Autoprocessing of *Helicobacter pylori* γ-Glutamyltranspeptidase Leads to the Formation of a Threonine-Threonine Catalytic Dyad

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*Helicobacter pylori* γ-glutamyltranspeptidase (HpGT) is a glutathione-degrading enzyme that has been shown to be a virulence factor in infection. It is expressed as a 60-kDa inactive precursor that must undergo autocatalytic processing to generate a 40-kDa/20-kDa heterodimer with full γ-glutamyl amide bond hydrolase activity. The new N terminus of the processed enzyme, Thr-380, is the catalytic nucleophile in both the autoprocessing and enzymatic reactions, indicating that HpGT is a member of the N-terminal nucleophile hydrolase superfamily. To further investigate activation as a result of autoprocessing, the structure of HpGT has been determined to a resolution of 1.9 Å. The refined model contains two 40-kDa/20-kDa heterodimers in the asymmetric unit and has structural features comparable with other N-terminal nucleophile hydrolases. Autoprocessing of HpGT leads to a large conformational change, with the loop preceding the catalytic Thr-380 moving >35 Å, thus relieving steric constraints that likely limit substrate binding. In addition, cleavage of the proenzyme results in the formation of a threonine-threonine dyad comprised of Thr-380 and a second conserved threonine residue, Thr-398. The hydroxyl group of Thr-398 is located equidistant from the α-amino group and hydroxyl side chain of Thr-380. Mutation of Thr-398 to an alanine results in an enzyme that is fully capable of autoprocessing but is devoid of enzymatic activity. Substrate docking studies in combination with homology modeling studies of the human homologue reveal additional mechanistic details of enzyme maturation and activation, substrate recognition, and catalysis.

Helicobacter pylori γ-glutamyltranspeptidase (HpGT) catalyzes the first step in glutathione degradation, removing glutamate from the tripeptide and its conjugates via a γ-glutamyl enzyme intermediate. The γ-glutamyl group can be transferred to water (hydrolysis) or an amino acid or short peptide (transpeptidation) (1–5). The enzyme is located in the periplasmic space of the bacterium and has been shown to be a virulence factor in infection (6, 7). However, the precise functions of HpGT in colonization and persistence have not been defined.

γ-Glutamyltranspeptidases (γGT) are fairly ubiquitous, with homologues observed in all kingdoms. Distant γGT often share considerable sequence identity (e.g. bacterial versus mammalian enzymes are >20% identical), suggesting a strong conservation of structure and function. Despite considerable sequence homology, significant catalytic differences exist between bacterial and non-bacterial homologues. For example, human γGT is >100-fold more effective than HpGT at catalyzing transpeptidation, even though the two enzymes have comparable hydrolase activities (8–12). Undoubtedly, structural variations in the enzyme active sites are responsible for these differences in substrate specificities.

HpGT is a member of the N-terminal nucleophile hydrolase superfamily (13, 14). N-terminal nucleophile hydrolases share a common core structure, undergo autoprocessing and activation, and catalyze amide bond hydrolysis, with the new N-terminal residue serving as a catalytic nucleophile in the enzymatic reaction. HpGT is synthesized as an inactive 60-kDa polypeptide. Intramolecular autocatalytic cleavage of the proenzyme results in a fully active heterodimer comprised of a 40-kDa and a 20-kDa subunit. The resultant N terminus, Thr-380, has been shown to be the catalytic nucleophile in both the autoprocessing and enzymatic reactions (6, 9). However, the residues responsible for increasing the nucleophilicity of Thr-380 in either reaction have not been identified. This perfectly conserved threonine residue has been shown to be essential for catalysis.

In this report, we have determined the crystal structure of recombinant *H. pylori* γ-glutamyltranspeptidase to 1.9 Å resolution using the recently reported *E. coli* enzyme structure (19)
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as a molecular replacement probe. Examination of these structures in combination with molecular docking and homology modeling studies reveals mechanistic details of enzyme maturation and activation, substrate recognition, and catalysis. Based on this analysis, we have used kinetic and mutagenesis studies of *H. pylori* \(\gamma\)-glutamyltranspeptidase to demonstrate that a threonine-threonine dyad is required for efficient hydrolysis of the \(\gamma\)-glutamyl peptide bond of glutathione.

