to the *E. coli* lid-loop region. In the second step the *C*-terminal part of *B. subtilis ggt* gene was also amplified from 168 genome, using primers MTF2ggtBs (5'-AAggtctcGaccccgacgattttatttaagg-3') and ggtRB (5'-CCGGATCCTTATTTACGTTTTAAATTAATGCCG-3') and ligated to the joined product obtained in the previous step upon digestion of both parts with *Bsa*I. The three-fragments product was finally amplified with ggt4X and ggtRB primers and inserted in the *Xho*I and *Bam*HI sites of pET16b to give the plasmid pETLL-GGT.

All plasmids, pETGGT$_{Bs}$, pETGGT$_{Ec}$ and pETLL-GGT were verified by sequencing.

The recombinant proteins were over-expressed and purified from *E. coli* BL21(DE3) cells as previously described [22]. Briefly, *N*-terminal His-tagged proteins were produced in autoinducing medium following the published protocol [41]. The cell pellet was resuspended in K buffer (50 mM Tris-HCl pH 7.5, 20 mM imidazole) and disrupted by sonication. After centrifugation, the cleared lysate was applied to Ni-agarose beads. After extensive washing with K buffer, proteins were eluted with the same buffer supplemented with 500 mM imidazole. Purified GGTs were dialyzed against 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 30 % Glycerol. Protein purity and efficiency of the autocatalytic cleavage [42] were verified by SDS-PAGE analysis (Fig. S1). Aliquots were stored at -80°C till use.

2.3 Measurement of enzyme activity

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Concentration of enzymes was determined by the method of Bradford using bovine serum albumin as standard. A 30 μL aliquot of each of the three enzymes (GGT_{Bs}, $GGT_{LL\text{-}Bs}$ and GGT_{Ec}) was diluted to 300 μL with 0.1 M sodium hydrogen carbonate, pH 8.2. Reactions (final volume 2 mL) were carried out in cuvettes in 0.1 M sodium hydrogen carbonate buffer containing 200 μL of 200 mM glycylglycine and 200 μL of 2.5 mM GPNA, both dissolved in 0.1 M sodium hydrogen carbonate. Reactions were initiated by adding 20 μL of the enzyme solution. The release of *p*-nitroaniline was followed continuously with a spectrophotometer at λ = 410 nm, recording data every 10 sec for 3 min. The slope of the resulting curves in their initial linear range represented the initial velocities of the reactions. A unit of enzyme was defined as the amount of enzyme that liberates 1 μmol/min of *p*-nitroaniline in the presence of glycylglycine. Specific activities of the enzymes were calculated from the protein concentration previously determined and are the following: GGT_{Bs} 256 U/mg, GGT_{Ec} 64 U/mg, $GGT_{LL\text{-}Bs}$ 36 U/mg.

2.4 Pre-column derivatization procedure with 1-fluoro-2,4-dinitrobenzene (Sanger's reagent)

100 μL of solution to be derivatized (reaction mixture or standard solution for calibration curve) was diluted with 850 μL of borate buffer at pH 8.5, then 50 μL of L-serine 50 mM in water was added as the internal standard. After shaking, 100 μL of the mix was transferred into a pyrex tube equipped with a perforated screw cap fitted with a forcible sealing septum. 400 μL of borate buffer pH 8.5 was added, followed by 500 μL of 10 mM 1-fluoro-2,4-dinitrobenzene solution in acetone. The tube was sealed and heated at 70 °C in a pre-heated water bath for 45 min in the dark. A needle was then inserted into the forcible septum and heating was continued for further 10 min, during which time most of the acetone evaporated. The tube was cooled under running water; 500 μL of the resulting yellow mixture was withdrawn, diluted with 500 μL of 0.1% TFA solution and used for HPLC analysis. The derivatized solution is stable and can be stored in a refrigerator.

