Contribution of Ser463 residue to the enzymatic and autoprocessing activities of *Escherichia coli* γ-glutamyltranspeptidase

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A serine residue Ser463, required for proper function of *E. coli* γ-glutamyltranspeptidase (*EcGGT*) was identified by site-directed mutagenesis on the basis of sequence alignment of human, pig, rat, and three bacterial enzymes. Thr-, Asp-, and Lys-substituted variants were overexpressed in *E. coli* M15 cells and the recombinant proteins were purified to near homogeneity by nickel-chelate chromatography. With the exception of S463T, the other two variants completely lost GGT activity, implying the importance of this residue in enzyme maturation. Measurements of intrinsic tryptophan fluorescence revealed alteration of the microenvironment of aromatic amino acid residues in S463D and S463K, while circular dichroism (CD) spectra were nearly identical for wild-type and all mutant enzymes. The temperature-dependent signal in the far-UV region for S463T was consistent with that of wild-type enzyme, but S463D and S463K showed a different sensitivity towards temperature-induced denaturation. These results implied that a significant conformational change occurred as a result of Asp- and Lys-substitution.

**Keywords:** *E. coli*, γ-Glutamyltranspeptidase, Site-specific mutagenesis, Autocatalytic processing, Tryptophan emission fluorescence, Circular dichroism

γ-Glutamyltranspeptidase (GGT; EC 2.3.2.2) is a heterodimeric enzyme that catalyzes the cleavage of the glutamyl linkage of γ-glutamyl compounds and the transfer of their γ-glutamyl group to other amino acids and peptides. The reaction catalyzed by GGT is known to proceed through a modified ping-pong mechanism. GGT is found from bacteria to mammals and is generally considered to be involved in the metabolism of glutathione and in salvaging of cysteine. In mammals, the enzyme is located on the exterior surface of cells and is composed of a membrane-bound α subunit of 42 kDa, linked non-covalently to a β subunit of 20 kDa.

In *E. coli*, the α subunit is somewhat smaller (39 kDa) and the entire enzyme is present in the periplasmic space. Both the α and β subunits of GGT are encoded by a common messenger RNA. The enzyme is translated as a single chain precursor, which yields subunits post-translationally by autoproteolytic processing. GGT plays a role in cellular detoxification through formation of mercapturic acids and confers resistance against antitumor drugs. It is also involved in many physiological disorders, such as Parkinson’s disease, diabetes, and inhibition of apoptosis.

GGTs belong to the structural superfamily of the N-terminal nucleophilic (Ntn) hydrolases. Members of this superfamily, despite lacking any discernible sequence similarity, share the same tertiary fold. The core three-dimensional (3D) folding pattern shared by Ntn-hydrolases consists of a four-layer αββα structure with two anti-parallel β-sheets between α-helical layers. Recently, the X-ray structures of two bacterial GGTs have contributed greatly to our understanding of the enzyme function. The structure of the acyl-enzyme intermediate of *E. coli* GGT (*EcGGT*) confirms the role of Thr391 and the location of the donor substrate binding site. Structural...
evidence has also been provided for the autocatalytic processing of EcGGT, and the recent structure of Helicobacter pylori GGT has shed still more light on this mechanism.

The availability of both a high resolution crystal structure and a cloned over-expressed gene for EcGGT offers the opportunity to investigate the role of individual amino acids. The recombinant EcGGT is translated as an inactive 62.4 kDa polypeptide precursor that undergoes intramolecular autocatalytic cleavage to generate a fully active heterodimer composed of a 41.2 kDa and a 21.2 kDa subunit. Recently, we have identified the essential residues for the enzymatic reaction of EcGGT using site-directed mutagenesis on the conserved Thr407, Asp433 and Met464 residues. As the replacements Thr407 and Asp433 significantly abolish the enzymatic activity, we presumed both residues to be important for the proper function of EcGGT.