**EXPERIMENTAL PROCEDURES**

**Overexpression and Purification of HpGT**—The expression and purification of HpGT have been described previously (6, 9). Briefly, a PET28 plasmid containing the coding sequence for mature HpGT was used to transform *E. coli* strain Rosetta (DE3) pLySS (Novagen). Cultures, grown to an \(A_{600}\) of 0.6–0.8 in a \(2 \times \) YT medium containing 34 mg/liters chloramphenicol and 30 mg/liters kanamycin, were induced for 4 h by the addition of isopropyl thio-\(\beta\)-D-galactoside to a final concentration of 500 \(\mu\)M. The cells were harvested by centrifugation and stored at \(-80^\circ\)C. Thawed cells were lysed by sonication, nucleic acids were precipitated, and the mixture was cleared of particulates by centrifugation. Recombinant HpGT was purified from the supernatant by affinity chromatography using a nickel-chelating column (Novagen).

**Crystallization and Data Collection**—Crystals of HpGT were grown at 18 °C by the sitting drop method out of a solution of 200 mM HEPES, pH 7.5, within the precipitant range of 16–25% polyethylene glycol 2000 monomethyl ether at a final protein concentration of 5 mg/ml. Crystals reached a final size of 0.1 \(\times\) 0.1 \(\times\) 0.05 mm in 1 to 2 weeks. To collect data at cryogenic temperatures, crystals were transferred to artificial mother liquor containing additional cryoprotectant (20). This was accomplished in a stepwise fashion by soaking the crystals for 1–2 min in artificial mother liquor containing increasing amounts of polyethylene glycol 2000 monomethyl ether (5% increments) until a final concentration of 30% was attained.

Diffraction data were collected under cryogenic conditions (100 K) on Beamline 14-BM-C (\(\lambda = 0.9 \text{ Å}\)) of BioCARS at the Argonne National Laboratory Advanced Photon Source and analyzed using the HKL2000 software package (21). The structure of HpGT was determined by molecular replacement using the Crystallography and NMR System software package (22) with the structure of *E. coli* \(\gamma\)-glutamyltranspeptidase (PDB ID 2DBX; Ref. 19) as the search molecule. The resulting model, which included residues 29–375 of the 40-kDa subunit and residues 1–213 of the 40-kDa subunit, was assessed by SDS-PAGE (9). Percent processing was determined by densitometric analysis of the gels subjected to a global energy minimization in CNS (22). The cleavage of the precursor (60 kDa) to its mature form (20- and 40-kDa subunits) was assessed by SDS-PAGE (9). Percent processing values were determined by densitometric analysis of the gels using Quantity One Software (Bio-Rad), plotted versus time, and fit to a first-order monoexponential curve using Prism (Graph Pad Software). Representative data from three or more determinations are presented with experimental errors determined from individual fits of the kinetic data.

**Structure Determination and Model Quality**—X-ray diffraction data were collected on Beamline 14-BM-C of BioCARS and extended to 1.9 Å resolution with an \(R_{sym} = 4.6\%\) (Table 1). The structure of HpGT was determined by molecular replacement using the related EcGT as a probe (19), with non-conserved residues replaced by alanine. An unambiguous solution was obtained, and inspection of the initial electron density map revealed numerous positive peaks corresponding to the omitted amino acid side chains. HpGT crystals belong to space group P2\(_1\) and contain two heterodimers in the asymmetric unit. For the first heterodimer (subunits A and B), the model includes residues 29–375 of the 40-kDa subunit and residues
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| TABLE 1 | Data collection and refinement statistics for the *H. pylori* γ-glutamyltranspeptidase structure |
|-----------------|------------------------------------------------------------------------------------------------|
| Data collection statistics | P2₁ |
| Space group | P2₁ |
| Cell dimensions (Å) | 54.35, 105.21, 91.06, β = 91.99 |
| Resolution | 28.20-1.90 Å (2.02-1.90 Å) |
| Number of unique reflections | 72974 |
| Average redundancy | 4.8 (4.8) |
| (I/|a|) | 32.3 (3.8) |
| Completeness | 91.5% (77.6%) |
| R₁ | 4.6% (46.6%) |