2.5 Pre-column derivatization procedure with dabsyl chloride

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100 μL of solution to be derivatized (reaction mixture or standard solution for calibration curve) was diluted with 870 μL of borate buffer at pH 8.5, then 30 μL of L-serine 50 mM in water was added as the internal standard. After shaking, 300 μL of the mix was transferred into a pyrex tube equipped with perforated screw cap fitted with a forcible sealing septum. 300 μL of borate buffer pH 8.5 was added, followed by 400 μL of 10 mM dabsyl chloride solution in acetone. The tube was sealed and heated at 70 °C in a pre-heated water bath for 10 min. A needle was then inserted into the forcible septum and heating was continued for further 5 min, during which time most of the acetone evaporated. The tube was cooled under running water; 500 μL of the resulting red solution was withdrawn, diluted with 300 μL of 0.1% TFA solution and used for HPLC analysis. The derivatized solution is stable if stored at -20 °C.

2.6 Enzymatic hydrolysis of γ-PGA

A stock solution containing 20 mg/mL γ-PGA was prepared and the pH was adjusted to 7.5. 750 μL of the γ-PGA solution was placed in an eppendorf tube; 150 μL water was added, and the reaction was initiated by adding 100 μL of the standardized enzyme (50 U). Final concentration of γ-PGA in the reaction mixture was 15 mg mL^{-1}. Reactions were carried out at 23 °C. At time intervals (0, 1, 2, 3, 5, 7 and 24 hours) 100 μL of the reaction mixture was withdrawn and derivatized with Sanger's reagent as described, but instead being diluted with 0.1% TFA, 500 μL of the derivatized mixture was transferred into a pyrex tube with screw cap, 500 μL 6 M HCl was added, the tube was sealed and heated at 105 °C for 24 h. After cooling, the mixture was analyzed by HPLC with no further dilution.

2.7 Enzymatic hydrolysis of oligo-γ-glutamylglutamines

Stock solutions of the starting material (γ-glutamylglutamine **1**, γ-glutamyl-γ-glutamylglutamine **2** or γ-glutamyl-γ-glutamyl-γ-glutamylglutamine **3**) were prepared at 40 mM concentration in HEPES buffer at pH 7.5. 750 μL of the stock solution was placed in an eppendorf tube and diluted with 150 μL of HEPES buffer. Reactions were initiated by adding 100 μL of the standardized enzyme

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solution (50 U) and were carried out at 23 °C. Final volume was 1 mL; final concentration of the starting material in the reaction mixtures was 30 mM. At fixed time intervals (0, 1, 2, 3, 5, 7 and 24 h reaction time) 100 μL of the reaction mixtures was withdrawn, derivatized with dabsyl chloride as described and analyzed by HPLC.

3. Results and discussion

3.1 Enzyme design, expression and purification

The amino acid sequences of the enzymes from *B. subtilis* and *E. coli*, the Gram positive and Gram negative model organisms, respectively, were aligned. The EMBOSS Needle web tool confirmed their high similarity (56.9%) and identity (39.9%). As expected a gap in GGT_{Bs} corresponds the lid loop region of GGT_{Ec}, which is however surrounded by conserved amino acids (Figure 1). Exploiting such sequence conservation, the lid loop region in GGT_{Ec} was inserted in the backbone of the *B. subtilis* enzyme together with 5 and 6 codons at the *N*-terminal and *C*-terminal ends, respectively, producing a soluble recombinant protein regularly exhibiting autocatalytic processing (Fig. S1) [42]. The mutant enzyme was named lid loop *B. subtilis* GGT ($GGT_{LL\text{-}Bs}$).

3.2 Enzymes standardization

To assess the different behavior of the three enzymes towards the polymeric γ-PGA substrate, activities of the enzymes were standardized with respect to a common reaction. The standard method based on the chromogenic substrate γ-glutamyl-*p*-nitroanilide as the donor and glycylglycine as the acceptor compound was used [43]. The mutant $GGT_{LL\text{-}Bs}$ efficiently catalyzed the transpeptidation reaction, thus proving that the modified enzyme is not only soluble and auto-catalytically competent but also functionally active.

Taking into account the relative activities thus determined, the initial batches of the three enzymes were differentially diluted in order to have the same concentration in enzyme units [40]. Differences in activity in the reactions in which γ-PGA and oligo γ-glutamylglutamines were the substrates could therefore be only attributable to the nature of the substrates and would be easily comparable.

