Uncoupling the Enzymatic and Autoprocessing Activities of *Helicobacter pylori* γ-Glutamyltranspeptidase*

Received for publication, April 10, 2006, and in revised form, May 1, 2006. Published, JBC Papers in Press, May 3, 2006, DOI 10.1074/jbc.M603381200

Gina Boanca1, Aaron Sand1, and Joseph J. Barycki2

From the Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588-0664

γ-Glutamyltranspeptidase (γGT), a member of the N-terminal nucleophile hydrolase superfamily, initiates extracellular glutathione reclamation by cleaving the γ-glutamyl amide bond of the tripeptide. This protein is translated as an inactive proenzyme that undergoes autoprocessing to become an active enzyme. The resultant N terminus of the cleaved proenzyme serves as a nucleophile in amide bond hydrolysis. *Helicobacter pylori* γ-glutamyltranspeptidase (HpGT) was selected as a model system to study the mechanistic details of autoprocessing and amide bond hydrolysis. In contrast to previously reported γGT, large quantities of HpGT were expressed solubly in the inactive precursor form. The 60-kDa proenzyme was kinetically competent to form the mature 40- and 20-kDa subunits and exhibited maximal autoprocessing activity at neutral pH. The activated enzyme hydrolyzed the γ-glutamyl amide bond of several substrates with comparable rates, but exhibited limited transpeptidase activity relative to mammalian γGT. As with autoprocessing, maximal enzymatic activity was observed at neutral pH, with hydrolysis of the acyl-enzyme intermediate as the rate-limiting step. Coexpression of the 20- and 40-kDa subunits of HpGT uncoupled autoprocessing from enzymatic activity and resulted in a fully active heterotrimer with kinetic constants similar to those of the wild-type enzyme. The specific contributions of a conserved threonine residue (Thr380) to autoprocessing and hydrolase activities were examined by mutagenesis using both the standard and coexpression systems. The results of these studies indicate that the γ-methyl group of Thr380 orients the hydroxyl group of this conserved residue, which is required for both the processing and hydrolase reactions.

*Helicobacter pylori* is a Gram-negative bacterial pathogen that colonizes the gastric mucosa. Infection puts the individual at greater risk for developing gastritis, peptic ulcer disease, and gastric cancer (1, 2). *H. pylori* γ-glutamyltranspeptidase (HpGT)3 is a glutathione-degrading enzyme that has been shown to be a virulence factor in infection (3, 4). *H. pylori* lacking γ-glutamyltranspeptidase has been shown to grow normally *in vitro*, but exhibits diminished growth rates within the gut in animal model systems. Bacterial loads of the HpGT-deficient strains are reduced by nearly 70% relative to the parental strain. Although not essential for colonization, HpGT clearly confers a growth advantage to the bacteria *in vivo* by mechanisms that remain unclear. HpGT has also been shown to up-regulate COX-2 and epidermal growth factor-related peptides in human gastric mucosal cells (5) and to induce apoptosis in human gastric epithelial cells (6). Both these activities are abolished by inactivation of the enzyme with mechanism-based inhibitors. Despite its demonstrated involvement in *H. pylori* colonization, persistence, and disease progression, biochemical characterizations of HpGT have been limited.

The reclamation of extracellular glutathione and its conjugates is initiated by γ-glutamyltranspeptidase (γGT). The enzyme cleaves the γ-glutamyl amide bond to liberate cysteinylglycine, and the catalytic mechanism proceeds via a γ-glutamyl-enzyme intermediate (7–11). The γ-glutamyl group can be transferred to water (hydrolysis) or to an amino acid or short peptide (transpeptidation). Whereas mammalian γGTs are embedded in the plasma membrane by a single N-terminal transmembrane anchor and are heterologously glycosylated, bacterial homologs are soluble and localized to the periplasmic space. Overall, the γGTs are highly conserved, with mammalian and bacterial homologs often sharing >25% sequence identity.