Among the serine residues in the β subunit, Ser463 of EcGGT is conserved across the GGT family (Fig.1) and has been proposed to be involved in the substrate binding. In the present study, to better understand its role, we used a set of three variants has been constructed by replacing this residue with Thr, Asp or Lys. Mutational analysis has also been performed to investigate to show the importance of Ser463 in EcGGT and Lys and Asp replacements on this position.

Materials and Methods

Materials

The growth medium components from Difco Laboratories (Detroit, MI, USA), restriction and DNA modification enzymes from Promega Life Sciences (Madison, WI, USA), nickel nitriolriacetate (NiNTA) resin from Qiagen Inc. (Valencia, CA, USA), acrylamide, bisacrylamide, ammonium persulfate, TEMED and low-molecular-mass protein markers from Bio-Rad (Richmond, CA, USA), L-γ-glutamyl-p-nitroanilide (L-γ-Glu-p-NA), Gly-Gly and p-nitroaniline (p-NA) from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA) were procured. All other chemicals were commercial products of analytical or molecular biological grade.

Bacterial strains and growth conditions

E. coli novablue (Novagen Inc., Madison, WI, USA) was used for the preparation of plasmids, while E. coli XL-1 blue (Stratagene, La Jolla, CA, USA) was used for the mutational experiments. T5 RNA-polymerase-mediated gene expression was performed in E. coli M15 (pRep4) (Qiagen). E. coli strains were grown aerobically in Luria-Bertani (LB) medium at 20°C or 37°C.

Molecular techniques

Restriction enzymes and T4 DNA ligase were used according to the manufacturer’s instructions. E. coli cells were made competent for transformation by the method of Dagert and Ehrlich. Amino acid sequences were analyzed with the programs BLAST-X from the National Center for Biotechnology Information (National Library of Medicine, National Institute of Health, MD) and Alignment from the ExPASy molecular biology server (Swiss Institute of Bioinformatics, Basel, Switzerland). The peptide sequences from EcGGT (TrEMBL P18956), H. pylori GGT (TrEMBL O25743), B. licheniformis GGT (TrEMBL Q65KZ6), Homo sapiens GGT (TrEMBL P19440), Sus scrofa (TrEMBL P20735), and Rattus norvegicus GGT (TrEMBL P07314) were included in this study.

Mutations were introduced into pQE-EcGGT plasmid using the Quick Change XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The kit employs double-stranded DNA as template, two complementary oligonucleotide primers containing the desired mutation, and DpnI endonuclease to digest the parental DNA template. Oligonucleotides were synthesized by Mission Biotechnology (Taipei, Taiwan) and listed in Table 1. The coding regions of the mutated Ecggt gene were sequenced to confirm the mutations and then E. coli M15 (pRep4) cells were transformed and used for expression.

Expression and purification of recombinant proteins

His leader-tagged recombinant proteins were expressed and purified as described previously. Briefly, E. coli M15 carrying pQE-EcGGT or each of the mutated plasmids was cultured in LB medium containing 100 µg ampicillin/ml and 25 µg kanamycin/ml at 37°C overnight. Protein expression was induced by dilution of the culture with two volumes of LB broth containing 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) and incubation at 20°C for 12 h. Bacterial cells were harvested by centrifugation and resuspended in 3 ml of binding buffer (5 mM imidazole, 0.5 M NaCl, and 50 mM Tris-HCl; pH 7.9). Recombinant proteins were purified from the cell lysate fraction by affinity chromatography with NiNTA resin according to the manufacturer’s protocol. After extensive washing, the
bound proteins were eluted with 50 mM Tris-HCl buffer (pH 7.9) containing 0.5 M imidazole and 0.5 M NaCl.