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3.3 Enzymatic hydrolysis of γ-PGA

The ability of GGT_{Bs}, $GGT_{LL\text{-}Bs}$ and GGT_{Ec} to degrade γ-PGA was first tested by checking the formation of free glutamic acid in the reaction mixtures through TLC (Figure S2). In line with previous data, the appearance of a spot attributable to glutamic acid was evident in reactions catalyzed by GGT_{Bs} and absent in reactions carried out with GGT_{Ec}. Several attempts carried out with increasing amounts of GGT_{Ec} enzyme or longer incubation times never allowed detection of glutamic acid released from γ-DL-PGA. Despite the presence of the lid loop, $GGT_{LL\text{-}Bs}$ was able to release glutamic acid from γ-PGA. A quantitative comparison of the activities of the three enzymes towards γ-PGA was thus obtained by monitoring the release of glutamic acid by HPLC (Scheme 1). It is to note that the number of chain ends does not increase with time being GGTs exo-hydrolases, i.e. removing one residue at time from the *N*-terminal end of the polymeric chains. These experiments (Figure 2) showed that the γ-PGA-hydrolysis reaction carried out by the mutant $GGT_{LL\text{-}Bs}$ was efficient although it proceeds at a lower initial rate than the reaction catalyzed by GGT_{Bs}. The inability of GGT_{Ec} to accept γ-PGA as a substrate was also confirmed.

From this result it can be concluded that the lack of the lid-loop is not the sole structural feature that enables GGT_{Bs} to accept a polymeric material as a substrate, even if the possibility that the lid-loop inserted into $GGT_{LL\text{-}Bs}$ is not completely functional cannot be ruled out.

In the search of a possible role of the lid-loop in substrate selection, γ-glutamyl oligos of increasing length were tested in hydrolysis reactions catalyzed by $GGT_{LL\text{-}Bs}$ and results were compared with those obtained with the wild type (wt) GGTs, from *B. subtilis* and *E. coli*.

3.4 Enzymatic hydrolysis of oligomers of increasing length

γ-Glutamylglutamine (**1**), γ-glutamyl-γ-glutamylglutamine (**2**) and γ-glutamyl-γ-glutamyl-γ-glutamylglutamine (**3**) (Figure 3) were selected as substrates of increasing length and subjected to enzyme-catalyzed hydrolysis in the presence of the three GGTs. Although γ-glutamylglutamine is not a physiological substrate for GGT_{Ec}, it is nevertheless accepted by the enzyme (see below). Indeed, it is transiently formed in GGT_{Ec}-catalyzed reactions through autotranspeptidation of

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glutamine and becomes a donor substrate in later stages of the reaction [44]. Moreover, glutamine is recognized as a donor substrate both by *B. subtilis* and *E. coli* GGT [4,5], therefore complete hydrolysis of the starting materials is expected to produce glutamic acid as the sole ultimate product. Indeed glutamic acid can only arise from the hydrolysis of the γ-glutamyl enzyme intermediate by means of a water molecule. This represents an irreversible step of the entire process, as GGTs do not accept glutamic acid as a γ-glutamyl donor substrate [5].

Hydrolysis of γ-glutamylglutamine **1** by GGT_{Bs} was accompanied by the expected formation of free glutamic acid and glutamine (Fig. 4A). The concentration of glutamine in the reaction rose up to ca 10 mM within three hours, when it equaled the concentration of residual compound **1**; then gradually decreased due to its enzymatic hydrolysis to glutamic acid.

When the hydrolysis of compound **1** was catalyzed by GGT_{Ec}, glutamine concentration never reached a substantial level but remained low and rather constant throughout the entire reaction course. The low level of free glutamine is accompanied by a lower rate of decrease of γ-glutamylglutamine concentration in the reaction, which indicates that GGT_{Ec} has a non-negligible transpeptidase activity, that mediates the re-synthesis of compound **1**, indistinguishable from the starting material, which indeed was still present after 24 hours reaction time. In fact the concentration of glutamic acid, the ultimate reaction product, never reached the expected value (Fig. 4B). The recognition of a little peak attributable to γ-glutamylglutamic acid in the HPLC chromatograms further supports the hypothesis of moderate transpeptidase activity.