A required post-translational modification is the maturation of the precursor protein. γGT is synthesized as a 60-kDa polypeptide, and cleavage of the proenzyme yields a heterodimer composed of a 40- and a 20-kDa subunit (see Scheme 1) (3, 12–14). Processing of γGT is thought to be an intramolecular autocatalytic event, and a mechanism has been proposed in which processing proceeds via a nitrogen → oxygen acyl shift, with a conserved threonine residue serving as the nucleophile (15). Based on its enzymatic function and autoprocessing activity, γGT has been classified as an Ntn (N-terminal nucleophile) hydrolase (15). Members of the Ntn hydrolase family contain an αβαα-core structure, are autocatalytically processed to yield an active enzyme, and catalyze amide bond hydrolysis (16, 17). The new N-terminal residue of the processed enzyme, typically a serine, threonine, or cysteine residue, then serves as a nucleophile in the catalytic mechanism. Although the general features of the function of HpGT can be inferred based on its classification as an Ntn hydrolase, many mechanistic details of the autoactivation and catalytic function of HpGT have not been addressed.
Characterization of H. pylori γ-Glutamyltranspeptidase

In this study, we isolated and biochemically characterized recombinant H. pylori γ-glutamyltranspeptidase. In contrast to previous reports of γGT purified from other organisms, the solubly expressed protein was isolated primarily as the 60-kDa precursor. This allowed us an opportunity to examine the auto-processing of the protein. The rate of maturation was maximal at neutral pH, as was the enzymatic activity of the mature α₂β₂ heterotetramer. We used biochemical measurements of enzymatic activity in conjunction with site-directed mutagenesis and a coexpression system to investigate the involvement of a conserved threonine residue in the processing and catalytic activities.

EXPERIMENTAL PROCEDURES

Generation of Wild-type and Mutant HpGT Expression Constructs—The isolation of HpGT has been described (3), and its sequence corresponds to a predicted open reading frame within the H. pylori genome (KEGG Data Base entry HP1118) (18, 19). Although encoded by a single gene, the protein was isolated as two polypeptides of ~40 and ~20 kDa. Primers were designed to amplify HpGT, excluding a 26-amino acid signal sequence that targets the enzyme to the periplasmic space (3). Using H. pylori genomic DNA (American Type Culture Collection) as a template, a 1.6-kb DNA fragment was amplified by PCR and inserted into a pET-28a expression vector (Novagen) incorporating a thrombin-cleavable N-terminal histidine tag. The resulting expression construct (pET-28/HpGT) was sequenced at the Genomics Core Facility of the University of Nebraska (Lincoln, NE) and confirmed to be identical to HP1118 excluding the signal sequence.

Expression constructs for individual subunits of the processed enzyme were generated. The gene sequence for the N-terminal 40-kDa subunit was amplified by PCR, incorporating SpeI and Sall restriction sites. The insert was digested with the appropriate restriction enzymes and ligated into the pET-28a expression vector digested with NheI and SalI. Similarly, the gene sequence for the C-terminal 20-kDa subunit was amplifed by PCR, incorporating Ndel and Sall restriction sites. The resultant 0.6-kb PCR product was digested with the relevant enzymes and ligated into a similarly digested pET-24a expression vector (Novagen), thus incorporating a C-terminal histidine tag. A biocistronic construct was also generated to express the N-terminal 40-kDa and C-terminal 20-kDa subunits separately but concurrently. The full-length HpGT expression construct was used as a template, and the HpGT sequence corresponding to the 20-kDa subunit was amplified by PCR, incorporating Ndel and XhoI restriction sites. The product was digested with the relevant enzymes and ligated into a similarly digested pETDuet expression vector (Novagen). The dual expression vector containing the 20-kDa coding sequence was amplified and isolated. Next, the coding sequence for the 40-kDa subunit was excised from the above 40-kDa expression vector using Ncol and Sall. The resultant 1-kb fragment was gel-purified and ligated into a similarly digested pETDuet expression vector containing the 20-kDa subunit sequence. The completed construct (HpGT-Duet) contained an N-terminal histidine tag on the 40-kDa subunit and an N-terminal methionine on the 20-kDa subunit. Point mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s protocol, and all constructs were verified by sequencing at the Genomics Core Facility of the University of Nebraska.