**Enzyme assay and kinetic analysis**

Enzymatic activity for transpeptidation was determined at 37 °C as described previously\(^29\) and the formation of \(p\)-NA was recorded by monitoring the corresponding color changes at 410 nm. The reaction mixture contained 1.25 mM \(L\)-\(\gamma\)-Glu-\(p\)-NA, 30 mM Gly-Gly, 1 mM MgCl\(_2\), 50 mM Tris-HCl buffer (pH 9.0), and an appropriate amount of the purified enzyme in a final volume of 1 ml. One unit of GGT activity was defined as the amount of enzyme that produces 1 \(\mu\)mol of \(p\)-NA per min under the assay conditions.

A Lineweaver-Burke plot was used to obtain the kinetic parameters \(K_M\) and \(V_{\text{max}}\), which were subsequently used to estimate enzyme turnover and catalytic efficiency. The kinetic parameters were determined in 50 mM Tris-HCl buffer (pH 9.0) containing 1 mM MgCl\(_2\), 30 mM Gly-Gly, and 1-100 \(\mu\)M \(L\)-\(\gamma\)-Glu-\(p\)-NA.

**Gel electrophoresis and determination of protein concentrations**

SDS-PAGE was performed in a vertical mini-gel system (Mini-Protean III system, Bio-Rad) with 4% polyacrylamide stacking and 12% polyacrylamide separating gels. Prior to electrophoresis, the purified enzymes were mixed with 2x SDS-sample buffer, heated at 100°C for 5 min and centrifuged at 12,000 \(g\) for 10 min. Proteins bands were stained with 0.25% Coomassie brilliant blue dissolved in 50% methanol-10% acetic acid, and destained in a 30% methanol-10% acetic acid solution. The protein size markers were phosphorylase \(b\) (97.4 kDa), bovine serum albumin (66.3 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa).

Detection of proteins with GGT activity on 10% non-denaturing PAGE gels was carried out as described previously\(^22\). The protein size markers were thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa).

Protein concentrations were measured by the Bradford method\(^30\) with the Bio-Rad protein assay reagent and using bovine serum albumin as the standard.

**Computer modeling of the enzymatic pockets and precursor structures**

Construction of molecular models, structure optimization and conformational analysis were carried out on a Windows-XP-based dual-core workstation using the Discovery Studio 1.7 program (Accelrys Inc., Burlington, MA, USA)\(^31\). Computer modeling of the 3D structure of wild-type precursor \(E_{\text{c}}\)GGT was performed in accordance with the X-ray crystal structure of T391A precursor mutant enzyme (2E0W.pdb). The 3D structures of pro-S463K and pro-S463D were further simulated based on the structure of wild-type pro-\(E_{\text{c}}\)GGT with the Build Mutants and Standard Dynamics Cascade pipelines set into the Discovery program. The energy minimization of the models was iterated over 500 cycles using the Steepest Descent methods with final convergence set at 0.002 kcal/mol Å.

**Recording of fluorescence emission spectra**

Tryptophan emission fluorescence spectra of wild-type and mutant enzymes were measured on a Hitachi F-4500 spectrophotometer at an excitation wavelength of 290 nm using cuvettes with an optical path length of 1 cm. The emission spectra of protein samples with a concentration of 0.35 \(\mu\)M in 50 mM Tris-HCl buffer (pH 9.0) were measured from 300 to 400 nm (excitation and emission slit width = 5 nm) under the scanning speed of 240 nm/min. The temperature was maintained constantly at 37°C using an external bath circulator. All fluorescence spectra were corrected for background scattering with pure buffer.
Circular dichroism (CD) spectroscopy

Far-UV CD was recorded at 22°C on a JASCO J-815 spectropolarimeter equipped with a temperature-controlling liquid nitrogen system. Samples were diluted in 50 mM Tris-HCl buffer to a final concentration of 6 μM. Spectral analysis was performed over the wavelength range from 190 to 250 nm in cuvettes with a 2 mm path length at 0.2 nm intervals with 4 s integration time and a bandwidth of 2.0 nm. Photomultiplier absorbance was kept below 600 V in the analyzed spectral region. Each scanning was repeated ten-times and an average was obtained. Data were corrected for the effect of buffer, and the results were expressed as mean residue ellipticity $\theta$ in the unit of degrees cm$^2$ dmol$^{-1}$, which was defined as $\theta = 1000\theta_{obs}(l\epsilon)^{-1}$, where $\theta_{obs}$ is the moles per liter, $c$ is the concentration in residue moles per liter, and $l$ is the length of the light path in centimeters.