When the hydrolysis of compound **1** was catalyzed by the mutant $GGT_{LL\text{-}Bs}$ (Figure 4C) the concentration of the intermediate glutamine rose at the beginning of the reaction and then remained fairly constant up to 24 hours. At each time point the concentrations of the hydrolysis products glutamine and glutamic acid did not account for the amount of the starting material consumed, as the glutamic acid concentration increased very slowly. This can be due to a substantial transpeptidase activity, witnessed by the appearance in the chromatograms of several little peaks

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corresponding to oligomers containing up to four γ-glutamyl residues linked to a single glutamine molecule. The peak of γ-glutamylglutamic acid was also identified.

From the above data a first conclusion can be envisaged; both lid-containing enzymes, GGT_{Ec} and mutant $GGT_{LL\text{-}Bs}$, preferentially accept the smaller substrate glutamine rather than the larger γ-glutamylglutamine, while GGT_{Bs} shows a lower substrate preference. Such a substrate preference for GGT_{Ec} was verified in an independent experiment, in which glutamine was the only substrate present. GGT_{Ec} completed glutamine hydrolysis within 40 min in the same experimental conditions (Fig. 4I). A small peak attributed to the autotranspeptidation product γ-glutamylglutamine (**1**) appeared transiently in the chromatograms prior to complete hydrolysis, thus confirming a residual transpeptidase activity of this enzyme.

In the experiments where γ-glutamyl-γ-glutamylglutamine (**2**) and γ-glutamyl-γ-glutamyl-γ-glutamylglutamine (**3**) were used as substrates the differential preference for the type of substrate shown by the three GGTs was further confirmed. In the GGT_{Bs}-catalyzed hydrolysis of compound **2** a clear time-dependent appearance of the intermediate products was observed during the reaction (Figure 4D). The same trend, characterized by the stepwise appearance of intermediates, was observed also for the hydrolysis of compound **3** (Figure 4G).

When compounds **2** and **3** were used as substrates for the GGT_{Ec}-catalyzed reaction, the concentration of glutamic acid rose very rapidly while the concentrations of the intermediate products remained low, fairly constant and similar to each other during the whole reaction courses (Figures 4E and 4H).

Similarly, in the hydrolysis reaction of compound **2** catalyzed by $GGT_{LL\text{-}Bs}$ the concentration of intermediate hydrolysis products rose at the beginning of the reaction and then reached a fairly constant level up to 24 hours (Figure 4F). This reaction trend is different from that observed for the wt counterpart, for which a maximum concentration of intermediate products is reached prior to their slow decrease (cfr Figure 4D and Figure 4F). In the $GGT_{LL\text{-}Bs}$-catalyzed reaction the peak attributable to the transpeptidation product γ-glutamyl-γ-glutamyl-γ-glutamylglutamine (**3)** was

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clearly visible in the chromatograms and easily integrated, therefore the concentration of this species was quantified. The concentrations of the intermediate hydrolysis product γ-glutamylglutamine **1** and of the transpeptidation product **3** were similar and remained nearly constant up to 24 hours.