Expression and Purification of HpGT—The pET-28/HpGT expression construct was used to transform Escherichia coli strain Rosetta(DE3)pLysS (Novagen). Cultures grown at 30 °C to A₆₀₀ₙₐₙ = 0.6–0.8 in 2× YT medium containing 34 mg/liter chloramphenicol and 30 mg/liter kanamycin were induced for 4 h by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 500 μM. The cells were harvested by centrifugation and stored at ~80 °C. Cells were resuspended in lysis buffer (50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, and 10 mM imidazole), lysed by sonication, and centrifuged. The supernatant was treated with 0.35% polyethyleneimine at 4 °C and centrifuged to separate precipitated nucleic acids from the protein-containing supernatant. The protein was purified by affinity chromatography using a nickel-chelating column (Novagen) following the manufacturer’s protocol. Recombinant HpGT was concentrated, dialyzed against 20 mM Tris (pH 7.4), and stored at 4 °C. Protein concentrations were estimated using a calculated extinction coefficient based on aromatic residue content (A₂₈₀ nm of 1 mg/ml solution = 0.766) (20). The histidine tag used for affinity purification was removed by proteolytic cleavage with thrombin (1 unit of thrombin/mg of HpGT, incubated overnight at 18 °C), which left an additional seven residues (GSHMASA) on the N terminus of HpGT. Following thrombin incubation, >50% of HpGT was found in the processed form. To ensure complete maturation of the enzyme, it was incubated at 37 °C for 6 h. The sample was centrifuged to remove precipitated proteins, concentrated, and dialyzed against 20 mM Tris (pH 7.4).

A similar protocol was followed for each of the expression constructs with the following modifications. After ascertaining that the N-terminal histidine tag did not impact the processing or enzymatic activity of the wild-type enzyme, the thrombin cleavage step was omitted from all subsequent enzyme purifications. The HpGT-Duet construct confers resistance to ampicillin, and kanamycin was thus replaced with 100 mg/liter ampicillin. For HpGT generated using the pETDuet expression construct (HpGT-Duet), the enzyme was isolated in its mature form, and additional incubations were not required.

Kinetic Characterizations of HpGT—To determine the rate of processing and its effects on catalytic activity, unprocessed HpGT was purified as described above with the following modifications. After concentration and dialysis of the enzyme, it was flash-frozen and stored at −80 °C to limit processing. It is important to note that <10% of the protein was cleaved after purification (see Fig. 1A, lane 1). The following day, HpGT (1 mg/ml) was thawed and incubated in 20 mM Tris (pH 8.0) at 37 °C. At the indicated times, an aliquot was removed, assayed for hydrolase activity as described below, and denatured by boiling in SDS loading buffer for 5 min. Samples were analyzed by SDS-PAGE, and the gel was stained using GelCode Blue (Pierce). To quantify the degree of processing, a digital image of the gel was taken, and densitometric measurements were made on the 20-, 40-, and 60-kDa bands at each time point. For wild-type HpGT, bands at 30 h (see Fig. 1A, lane 8) were taken to

19030 JOURNAL OF BIOLOGICAL CHEMISTRY
represents complete cleavage, and the percent processing was calculated for the 40-kDa band at a given time point. The percent processing values were plotted versus time to obtain a processing rate (see Fig. 1B). A similar analysis was performed with the T380S mutant and required an extended incubation of 30 days. Representative plots from three or more determinations are shown. To examine the pH profile of autoprocessing, a citrate/phosphate buffer system of constant ionic strength (21) was used, and the percent processing was calculated for each band at a given time point and averaged.

The cleavage of the γ-glutamyl group of the physiological substrate glutathione was assessed using a coupled assay system to detect the release of glutamate. HpGT (10 μg/ml) in 0.1 M Tris (pH 8.0) was incubated with 20 mM glutathione at 37 °C for 15 min. The reaction was stopped by boiling, and precipitated protein was removed by centrifugation. Parallel incubations were performed in the absence of HpGT to establish the nonenzymatic rate of hydrolysis. The amount of glutamate released was determined using a glutamate dehydrogenase assay following the conversion of glutamate to α-ketoglutarate concomitant with the production of NADH. Aliquots of the HpGT reaction mixtures were withdrawn, incubated at 37 °C with glutamate dehydrogenase (0.1 mg/ml; Sigma) and NAD+ (2 mM), and allowed to come to equilibrium (2 h). The absorbance at 340 nm was measured, and a standard curve for glutamate (0–100 μM) was determined. Similar experiments were performed using glutamine as the HpGT substrate.