| Refinement statistics | |
|----------------------|------|
| Number of atoms | 8682 |
| Protein atoms | 8092 |
| Solvent atoms | 590 |
| R-factor | 19.7% (24.7%) |
| R-free | 23.9% (29.5%) |
| Overall B factor | 31.9 Å² |
| Protein B factor | 31.6 Å² |
| Solvent B factor | 35.3 Å² |
| RMSD from ideal values | |
| Bond lengths | 0.005 Å |
| Bond angles | 1.3° |
| Estimated coordinate error (Luzzati) | 0.22 |

* R₁ = Σ/I (I − <I>)/Σ <I>.

R-free = Σ/I (I − <I>)/Σ <I>.

RMSD, root mean square deviation.

380–565 of the 20-kDa subunit. For the second heterodimer (subunits C and D), residues 31–379 of the 40-kDa subunit and residues 380–565 of the 20-kDa subunit were observed. The final model has 96.7% of its residues in the favored region of the Ramachandran plot and 99.6% in the allowed, as assessed by MOLPROBITY (24). Complete data collection and refinement statistics are provided in Table 1.

Overall Structure of HpGT—The refined structure of HpGT is presented as a ribbon diagram in Fig. 1A. The 40- and the 20-kDa subunits of the enzyme are colored in purple and green, respectively. The two heterodimers in the asymmetric unit are nearly identical with a root mean square deviation of 0.39 Å for main chain atoms. The overall HpGT structure is comparable with that of the *E. coli* enzyme, which also contained two heterodimers in the asymmetric unit (root mean square deviation 0.95 Å; Fig. 1B). Interestingly, as in the *E. coli* structure, the C terminus of the 40-kDa subunit (Asn-379) is >35 Å from the N terminus of the 20-kDa subunit (Thr-380), suggesting that a significant conformational change accompanies autoprocessing.

Previously we have shown that HpGT is a heterotetramer comprised of two 40- and two 20-kDa subunits (α₂β₂) both by gel filtration and dynamic light scattering (9). Although two HpGT heterodimers are contained within the asymmetric unit, the buried surface area between them is only ~1600 Å². This significant, but not extensive, interaction could indicate that the heterotetramer is not the biologically relevant form. However, despite crystallizing in a different space group, EcGT has a nearly identical arrangement of heterodimers in the asymmetric unit (19), as does a P1 crystal form of HpGT.⁴ These observations suggest that crystal contacts alone are not responsible for the observed arrangement of heterodimers, and further studies of this potential interface are warranted.

Substrate Binding Site of HpGT—HpGT was crystallized in the absence of substrates or inhibitors, and thus the active site was identified by comparison with the glutamate-bound form of the closely related EcGT (19). Glutathione, the presumed physiological substrate, was docked into the active site of HpGT by superimposing its γ-glutamyl group onto the corresponding atoms of the EcGT-bound glutamate. The modeled complex was then subjected to local and global energy minimization. Several potential orientations of the cysteinylglycine were observed, and a representative schematic is provided in Fig. 2. The glutamate binding site is inaccessible to solvent, whereas the remainder of the active site is surface exposed (Fig. 2A). Because the cysteinylglycine binding site is shallow and solvent accessible, direct binding contacts are minimal. This probably

⁴ A. Sand and J. J. Barycki, unpublished observation.
accounts for the ability of the enzyme to accommodate its diverse glutathione-conjugated substrates as well as numerous substrate analogues.