Thermal denaturation of wild-type and mutant enzymes was explored by monitoring the ellipticity at 222 nm. The temperature was increased with a heating rate of 2°C per min from 20 to 90°C.

Results and Discussion

Amino acid sequence alignment

Amino acid sequences of E. coli, H. pylori, Bacillus licheniformis, human, pig and rat GGTs were aligned with the program BLASTX. The alignment of these six species is shown in Fig. 1. EcGGT had sequence identity between 30 and 49% with H. pylori, B. licheniformis, rat, pig and human enzymes and the alignment of six proteins showed a strict conservation of residues implicated in substrate binding and catalytic reaction. Extensive studies for human GGT show that residues Arg107, Asp423, Ser451 and Ser452 are critical for enzymatic activity. Asp433, Ser462, and Ser463 residues of EcGGT have been proposed to be involved in the binding of the $\alpha$-amino and $\alpha$-carboxyl groups of the $\gamma$-glutamyl moiety of glutathione, suggesting that the mechanism of substrate binding is conserved in bacterial and mammalian enzymes. As shown in Fig. 1, the serine residue at position 463 of EcGGT is ideally conserved and Ser463 N has been shown to bind the carboxyl group of the $\gamma$-glutamyl moiety of glutathione. Based on these observations, it can be concluded that any change in this residue would affect the catalytic reaction of the enzyme.

Purification and biochemical characterization of wild-type and mutant enzymes

Due to the potential importance of the conserved Ser463 residue in EcGGT, this residue was chosen for site-specific mutagenesis. After verification of the altered sequences, pQE-EcGGT and each of the mutated plasmids were transformed into E. coli M15 cells for IPTG-induced gene expression. To characterize wild-type EcGGT and each mutant enzyme, the expressed proteins were purified to near homogeneity by a Ni$^{2+}$-NTA resin. As shown in Fig. 2A, the molecular masses of the subunits of the purified wild-type EcGGT were determined to be 41 and 23 kDa, respectively. These two bands were also observed in S463T. However, the purified S463K and S463D were predominantly present in a precursor form, indicating that the substitutions of Ser463 with Lys or Asp impaired the autocatalytic processing of the enzyme.

As shown in Fig. 2B, the migration behavior of S463T was consistent with that of the wild-type enzyme. However, a lower mobility was observed in S463K and S463D. In these three mutant enzymes, only S463T retained GGT activity on the gel (Fig. 2C), indicating that the hydroxyl group in the side chain played a critical role in this position. Recently, the crystal structure of the EcGGT precursor protein has been determined at 2.55 Å resolution. In the precursor protein, the number of segments with high mobility is increased relative to the mature EcGGT and residues 375-390, known as the P-segment, takes on an extended conformation on the molecular surface. Except for the P-segment and the residues located in its vicinity, the structure of precursor protein is similar to the mature EcGGT. Accordingly, the mobility shift of Ser463 variants in the non-denaturing gel might result from the major structural perturbation around the P-segment. It was worth noting that a minor activity band was also observed in both wild-type EcGGT and S463T (Fig. 2C, lines 1 and 2). The minor GGT bands might result from a higher order of EcGGT quaternary structure with respective to the tetrameric form.