For routine enzymatic characterizations, the substrate analog l-glutamic acid γ-(4-nitroanilide) (GNA) was used (11, 22). The release of 4-nitroaniline can be monitored continuously at 412 nm, and the enzymatic rate of hydrolysis. The amount of glutamate released using the manufacturer’s DYNAMICS software package, and the molecular mass was estimated based upon the hydrodynamic radius of the protein assuming a spherical model. Electrospray mass spectrometric analyses were performed at the Nebraska Redox Biology Center Metabolomics Core Facility of the University of Nebraska (Lincoln).

RESULTS

Protein Expression and Purification—HpGT is expressed as an ~60-kDa precursor that undergoes autocatalytic processing to an ~40 kDa α-subunit and an ~20-kDa β-subunit (Scheme 1). HpGT has been implicated as a virulence factor in colonization (3, 4), an inducer of apoptosis (6), and a modulator of COX-2 and epidermal growth factor-related peptides (5). However, the autoprocessing activity, enzymatic profile, and quaternary structure of HpGT have not been rigorously characterized. To examine these properties, we generated a prokaryotic expression vector containing the coding sequence of HpGT and overexpressed the protein in E. coli. HpGT was purified by affinity chromatography exploiting an engineered N-terminal hexahistidine tag, with typical yields of 50 mg of purified protein/liter of bacterial culture. The enzyme was >95% pure based on SDS-PAGE, and the vast majority of HpGT exhibited an apparent molecular mass of ~60 kDa (Fig. 1A, lane 1), corresponding to the inactive precursor. The precursor could be induced to catalytically cleave itself to produce the active heterodimer composed of an ~40- and an ~20-kDa subunit (Fig. 1A, lane 8).

Autocatalytic Processing of HpGT—To characterize the autocatalytic processing of the enzyme, we monitored the conversion of precursor HpGT to its mature form as a function of time (Fig. 1). At the indicated times, an aliquot of HpGT was removed and denatured. Samples were analyzed by SDS-PAGE (Fig. 1A), and the percent processing was plotted versus time (Fig. 1B). Under the indicated conditions, the processing of HpGT exhibited t1/2 = 1.73 ± 0.22 h (Table 1). Catalytic activity was also plotted as a function of processing (Fig. 1B, inset). Hydrolysis activity was strongly dependent on autocatalytic processing of the enzyme, exhibiting a nearly 1:1 relationship. Extrapolation to the completely unprocessed enzyme suggested that the uncleaved enzyme exhibited ~7% activity. However, the uncertainty in the densitometric measurements may underestimate the extent of cleavage in the early time points. Subsequent studies of HpGT mutants (discussed below) with diminished processing activities indicated that processing is required for enzymatic activity. Thus, it is likely that the unprocessed wild-type enzyme is also completely inactive.

The maturation of HpGT was further characterized to gain insights into the autoprocessing reaction. To verify that matu-
Characterization of H. pylori γ-Glutamyltranspeptidase

The pH dependence of HpGT processing was also examined and found to be most efficient in the neutral pH range (data not shown). Because of the inherent uncertainty in the densitometric measurements and prolonged incubation times, considerable variability in rates was observed. Most notably, reliable rates for processing could not be determined below pH 5.0 and above pH 9.0, as incomplete maturation of the enzyme was observed. At these extreme pH values, HpGT was prone to aggregation as judged by dynamic light scattering experiments.