The reactions catalyzed by the two enzymes showed typical profiles of consecutive reactions (Figure 5), in which the relative size of the rate constants is responsible for the observed trends. A rigorous mathematical description of the observed kinetics is out of the scope of this work, yet it should take into account several complicating factors, such as the liberation of one molecule of glutamic acid for each γ-glutamylglutamine molecule hydrolyzed, the reversible and consecutive nature of the involved reactions [45], potential inhibitory phenomena [46] and the formation of the competitive substrate glutamine during the reaction [47]. Nevertheless, the relative size of the rate constants here involved may be hypothesized in order to give a picture of the observed trends. In the GGT_{Bs}-catalyzed reaction, accumulation of glutamine is observed prior that its enzyme-catalyzed hydrolysis becomes appreciable. This reaction profile suggests that the rate constant for the two consecutive reactions should be similar ($k_1 \approx k_2$). On the other hand, the low concentration of glutamine observed throughout the reaction course in the GGT_{Ec}-catalyzed reaction implies that consumption of glutamine, mainly by hydrolysis and, at a minor extent, as acceptor substrate in autotranspeptidation reaction, is faster than the hydrolysis of the starting material (k_{-1}, $k_2 > k_1$). Therefore, the time course observed in Figure 4B is better explained by a steady state kinetics. The general scheme for consecutive reactions in Figure 5 can then be rewritten as in Figure 6A, in order to take into account that the reactions involved are enzyme-catalyzed and proceed through a ping-pong mechanism [1]. Since the rate of hydrolysis of the γ-glutamyl enzyme intermediate, represented by the rate constant k_{hydr}, does not change in the reactions catalyzed by the same enzyme, the different rate profiles observed for the two enzymes must depend from the preceding steps. It can then be concluded that the rate-determining step is most likely the formation of the

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enzyme-substrate complex or the acylation step leading to the γ-glutamyl-enzyme intermediate, or a combination of the two.

The observed trends reported in Fig. 4A,D,G for GGT_{Bs}-catalyzed reactions imply that the apparent rate constants k_{obs} (Figure 6B) for the various reaction steps, which incorporate also the rate constant for the reverse reactions, have to be very similar to each other (Figure 6B, in which $k_{obs1} \approx k_{obs2} \approx k_{obs3}$). It follows that GGT_{Bs} shows no preferences in substrate selection, rather it acts preferentially on the more abundant compound in the reaction mixture. As a consequence, in the hydrolysis of oligomeric γ-glutamylglutamines the intermediate products of the reactions were formed in a sequential fashion, in such a way that the maximum concentration of each intermediate was reached after the concentration of its immediate precursor started to decrease. On the contrary, the lid loop-containing GGT_{Ec} seems to preferentially hydrolyze glutamine over the di-, tri- and tetrapeptide, i.e. the rate of glutamine hydrolysis is higher than the rate of hydrolysis of the oligomeric poly-γ-glutamylglutamines ($k_{obs3} >> k_{obs2}$ and k_{obs1} in Figure 6B). The higher k_{obs3} is confirmed by the glutamine hydrolysis rate shown in Figure 4I. The net result is a low and stationary concentration of glutamine and other intermediate products in the reaction mixtures, while the concentration of the ultimate product, glutamic acid, raises very rapidly with respect to a moderate decrease in the concentration of polymeric compounds.

Surprisingly, the lid loop-containing mutant $GGT_{LL\text{-}Bs}$ showed reaction profiles reminiscent of those catalysed by GGT_{Ec}, rather than those catalysed by the *Bacillus* wt counterpart, characterized by fairly stationary concentrations of the intermediate products throughout the reaction course, rather than their time-dependent formation.

4. Conclusions

Through the construction of the mutant enzyme $GGT_{LL\text{-}Bs}$, obtained by inserting the lid loop of GGT_{Ec} into the structure of GGT_{Bs}, we were able to gain some experimental evidences about the role of the lid loop in substrate selection.

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In both GGT_{Ec} and $GGT_{LL\text{-}Bs}$ the lid loop behaves as a gating structure at the entrance of the active site, able to orient the enzyme towards small glutamine with respect to bulkier derivatives. It is therefore conceivable that the presence of the lid loop poses a limit in substrate size accepted by GGT_{Ec}, thus preventing the high molecular weight γ-PGA from being a substrate for GGT_{Ec} [31]. This is in line with the evidence that the lack of the lid loop is shared by enzymes able to accept polymeric γ-PGA as a substrate (GGT_{Bs} and Cap D from *B. anthracis*) [31]. However, our results show that this cannot be the sole structural feature preventing the processing of larger substrates, since $GGT_{LL\text{-}Bs}$ was still able to hydrolyze long γ-PGA chains. In addition, the presence of the lid loop favors the transpeptidase activity, slightly in GGT_{Ec} and more evidently in $GGT_{Bs\text{-}LL}$, probably by shielding the reactive carboxyl group of the γ-glutamyl enzyme intermediate from the bulk water.

