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Low resolution X-ray structure of γ-glutamyltranspeptidase from *Bacillus licheniformis*: Opened active site cleft and a cluster of acid residues potentially involved in the recognition of a metal ion

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ABSTRACT

γ-Glutamyltranspeptidases (γ-GTs) cleave the γ-glutamyl amide bond of glutathione and transfer the released γ-glutamyl group to water (hydrolysis) or acceptor amino acids (transpeptidation). These ubiquitous enzymes play a key role in the biosynthesis and degradation of glutathione, and in xenobiotic detoxification.
Here we report the 3 Å resolution crystal structure of *Bacillus licheniformis* γ-GT (*Bl*GT) and that of its complex with L-Glu. X-ray structures confirm that *Bl*GT belongs to the N-terminal nucleophilic hydrolase superfamily and reveal that the protein possesses an opened active site cleft similar to that reported for the homologous enzyme from *Bacillus subtilis*, but different from those observed for human γ-GT and for γ-GTs from other microorganisms. Data suggest that the binding of L-Glu induces a reordering of the C-terminal tail of *Bl*GT large subunit and allow the identification of a cluster of acid residues that are potentially involved in the recognition of a metal ion. The role of these residues on the conformational stability of *Bl*GT has been studied by characterizing the autoprocessing, enzymatic activity, and chemical and thermal denaturation of four new Ala single mutants. The results show that replacement of Asp568 with an Ala affects both the autoprocessing and structural stability of the protein.

1. Introduction

γ-Glutamyltranspeptidases (γ-GTs, EC 2.3.2.2) are enzymes belonging to the class of N-terminal nucleophilic (Ntn) hydrolases which initiate the breakdown of extracellular glutathione (γ-Glu-Cys-Gly, GSH) into its constituent amino acids, glutamate, cysteine, and glycine and catalyze the transfer of its γ-glutamyl moiety to water, amino acids or small peptides [1–5]. They are widely distributed in nature from mammals to bacteria and involved in many crucial cellular events such as aging, senescence and drug detoxification [6,7]. In humans, γ-GT has been associated with several physiological disorders related to oxidative stress, such as Parkinson's disease and diabetes [8,9]; it is currently used as a marker of liver diseases [10], cardiovascular diseases [11,12] and cancer [13–16].

In mammalians, γ-GTs are membrane heterodimeric glycoproteins that are localized at the cell surfaces (mainly in the kidney); in bacteria, γ-GTs are found in the cytosol or in the periplasmic space and can be heterodimeric [1,7] or heterotetrameric [18]. Both mammalian and microbial proteins undergo an autoproteolytic post-translational cleavage (autoprocessing) into a heavy and a light chain [19], usually called large and small subunits, respectively [20].

The catalytic cycle of mature γ-GTs follows a ping-pong mechanism [1–3,20]. In the first step, the γ-glutamyl moiety of the donor substrate (e.g., GSH or GSH conjugate) reacts with the hydroxyl group of a conserved Thr in the active site to generate a tetrahedral intermediate [1,3,20]. In the case of the human enzyme (hGT), the essential catalytic residue is Thr380. Subsequent release of the free amine donor (e.g., Cys–Gly) from the intermediate produces a covalent γ-glutamyl enzyme intermediate. The cycle is finished when the γ-glutamyl moiety is transferred to an acceptor substrate, such as water (hydrolysis) or a dipeptide (transpeptidation), releasing glutamate or a γ-glutamyl dipeptide, respectively. Both hydrolysis and transpeptidation reactions are believed to have physiological importance, although the role of transpeptidation in bacteria is not clear [1,18]. Proteins from different sources are characterized by a broad substrate specificity, which includes a large variability of acceptors.

Beyond their physiological function, γ-GTs can be employed for the biosynthesis of various γ-glutamyl compounds, with great potential for pharmaceutical and biotechnological applications [1,5,21,22]. In fact, it has been shown that these proteins can be used as glutaminase in food industry [23,24], as cephalosporinacylases [25], in the production

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of theanine [26], and in the biosynthesis of flavor enhancers and medical compounds [27,28].

Although γ-GTs have been characterized from many different sources, crystal structures of just five members of their family have been solved [20,29,31–33]; two out of these have not been described in literature and one has been deposited in the Protein Data Bank when this manuscript was in preparation [33]. Biochemical studies and X-ray structures of γ-GTs have provided insights on the mechanisms of autoprocessing and hydrolysis reaction [19,20,30,34] and on their structural stability [35,36].

As a further step in the structural characterization of γ-GTs, here we report the crystal structure of the protein from *Bacillus licheniformis* (*Bl*GT) at 2.98 Å resolution, and that of its complex with L-Glu at 3.02 Å resolution. Data demonstrate that the protein possesses an opened active site cleft and show that the binding of L-Glu induces a reordering of the C-terminal tail of the large subunit of the protein. The results allow the identification of a cluster of acid residues that are potentially involved in the recognition of a metal ion. The role of these residues on the structure, stability and catalytic activity of the protein has been studied using alanine-scanning mutagenesis. The substitution of Asp568 significantly affects the biophysical behavior of the protein.