In the model, the glutamate binding site is defined by nine hydrogen bonds with highly conserved residues predominantly in the 20-kDa subunit (Fig. 2B). The α-carboxylate of glutamate is coordinated directly and indirectly (via a bridging water molecule) by Arg-103, Ser-451, and Ser-452. The α-amino group forms hydrogen bonds with the side chains of Asn-400, Glu-419, and Asp-422, with the negatively charged Glu-419 and Asp-422 likely balancing the positive charge of the α-amino group. Lastly, the γ-carbonyl can interact favorably with the backbone amide of Gly-472. Many of the corresponding residues in hGT have been implicated as active site residues by site-directed mutagenesis (10, 29–31). In EcGT, a nearly identical arrangement of glutamate binding site residues is observed (19). The only difference is the replacement of Glu-419 with a glutamine (Gln-430 in E. coli). This isosteric substitution suggests that the negative charge of Glu-419 is not required to counterbalance the presumed positive charge of the α-amino group of the γ-glutamyl portion of glutathione.

Homology Modeling of hGT—We have shown that HpGT is a poor transpeptidase relative to mammalian homologues when...
using glycylglycine as the acceptor molecule (9). Although human and H. pylori \( \gamma \)-glutamyltranspeptidases share \( \sim 30\% \) sequence identity and an additional 22\% sequence similarity, HpGT has <1\% of the transpeptidase activity of the human enzyme. To identify structural features that could account for the limited transpeptidase activity of the \( H. \ pylori \) enzyme, we have generated a homology model of hGT based on the two available bacterial structures, EcGT and HpGT (Fig. 3). The overall topology of the bacterial structures was maintained and side chain assignments were made based on the sequence alignment provided in supplemental materials.

With these constraints, the model closely resembles the initial bacterial templates and has reasonable geometries. To validate the model, we evaluated the structure with respect to available biochemical data. As indicated above, previous mutagenesis studies with the human enzyme suggested that Arg-107, Glu-108, Asp-422, Asp-423, Ser-451, and Ser-452 were located at or near the enzyme active site (10, 29–31). In the human model, these residues largely comprise the glutamate binding site of the enzyme (Fig. 3B). Because these are active site residues, structural conservation is expected. However, the prediction of two disulfide bonds within the human enzyme in regions remote from the active site (Fig. 3A) but of demonstrated significance in the rat enzyme (18) further validates the hGT model.

Hughey and coworkers (18) have shown in rat \( \gamma \)-glutamyltranspeptidase that disulfides likely exist between Cys-49 and Cys-73 and between Cys-191 and Cys-195. In the human model, the equivalent residues (Cys-50/Cys-74 and Cys-192/Cys-196) are positioned optimally for disulfide bond formation. In contrast, neither HpGT nor EcGT contains a cysteine residue in the mature enzyme. A more extensive sequence comparison (see supplemental materials) indicates that the Cys-192/Cys-196 pair is only found in a subset of mammalian \( \gamma \)-glutamyltranspeptidases but the Cys-50/Cys-74 pair is highly conserved in non-bacterial homologues. Because the Cys-50/Cys-74 pair is remote from the active site, its high sequence conservation suggests a structural and/or regulatory function. In rat \( \gamma \)-glutamyltranspeptidase, the Cys-49/Cys-73 pair is required for maturation and cell surface expression of the enzyme, whereas the Cys-191/Cys-195 pair appears to be required for enhanced enzyme stability (18). Examination of the human model indicates that the Cys-50/Cys-74 pair precedes a loop (residues 79–90; colored red in Fig. 3A) that contains a highly conserved Gly-Gly-Gly motif (residues 86–88 in hGT; see supplemental materials). Gly-88 and Leu-89 are within hydrogen bond distance of the peptide backbone of Thr-399 and Ala-106, respectively. As discussed below, Thr-399 is a critical active site residue, and Ala-106 precedes an

**FIGURE 4. Activation of the catalytic nucleophile, Thr-380.** A, \( 2F_o - F \) map contoured at 3\( \sigma \) (red cages) and 1\( \sigma \) (blue cages) illustrates the electron density quality near the threonine-threonine catalytic dyad. B, shown in the stereodigram are residues located near Thr-380, the nucleophile in both the autoprocessing and hydrolysis reactions. Potential hydrogen bonds were identified with Chimera and are indicated as solid black lines. The \( \alpha \)-amino group of Thr-380 can form a hydrogen bond with the hydroxyl group of a second conserved threonine, Thr-398, which in turn can form a hydrogen bond with the hydroxyl group of Thr-380. The free amino group of Thr-380 is also <3.2 \( \AA \) from the Thr-380 hydroxyl group, a tightly bound water molecule, and the backbone carbonyl of Asn-400. Based on substrate modeling studies with glutathione, the Thr-380 \( \alpha \)-amino group is in close proximity to the cysteinyl amide of glutathione (<3.8 \( \AA \)), suggesting that it may protonate the cysteinylglycine leaving group.
active site arginine residue, Arg-107, that coordinates the α-carboxylate of the glutamyl portion of glutathione. Thus, the oxidation state of the Cys-50/Cys-74 dithiol/disulfide pair may influence the position of this active site loop and thus impact autoprocessing and enzymatic activities.