Results here reported might be of interest for the choice of bacterial GGTs intended as biocatalysts for preparative purposes [48-50], as they can be helpful in designing the most appropriate reaction conditions. They can be also taken into account for the engineering of mutant enzymes with enhanced transpeptidase activity.

Conflicts of interest: Authors declare no conflicts of interest.

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Figure legends

Figure 1. Alignment of the amino acid sequence of GGTs from *B. subtilis* and *E. coli* and the recombinant LL-GGT. The GGT from *B. subtilis* (WP_003231470.1) and *E. coli* (WP_000595082.1) were aligned using T-coffee [51]. The sequence of the modified LL-GGT, in red, has been superimposed to the alignment. The region shadowed in grey is derived from *E. coli* GGT; the Lid Loop domain there contained is further highlighted in bold.

Figure 2. Time course of glutamic acid liberation during GGT-catalyzed hydrolyses of γ-PGA.

Figure 3. Chemical structures of oligo-γ-glutamylglutamines **1**-**3** subjected to GGT-catalyzed hydrolysis as model substrates.

Figure 4. Time-dependent profiles of GGT-catalyzed hydrolyses of oligo-γ-glutamylglutamines **1**-**3**. GGT-catalyzed hydrolysis of γ-glutamylglutamine **1** with (A) GGT_{Bs}; (B) GGT_{Ec}; (C) $GGT_{LL\text{-}Bs}$.

GGT-catalyzed hydrolysis of γ-glutamyl-γ-glutamylglutamine **2** with (D) GGT_{Bs}; (E) GGT_{Ec}; (F) $GGT_{LL\text{-}Bs}$.

GGT-catalyzed hydrolysis of γ-glutamyl-γ-glutamyl-γ-glutamylglutamine **3** with (G) GGT_{Bs}; (H) GGT_{Ec}.

(I) GGT_{Ec}-catalyzed hydrolysis of glutamine. All substrates were used at 30 mM initial concentration. Concentration axis in graphs D-H is limited to 30 mM in order to appreciate the variations in the concentrations of the intermediates during the reaction, in particular the time-dependent appearance of intermediates in the GGT_{Bs}-catalyzed reactions. Concentration of glutamic acid in graphs D-H is therefore out of range.

Figure 5. Consecutive reactions in the GGT-catalyzed conversion of γ-glutamylglutamine **1** to glutamic acid with liberation of intermediate glutamine.

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Figure 6. GGT-catalyzed consecutive hydrolysis reaction proceeding through the ping-pong mechanism involving a γ-glutamyl-enzyme intermediate. (A) General scheme for a GGT-catalyzed hydrolysis. The transfer of the γ-glutamyl moiety to a water molecule affording free glutamic acid is an irreversible step of the process. (B) Relative size of the observable rate constants k_{obs1-3} in the enzyme-catalyzed hydrolyses of γ-glutamyl-γ-glutamylglutamine **2**. Values of k_{obs1-3} close to each other explain the profiles observed in Figures. 4A,D,G for GGT_{Bs}-catalyzed reactions. A substantial higher rate constant k_{obs3} in comparison with k_{obs2} and k_{obs1} leads to reaction profiles similar to those observed in the GGT_{Ec} (Figures 4B,E,H) and $GGT_{LL\text{-}Bs}$-catalyzed hydrolyses (Figures 4C,F).

Scheme 1. Pre-column derivatization of glutamic acid liberated during GGT-catalyzed γ-PGA hydrolysis. The amount of glutamic acid deriving from the *N*-terminal ends after acidic hydrolysis of the polymeric chains proved to be negligible with respect to the amount liberated in the enzyme-catalyzed hydrolysis.