**Determination of the Oligomeric State of HpGT**—To examine the quaternary structure of HpGT, the apparent molecular mass of the native enzyme was determined by gel filtration (Fig. 2). Compared with molecular mass standards, mature HpGT exhibited an estimated molecular mass of ~90 kDa, suggesting that the enzyme is either an extended heterodimer (αβ, 60 kDa) or a compact heterotetramer (αββ, 120 kDa). To differentiate between these two possibilities, dynamic light scattering experiments were performed. Measurements of mature HpGT indicated a molecular mass between 109 and 122 kDa, suggesting that the enzyme is an αββ-heterotetramer. Although SDS-PAGE analysis suggested equivalent concentrations of 20- and 40-kDa subunits, the possibility of an unequal combination of α- and β-subunits in the mature enzyme, such as αβ or α2β2, was excluded by studies with the unprocessed precursor and an HpGT mutant incapable of forming the α- and β-subunits (T380A; discussed below). Both of these proteins ran nearly identically to mature HpGT on the gel filtration column, and both had comparable molecular masses (110–120 kDa) as judged by dynamic light scattering experiments.

**Kinetic Characterization of HpGT**—A coupled assay system was used to detect the release of glutamate from potential physiological substrates. HpGT exhibited a specific activity of 3.59 ± 0.17 μmol of glutathione hydrolyzed per min/mg of enzyme. Similar experiments were performed using glutamine as the substrate, and a comparable rate of hydrolysis (2.77 ± 0.04 μmol of glutamine hydrolyzed per min/mg of enzyme) was observed. Cleavage of the γ-glutamyl bond in both glutathione and glutamine was verified by mass spectrometry. The coupled assay system is cumbersome, and therefore, most studies of γGT have employed the substrate analog GNA. To obtain kinetic constants (Km and Vmax) for the artificial substrate, the steady-state release of 4-nitroaniline was monitored by its absorbance at 412 nm. Measurements to determine the dependence of the reaction on substrate concentration were done using purified HpGT incubated with increasing concentrations of GNA. Saturation kinetics were observed, and data were fit to the Michaelis-Menten equation to obtain Km and Vmax for the enzyme-catalyzed reaction (Table 1). The apparent Km for GNA was 12.5 ± 1.2 μM, and the apparent Vmax was 5.81 ± 0.13 μmol/min/mg of enzyme.

The Km and Vmax values for the hydrolysis of GNA as catalyzed by HpGT are in good agreement with those reported for the human enzyme (Table 1) (24). However, there are considerable differences in transpeptidation. For human γGT, the addition of 20 mM glycylglycine as an acceptor substrate resulted in a >100-fold rate increase in the release of 4-nitroaniline. Furthermore, the apparent Km for GNA increased substantially (Table 1), presumably because the donor- and acceptor-binding sites are at least partially overlapping, i.e. the acceptor site is likely coincident with the cysteinylglycine portion of the glutathione-binding site (9, 25). In contrast, HpGT was only marginally more active as a transpeptidase than a hydrolase, and the apparent Km for GNA was relatively unaffected by the presence of 20 mM glycylglycine (Table 1). HpGT did not exhibit saturation kinetics with respect to glycylglycine, whereas the human enzyme has a reported Km for glycylglycine of 2.5 mM. Furthermore, even at 100 mM glycylglycine, HpGT...
exhibited a modest ~2-fold rate increase relative to the hydrolase activity. Mass spectrometric analysis indicated that transpeptidase did occur, as a peak corresponding to the molecular mass of glutamylglycylglycine was observed. However, these data strongly indicate that HpGT is not an effective transpeptidase when using the standard acceptor glycylglycine.

The pH dependence of HpGT activity was also examined. At a given pH, steady-state kinetic constants were determined. No significant pH dependence for the $K_m$ values was observed (data not shown). However, a bell-shaped pH profile was observed for enzymatic activity (Fig. 3), suggesting that at least two ionizable groups contribute to catalysis. Similar to the pH dependence of autoprocessing, HpGT enzymatic activity with GNA as the substrate was greatest at neutral pH.

For mammalian $\gamma$GT, the rate-limiting step in catalysis was shown to be the breakdown of the acyl-enzyme intermediate (23). To ascertain whether this is also the case for HpGT, pre-steady-state kinetic studies were initiated. HpGT was rapidly mixed with GNA, and the absorbance at 412 nm was monitored using an Applied Photophysics stopped-flow apparatus. A burst phase proportional to the enzyme concentration was observed, followed by a slower steady-state rate (Fig. 4). The predicted absorbance change for a single turnover (~0.15 absorbance units) agreed closely with the observed burst phase.