The purified wild-type EcGGT had a specific activity of 6.8 ± 0.5 U per mg of protein (Table 2). S463T showed a comparable activity with that of wild-type enzyme, whereas the GGT activity was completely abolished in S463K and S463D. These results indicated that the conserved Ser463 residue played an important role in the catalytic activity of EcGGT.
Fig. 1—Amino acid sequence alignment of microbial and mammalian GGTs. [The deduced amino-acid sequence for *E. coli* GGT (*EcGGT*), *H. pylori* GGT (* HpGGT*), *B. licheniformis* GGT (*BlGGT*), *H. sapiens* GGT (*HsGGT*), *S. scrofa* GGT (*SsGGT*), and *R. norvegicus* GGT (*RnGGT*) are shown. Gaps in the aligned sequences (dashes) were introduced to maximize similarities. The critical substrate-binding and catalytic residues are marked by solid circles and a star, respectively]

Fig. 2—Analysis of the purified wild-type and mutant enzymes by (A): SDS-PAGE; (B): native-PAGE and (C): activity staining [Lanes: M, protein size markers; 1, wild-type *EcGGT*; 2, S463T; 3, S463K; 4, S463D]
Steady-state kinetic parameters of wild-type EcGGT and S463T were determined with Leu-p-NA. The $K_m$ and $k_{cat}$ values for wild-type enzyme were 45.1 µM and 37.4 min$^{-1}$, respectively (Table 2). It was notable that S463T exhibited 29% increase in $K_m$ value and 28% reduction in the catalytic efficiency. These results suggested that residue Ser463 was necessary for maximal activity of the enzyme by maintaining the affinity for the substrate. It has been proposed that the $\alpha$-carboxyl and $\alpha$-amino groups of the $\gamma$-glutamyl moiety of glutathione are situated at the bottom of the enzymatic pocket, and this moiety is held at the critical position by many hydrogen bonds and salt bridges. The carboxyl group is bonded with Arg114 $N_{\eta\eta}$, Ser462 $O_{\gamma\gamma}$, Ser463 $N$ and Ser463$O_{\gamma\gamma}$ via water 1, and the $\alpha$-amino group with Asn411 $O_{\delta}$, Gln430 $O_{\epsilon}$ and Asp433 $O_{\delta}$. Moreover, the $\gamma$-glutamyl carbonyl oxygen is hydrogen-bonded with two main-chain amino groups of Gly483 and Gly484. The large side chain of Tyr444 forms a wall that shields the pocket from solvent and the hydrogen bond between Tyr444 $O_{\eta}$ and Asn411 $O_{\delta}$ may contribute to firm the wall formation. To elucidate the mutational impact, computer modeling of the pocket of S463T was performed on the basis of the X-ray crystal structure of wild-type EcGGT (2DBX.pdb). As shown in Fig. 3A, the model of S463T showed very little change in the arrangement of the substrate-binding residues. In the wild-type EcGGT, Ser463 binds to the carboxyl group of $\gamma$-glutamyl moiety via Ser463 N. The interactions between the carboxyl group and Thr463 increased to three hydrogen bonds in the S463T variant. These results indicated that either Ser or Thr at position 463 was accessible to the $\gamma$-carbonyl carbon of the $\gamma$-gluamyl substrate.

**Autocatalytic processing**

SDS-PAGE analysis showed that the purified S463K and S463D were present in a precursor form (Fig. 2A). To investigate the *in vitro* maturation of S463K and S463D, the purified enzymes were incubated in Tris-HCl buffer (pH 8.0) at 4°C. Samples from the reaction mixtures were taken periodically for

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**Table 2**—Specific activity and kinetic parameters of wild-type and mutant enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (U mg$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$ (µM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>6.8 ± 0.5</td>
<td>45.1 ± 0.2</td>
<td>37.4 ± 1.4</td>
<td>0.83</td>
</tr>
<tr>
<td>S463T</td>
<td>4.1 ± 0.2</td>
<td>58.3 ± 2.3</td>
<td>29.2 ± 3.7</td>
<td>0.50</td>
</tr>
<tr>
<td>S463K</td>
<td>ND$^a$</td>
<td>ND$^a$</td>
<td>ND$^a$</td>
<td>ND$^a$</td>
</tr>
<tr>
<td>S463D</td>
<td>ND$^a$</td>
<td>ND$^a$</td>
<td>ND$^a$</td>
<td>ND$^a$</td>
</tr>
</tbody>
</table>