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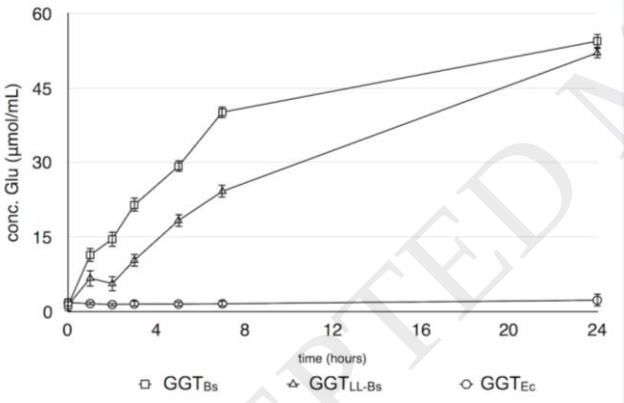
Figures:



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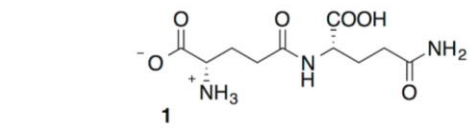

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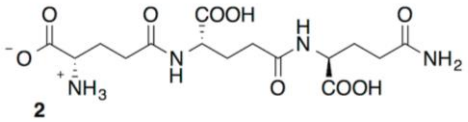

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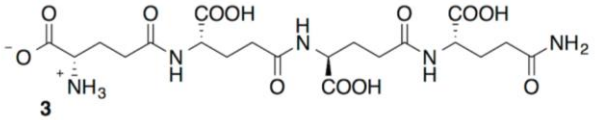

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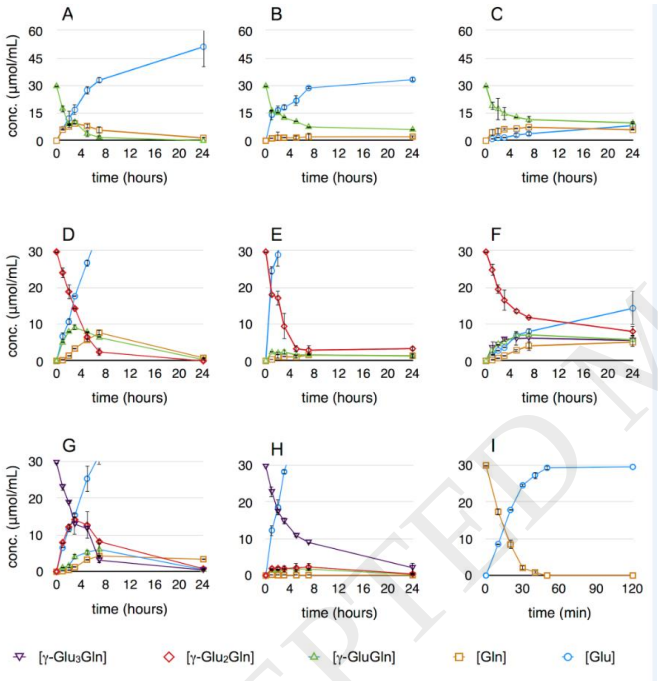

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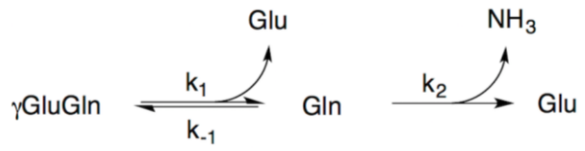

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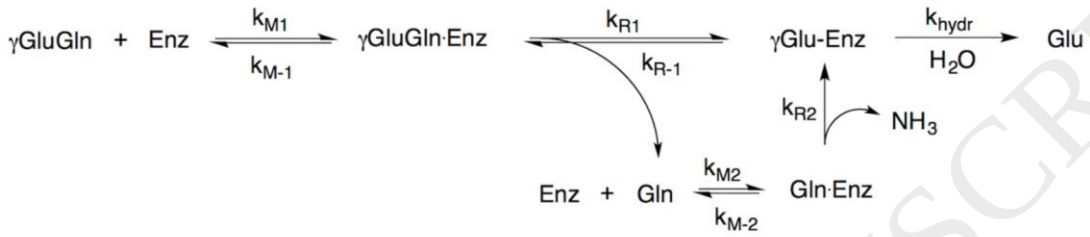

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