The slower rate of 3.54 $\mu$mol/min/mg of enzyme compared favorably with our steady-state kinetic measurement of 5.81 ± 0.13 $\mu$mol/min/mg of enzyme. This is consistent with the formation of an acyl-enzyme intermediate, the breakdown of which is rate-limiting overall.

Site-directed Mutagenesis of Thr380—Thr380 is the new N-terminal residue of the ~20-kDa $\beta$-subunit of the mature enzyme. To assess the contributions of Thr380 to autoprocessing and enzymatic activities, two point mutations were made at this position. Substitution with alanine (T380A) resulted in a protein that could not process even after 30 days at 37 °C and that was devoid of enzymatic activity (Table 2). Substitution with serine (T380S) resulted in an enzyme that was most active at neutral pH and likely has at least two ionizable groups that contribute to catalysis.

Saturation kinetics were not observed with glycylglycine as the varied substrate, and therefore, measurements were made using 20 mM glycylglycine as reported for the human enzyme. Therefore, saturation kinetics were observed with glycylglycine as the varied substrate with an apparent $K_m$ of 2.5 ± 0.3 mM. However, a bell-shaped pH profile was observed for enzymatic activity (Fig. 3), suggesting that at least two ionizable groups contribute to catalysis.
Characterization of H. pylori $\gamma$-Glutamyltranspeptidase

FIGURE 4. Burst phase kinetics observed with HpGT and the substrate analog GNA. HpGT was rapidly mixed with GNA, and the absorbance at 412 nm was monitored using a stopped-flow apparatus. The predicted absorbance change for a single turnover agrees closely with the observed burst. This observation is consistent with the rapid formation of an acyl-enzyme intermediate, followed by its slow hydrolysis.

The $\gamma$-methyl group of Thr$^{380}$ is also required for efficient enzymatic hydrolysis of GNA. Analysis of the kinetic constants for processed T380S indicated significant perturbations relative to the wild-type enzyme (Table 1). Overall, the rate of catalysis was reduced by $\sim$12-fold, whereas the apparent $K_{\text{m}}$ for GNA was increased by $\sim$2.5-fold. This reduced activity may result from the prolonged incubation (>30 days at 37 °C) required for maturation. However, the native enzyme exhibited a <5% loss of activity over the same 30-day period required for maturation of the T380S mutant. Furthermore, the T380S mutant did not precipitate during the maturation process, and the mature T380S protein had a similar tryptophan fluorescence profile compared with the wild-type enzyme (data not shown), suggesting that the overall fold and intrinsic stability of the mutant were uncompromised. Although the $\gamma$-methyl group of Thr$^{380}$ was required for optimal catalytic activity, its removal did not result in a dramatic loss of activity (Table 1). As opposed to the wild-type protein, completely autoprocessed T380S could be obtained. Analysis of enzymatic activity as a function of processing (Fig. 5B, inset) indicated that unprocessed T380S was devoid of enzymatic activity. In conjunction with similar observations with wild-type HpGT (Fig. 1B, inset), these data suggest that processing is required for HpGT activity.

Generation of a Coexpression Construct—Using the standard expression system, it is difficult to assess the precise role of Thr$^{380}$ in catalysis. As indicated above, the hydroxyl group of a serine residue could adequately substitute for the conserved threonine residue in the hydrolysis of GNA. Substitution with alanine resulted in a protein incapable of maturation (Fig. 6, lane 3) and devoid of enzymatic activity. However, it was unclear whether the T380A mutant lacked enzymatic activity because it could not autoprocess or because the hydroxyl group of Thr$^{380}$ was required for catalytic activity. To address whether the hydroxyl group of Thr$^{380}$ is required for autoprocessing, enzymatic activity, or both, we generated a system in which the catalytic activity of HpGT could be uncoupled from its processing.