Comparison of Human and H. pylori Active Sites—An inspection of the hGT model indicates that the glutamate binding site is very similar to that of the bacterial homologues. A minor difference is the conservative substitution of Tyr-433 in HpGT with a phenylalanine in hGT that is unlikely to affect glutamate binding significantly. Within the potential cysteinylglycine binding pocket, which is thought to be at least in part overlapping with the acceptor binding site for the transpeptidation reaction, there are several notable differences. Ala-78, Ala-79, Ala-401, and Ser-402 of HpGT are replaced by His-81, Ser-82, Leu-402, and Tyr-403, respectively, in hGT. These substitutions convert the rather open and non-polar binding pocket of HpGT to a more sterically constrained and polar site in the human enzyme and may account for the dramatic differences in donor and acceptor substrate specificities. Both Ser-82 and Tyr-403 are highly conserved in non-bacterial homologues (see supplemental materials) and may be important in recognition of the acceptor substrate during transpeptidation. Examination of the hGT model also suggests that the α-amino group of Thr-381 is the proposed general base that activates the peptide bond (17). An examination of the HpGT structure may be important in recognition of the acceptor substrate during transpeptidation. Examination of the hGT model also suggests that the α-amino group of Thr-381 is the proposed general base that activates the peptide bond (17). An examination of the HpGT structure may be important in recognition of the acceptor substrate during transpeptidation.

Activation of HpGT—HpGT must undergo autoprocessing in order to gain enzymatic activity, but the mechanism of activation has not been elucidated. Previous studies have shown that Thr-380 is the catalytic nucleophile in both the autoprocessing and hydrolysis reactions (9). For both processes, a general base mechanism has been proposed in which an adjacent residue would facilitate the attack of the threonine hydroxyl on the α-carboxylate of glutamate. Because the α-amino group is conserved in all γGT and appears to have similar hydrogen bond networks as judged by modeling studies, the increased transpeptidase activity in mammalian homologues may be the result of more efficient acceptor binding relative to bacterial γGT.

Both the HpGT and EcGT structures indicate that the C terminus of the 40-kDa subunit is a considerable distance from the conserved threonine residue at the N terminus of the 20-kDa subunit (Fig. 1B). In the unprocessed form of the enzyme, the mobile tail of the 40-kDa subunit would necessarily be positioned across the active site of the enzyme, possibly occluding the substrate binding site (19). Cleavage of the proenzyme would relieve this steric constraint. In addition, autoprocessing leads to activation of Thr-380 for the hydrolysis reaction (Fig. 4). The new N terminus, the α-amino group of Thr-380, is within hydrogen bond distance of its own side chain hydroxyl as well as that of Thr-380. This second highly conserved threonine residue can form a hydrogen bond with the catalytic nucleophile, the hydroxyl group of Thr-380, thus forming a threonine-threonine dyad. A nearly identical orientation of active site residues is observed in mature EcGT, including the placement of the active site water (W3 in the EcGT structure).