$^a$ND, not detected; $^b$–, not available.
determination of the concentration of precursor proteins by SDS-PAGE. However, no significant in vitro maturation was observed for both proteins in the duration of 2 weeks (data not shown). A base is generally believed to be important for autoproteolytic activation of Ntn-hydrolase precursors into α- and β-subunits. It has been reported that a water molecule acts as a base to improve the nucleophilicity of the catalytic residue during the autocatalytic processing of proteasome β subunit \(^{35}\) and cephalosporin acylase \(^{36-39}\). In the proposed mechanism for the autocatalytic processing of EcGGT, an unidentified base abstracts the proton from the hydroxyl group of Thr391 to form the reactive oxyanion \(^{15}\). The addition of Thr391 O\(_{\gamma}\) to Gln390 C results in the formation of a transitional tetrahedral intermediate. Based on this mechanism, it is obvious that both the generation of a reactive oxyanion and the formation of a transitional tetrahedral intermediate are critical for the autocatalytic processing in these two variants. The similar results have been demonstrated in Arg337 replacements of the same enzyme \(^{40}\).

### Structural analysis

Fig. 4A shows the intrinsic fluorescence spectra of the wild-type and mutant enzymes. The fluorescence intensities of these four proteins showed a quenching for the wild-type EcGGT, S463K, and S463D corresponding to about 97.4, 79.6, and 72.5%, respectively of the global fluorescence of S463T. Alteration in fluorescence emission maxima was also noticeable. The fluorescence emission maxima \((\lambda_{\text{max}})\) of wild-type EcGGT and S463T were at 335 nm. However, the \(\lambda_{\text{max}}\) of S463K and S463D exhibited a ~1.4 nm red shift, suggesting that the tryptophan microenvironment was somewhat slightly altered in these two variants.

We also probed the secondary structure of wild-type and mutant enzymes by far-UV CD (Fig. 4B). The CD spectra exhibited two minima at 222 nm and 208 nm, and a positive maximum at 190 (typical of α-helix). The spectra were quantitatively analyzed using the CONTINLL program (http://www.cryst.bbk.ac.uk). The estimated values suggested that there was no significant change in the secondary structure in any of the mutant proteins.
In a further attempt to probe the conformation of mutant enzymes, temperature-induced denaturation of secondary structure of the wild-type and mutant proteins was monitored by following the loss of ellipticity of the CD signal at 222 nm (Fig. 4C). Temperature-dependent CD signals in the far-UV region displayed sigmoidal curves. Although the thermal denaturation was irreversible, the transition curves indicated the apparent T_m values of 50.1, 48.9, 38.2, and 36.1°C for wild-type EcGGT, S463T, S463K, and S463D, respectively. Since the temperature-induced denaturation curves of wild-type and mutant enzymes were significantly different, it was implied that conformational changes occurred as a result of Lys- and Asp-substitution.

Conclusion

Residues that comprise the γ-glutamyl binding site are primarily located in the β subunit and make numerous hydrogen bonds with the α-amino and α-carboxylate groups of the substrate. In the present study, a serine residue Ser463, required for catalytic activity of EcGGT, has been identified by bioinformatics methods. It has been proposed that this residue plays a role to bind the γ-glutamyl moiety of the substrate within the enzymatic pocket. Our current investigations highlight the involvement of the invariant Ser463 in the enzymatic activity and provide a basis for further studies on structure-function to fully elucidate the amino acids residues involved in the autocatalytic processing of the enzyme.

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