We created constructs to express the 40-kDa $\alpha$-subunit and the 20-kDa $\beta$-subunit of mature HpGT independently in a bacterial expression system. Both the 40- and 20-kDa subunits were soluble when expressed separately in E. coli over a wide range of conditions, suggesting that both subunits must be present for proper folding. Therefore, to express both subunits in the same cell, the gene sequences of the 40-kDa $\alpha$-subunit and the 20-kDa $\beta$-subunit were subcloned into the pETDuet expression vector. In this construct, the 40-kDa subunit contained an N-terminal histidine tag, and the 20-kDa subunit had only the initiator methionine added (HpGT-Duet). The HpGT-Duet protein was purified by immobilized metal affinity chromatography with yields of 15–25 mg of HpGT/liter of bacterial culture. Similar molar ratios of $\alpha$- to $\beta$-subunits were observed.

The burst phase kinetics observed with HpGT and the substrate analog GNA. HpGT was rapidly mixed with GNA, and the absorbance at 412 nm was monitored using a stopped-flow apparatus. The predicted absorbance change for a single turnover agrees closely with the observed burst. This observation is consistent with the rapid formation of an acyl-enzyme intermediate, followed by its slow hydrolysis.

FIGURE 5. Autocatalytic processing of T380S. A, T380S (1 mg/ml) in 20 mM Tris (pH 8.0) was incubated at 37 °C for 0 h (lane 1), 2 days (lane 2), 4 days (lane 3), 6 days (lane 4), 8 days (lane 5), 10 days (lane 6), 15 days (lane 7), 20 days (lane 8), and 30 days (lane 9). At each time point, a sample was removed and treated as described in the legend to Fig. 1. B, the extent of processing was determined using densitometric measurements as described under “Experimental Procedures” and plotted as a function of time. The rate of processing exhibited $t_{1/2} = 5.75 \pm 0.82$ days. Inset, the catalytic activity of T380S plotted as a function of processing indicates a 1:1 correlation.

TABLE 2

| Enzyme          | Single polypeptide | Coexpressed subunits |
|-----------------|--------------------|-----------------------|
|                 | $K_{\text{m}}$   | $V_{\text{max}}$   | $K_{\text{m}}$   | $V_{\text{max}}$   |
| Wild-type       | 12.5 ± 1.2         | 5.81 ± 0.13          | 26.2 ± 3.4        | 5.11 ± 0.18        |
| T380A           | No activity        | No activity          |                     |                     |
FIGURE 6. Generation of a coexpression system to produce mature HpGT independent of autoprocessing. To determine the contributions of critical residues to autoprocessing and enzymatic activities, a coexpression system was developed. The gene sequences of the 40-kDa α-subunit and the 20-kDa β-subunit of HpGT were subcloned into a coexpression vector for concurrent bacterial expression as described under “Experimental Procedures” (HpGT-Duet). Purified proteins are as follows: mature wild-type HpGT (lane 1), HpGT-Duet (lane 2), T380A (lane 3), and T380A-Duet (lane 4). HpGT-Duet had electrophoretic properties comparable with those of the wild-type protein (compare lanes 1 and 2). The T380A mutant was incapable of maturation (lane 3), but the coexpression system allowed for generation of a mature T380A heterotetramer (lane 4).

In the wild-type and HpGT-Duet proteins (Fig. 6, compare lanes 1 and 2). Only the 40-kDa α-subunit had a hexahistidine tag for purification, suggesting that a properly folded α₂β₂-heterotetramer was formed. Analysis by electrospray mass spectrometry indicated that the initiator methionine had been removed from the 20-kDa subunit during expression in E. coli. The 20-kDa subunit generated via autoprocessing had an observed mass of 20,390.0 Da compared with 20,389.8 Da for that generated via the coexpression system (predicted molecular mass of 20,391.25 Da). HpGT-Duet had kinetic constants comparable with those of the wild-type enzyme (Table 2) and thus successfully uncoupled maturation from enzymatic activity.

Using the newly developed coexpression construct, Thr³⁸⁰ was again replaced with an alanine residue. Whereas the T380A mutant produced in the original construct persisted as an unprocessed 60-kDa protein (Fig. 6, lane 3), purified T380A-Duet contained both the α₂ and the β₂-subunits (Fig. 6, lane 4), suggesting that the protein was correctly assembled. Removal of the initiator methionine from the β₂-subunit of the mutant protein was confirmed by mass spectrometry. Like the wild-type and HpGT-Duet proteins, the T380A-Duet protein formed an α₂β₂-heterotetramer in solution as judged by dynamic light scattering experiments. The T380A-Duet protein did not exhibit any catalytic activity with the substrate analog GNA (Table 2), indicating that Thr³⁸⁰ is required for enzymatic activity.