![Figure 5. Autocatalytic processing of Thr-398 mutants. A, T380A (1 mg/ml; 20 mM Tris, pH 8.0) was incubated at 37 °C for the indicated times. At each time point, a sample was removed and subjected to SDS-PAGE. Percent processing values were determined by densitometric analysis of the gels, plotted versus time, and fit to a first-order monoexponential curve as described under “Experimental Procedures.” Representative data from three or more determinations are presented. The rate of processing exhibits a t1/2 = 2.34 ± 0.38 h. B, the same methodology was used for the T398S mutant and yields a t1/2 = 0.77 ± 0.07 h.](image)

**TABLE 2**

| Enzyme  | Hydrolysis | Processing | 
|---------|------------|------------|
|         | $K_m$ GNA  | $V_{max}$ | $T_{1/2}$ |
|         | μM         | μmol/min/mg| hours     |
| HpGT  | 12.5 ± 1.2 | 5.81 ± 0.13| 1.73 ± 0.22|
| T398S | 20.3 ± 1.9 | 1.14 ± 0.03| 0.77 ± 0.07|
| T398A | No activity | 2.54 ± 0.38|          |

Values for wild-type HpGT were previously reported (9).
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FIGURE 6. Proposed mechanism of γ-glutamyl peptide bond cleavage by HpGT. The proposed catalytic mechanism is based on available biochemical and structural data for HpGT. Additional biochemical and kinetic studies will be required to validate the mechanism, particularly with regard to protonation of the leaving group and activation of the nucleophilic water in the hydrolysis of the acyl enzyme intermediate.

...5-fold relative to wild-type enzyme and an overall catalytic efficiency, as measured by $V_{\text{max}}/K_m$, that was diminished by nearly an order of magnitude. Collectively, these results suggest that the γ-methyl group of Thr-398 may be necessary for optimal positioning of its side chain hydroxyl group. This hydroxyl, precisely positioned, is in turn required for activation of Thr-380 in the hydrolysis reaction but not in autoprocessing.

For several members of the N-terminal nucleophile hydrodase superfamily (13, 14), it has been suggested that cleavage immediately before the N-terminal nucleophile allows its free α-amino group to serve as a general base, leading to the activation of its own side chain. Alternatively, self-processing may orient a general base that increases the nucleophilicity of the conserved catalytic residue. For isoaaspartyl aminopeptidase (33) and glycosylasparaginase (34), a threonine hydroxyl group is found within hydrogen bond distance of the hydroxyl group of the catalytic nucleophile. Jaskolski and coworkers (33) have proposed that this threonine-threonine side chain interaction would be preferable to the intramolecular activation of the catalytic nucleophile by its free α-amino group, citing that the N-terminal amino group is a weak hydrogen bond abactor and easily protonated at neutral pH. Furthermore, an intramolecular hydrogen bond between the α-amino group and the side chain hydroxyl group would generate a five-membered ring with strained geometry (33). In the HpGT structure, the Thr-380 hydroxyl group is nearly equidistant from the α-amino of Thr-380 and the hydroxyl group of Thr-398. With the available data, we cannot unequivocally establish the identity of the base responsible for activation of the catalytic nucleophile. However, previous studies with rat γ-glutamyltranspeptidase have indicated that an imidazolium or primary ammonium ion with a perturbed $pK_a$ value is important in catalysis (35).

Implications for Catalysis—Additional kinetic studies are required to establish the details of HpGT catalysis, particularly with regard to the identity of many of the proton donors/acceptors. However, using the available structural and biochemical data, a plausible HpGT mechanism can be described (Fig. 6). In the resting enzyme, the Thr-398 hydroxyl group is positioned to form hydrogen bonds with both the α-amino and hydroxyl groups of Thr-380. These interactions help to position the catalytic nucleophile and increase its reactivity. Attack at the γ-glutamyl peptide bond leads to the formation of a tetrahedral transition state that is stabilized through interactions with the backbone amides of Gly-472 and Gly-473, similar to the oxyanion hole observed in serine proteases (19). Collapse of the tetrahedral intermediate leads to the cleavage of the γ-glutamyl peptide bond, dissociation of cysteinylglycine, and the formation of an acyl enzyme intermediate. We have indicated that the α-amino group of Thr-380 is the general acid responsible for protonation of the leaving group based on its proximity to the cysteine amide in the modeled glutathione-HpGT complex (Fig. 4; ~3.5 Å). Activation of a water molecule, potentially by the Thr-380 α-amino group, leads to hydrolysis and regeneration of the active site. Ongoing studies in the laboratory are aimed at clarifying the speculative aspects of this model on the basis of our new structural insights.

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