2. Materials and methods

2.1. Crystallization, structure determination and refinement

Protein preparation and materials and methods of crystallization were already described [37]. Briefly, the best crystals of *Bl*GT (UNIPROT code A9YTT0) have been grown using hanging drop vapor diffusion method, protein concentration 20 mg mL^{-1} and a precipitant solution consisting of 20% (w/v) PEG 3350, 0.2 M $MgCl_2$ hexahydrate, 0.1 M Tris–HCl (pH 8.2) [37]. Crystals of the complex between *Bl*GT and L-Glu have been obtained by performing a screening around the crystallization conditions previously used to grow crystals of *Bl*GT. The best crystals appeared in drops consisting of 22% (w/v) PEG 3350, 0.2 M $MgCl_2$ hexahydrate, 0.1 M Tris–HCl (pH 8.0), protein solution at a concentration of 10 mg mL^{-1} and 8.5 mM L-Glu. Crystals were flash-cooled in liquid nitrogen at 100 K. X-ray diffraction data were collected at the Institute of Biostructures and Bioimages, CNR, Naples, Italy using a Rigaku MicroMax-007 HF generator producing Cu Kα radiation (45 kV and 60 mA) and equipped with a Saturn 944 CCD detector. The crystal-to-detector distance was set to 70 mm, with all frames collected at 100 K. Diffraction data were recorded over a 350° rotation of the crystal with a width of 0.5° per image. Diffraction data were processed using the program HKL2000 [38] and CCP4 program suite [39]. Data collection statistics and scaling results are listed in Table S1. Crystals diffract at about 3 Å resolution and belong to the space group $P2_12_12_1$. Since the known structures of γ-GTs have been determined at high resolution (<2 Å), many attempts have been done to improve the diffraction power of *Bl*GT crystals, but unfortunately, all these trials failed. Different cryoprotection strategies, including the crystal dehydration [40] and the flash-cooling in the absence of cryoprotectant [41] have been used, but in all cases we are not able to improve the resolution of the collected data sets. Since the *Bl*GT crystals are very fragile, they do not resist to the transport to synchrotrons.

Initial phases were determined by a molecular replacement method with the program Phaser [42], using the structure of the protein from *Bacillus subtilis* (*Bs*GT) [32], which shares about 70% of sequence identity with *Bl*GT, as search model. The structures were refined using the programs Refmac5.7 [43] and Coot [44]. Mg^{2+} ions were introduced in the model in correspondence with peaks above 5σ in the Fo–Fc electron density map. The number of residues in the most favored regions of a Ramachandran plot [45] was determined by the program PROCHECK [46]. Cavities were identified using Fpocket [47].

The atomic coordinates and structure factors for *Bl*GT (with PDB ID: 4OTT) and its complex with L-Glu (PDB ID: 4OTU) were deposited in the Protein Data Bank (http://www.rcsb.org/).

2.2. Site-directed mutagenesis

Mutant proteins were constructed using a template plasmid pQE-*Bl*GGT that expresses an N-terminal His_6-tagged form of *Bl*GT. Mutations were created by a QuikChange site-directed mutagenesis kit obtained from Stratagene (La Jolla, CA, USA). The kit essentially employs the high fidelity *PfuTurbo* DNA polymerase and appropriate forward and reverse primers (Table S2) to replicate both strands of the supercoiled plasmid containing the *γ-GT* gene. Following the thermal cycling, the reaction mixture was treated with *DpnI* endonuclease that digests the hemimethylated template leaving behind the nicked DNA with the mutation of interest. After transformation of the digested mixture into *Eschericchia coli* XL-1 blue supercompetent cells, single colony was picked up to verify and confirm the mutations by DNA sequencing.

2.3. Protein expression and purification

For biochemical and biophysical analyses, wild-type and mutant *Bl*GTs were expressed in *E. coli* M15 (pRep4) as previously described [37,48]. Target proteins were purified by affinity chromatography with a Ni^{2+}–NTA agarose column under native conditions. The eluted fractions (total volume for each preparation = ~5 mL) were pooled and dialyzed overnight against 500 mL of 10 mM Tris–HCl buffer (pH 8.0) through a 10-kDa cutoff membrane to remove salts.

Protein purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) carried out on 12% acrylamide slabs using the buffer system of Laemmi [49]. Before electrophoresis, the samples were heated to 100 °C for 5 min in dissociating buffer containing 2% SDS and 5% 2-mercaptoethanol. Protein concentration was measured with a protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as the standard.

2.4. Autoprocessing of the enzymes

Autocatalytic processing studies on *Bl*GT and its mutants (1 mg mL^{-1}) were performed by incubating the proteins in 20 mM Tris–HCl, pH 8.0 at 45 °C up to two days. Aliquots were denatured by boiling in SDS loading buffer for 5 min. Samples were analyzed by SDS-PAGE 12%, and the gel was stained using Coomassie Brilliant Blue.

2.5. Enzyme activity assay and determination of kinetic parameters

The enzyme activity of wild-type and *Bl*GT variants towards substrate analog L-γ-glutamyl-*p*-nitroanilide (G*p*NA) was determined spectrophotometrically by monitoring the *p*-nitroaniline released from G*p*NA at 412 nm using the method assay by Tate and Meister [50]. One unit of γ-GT activity is defined as the amount of enzyme that produced 1 μmol of *p*-nitroaniline per min under the assay conditions. Catalytic activity was measured in 50 mM Tris–HCl buffer, pH 8.0, and containing 1.25 mM G*p*NA, 30 mM Gly–Gly, 1 mM $MgCl_2$, and 20 μL of enzyme solution at a suitable dilution, and enough distilled water to bring the final volume to 1 mL. The reaction mixtures were incubated at 25 °C for 10 min. The reaction was stopped by the addition of 100 μL of 3.5 N acetic acid.

To evaluate kinetic constant, dependence of the reaction on the substrate concentration was evaluated using fixed amounts of purified *Bl*GT (or of its mutants) incubated with increasing concentrations of G*p*NA from 1 to 1000 μM. Saturation kinetics were observed, and data were fit to the Michaelis–Menten equation to obtain K_M and V_{max} for the catalyzed reaction.

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