DISCUSSION

HpGT has been implicated in gut colonization and persistence by H. pylori as well as in the progression of diseases resulting from infection by these bacteria (3, 4). To gain insights into the mechanisms by which HpGT confers a growth advantage, we have biochemically examined the enzymatic and processing activities of the enzyme. We were able to isolate an unprocessed precursor protein and to characterize its maturation in detail. The precursor protein lacks hydrolase activity, indicating that autoprocessing is required for enzymatic activation. In complementary studies, we successfully uncoupled processing from enzymatic activity using a coexpression system. These recombinant protein expression systems allowed us to assess the contributions of a conserved threonine residue (Thr³⁸⁰) to autoprocessing independent of glutathione hydrolase activity.

In many organisms, γGT catalyzes the first step in the reclamation of extracellular glutathione and its conjugates by cleaving the γ-glutamyl peptide bond. HpGT and human γGT share >30% sequence identity and exhibit similar hydrolase activities. However, a search of the H. pylori genome did not reveal any proteins with significant similarity to known glutathione biosynthetic enzymes, suggesting that H. pylori does not produce glutathione to modulate cellular redox levels (18, 19). The bacteria are equipped with numerous peroxidases, catalase, and a functional thioredoxin system that may protect against oxidative damage (26). Perhaps, as suggested by in vivo studies (3, 4), HpGT provides the bacteria with a growth advantage within the gut by salvaging extracellular glutathione to obtain cysteine for subsequent protein synthesis. Both the autoprocessing and hydrolase activities of HpGT are maximal at neutral pH and suggest a functional role for the enzyme following colonization.

Studies in other model systems are consistent with γGT providing necessary precursors for protein and glutathione biosynthesis. Cysteine is often limiting in these processes and is obtained either via the transsulfuration pathway, which converts homocysteine to cysteine, or via the salvage of excreted glutathione and its conjugates. γGT-deficient mice appear normal at birth, exhibit slower growth, do not sexually mature, and have shorter life spans (27–34). They are also considerably more susceptible to cataract formation (29) and damage by reactive oxygen species (32). Supplementation with N-acetylcysteine largely restores the native phenotype, suggesting that the primary function of γGT in normal cells is the recovery of cysteine for use in protein and glutathione synthesis, which is important for adequate growth and oxidative protection (28). In agreement with these findings, γGT is found up-regulated in several cancer types and has been shown to accelerate tumor growth and to increase tumor resistance to damage induced by chemotherapy and radiation treatment (35–37). Thus, it is reasonable to suppose that expression of a homologous enzyme would also facilitate growth of the bacteria.

HpGT exhibits similar rates of hydrolysis with glutathione, glutamine, and the substrate analog GNA (Table 1), and these values are comparable with the rates observed for other γGTs isolated from E. coli (38), Bacillus subtilis (39), rat (40), and human (24). Pre-steady-state kinetic studies with HpGT (Fig. 4) indicated that hydrolysis of the γ-glutamyl-enzyme intermediate is rate-limiting, as has been demonstrated in the rat homolog (23). In the presence of 20 mM glycylglycine, HpGT exhibits a modest 1.2-fold rate increase relative to hydrolysis and does not exhibit saturation kinetics with respect to this model acceptor peptide (Table 1). Each of the 20 common L-amino acids, as

Characterization of H. pylori γ-Glutamyltranspeptidase
Characterization of H. pylori γ-Glutamyltranspeptidase

As observed in other systems, substitution of Thr380 with alanine (T380A) in HpGT results in a protein incapable of maturation. A serine residue can substitute for Thr380, but results in an ~80-fold reduction in the maturation rate (Fig. 5 and Table 1), suggesting that the γ-methyl group of Thr380 helps to position its hydroxyl group for optimal processing. Similarly, mature T380S has reduced hydrolyase activity (Table 1), underscoring the importance of the γ-methyl group in orienting the nucleophilic hydroxyl group for the enzymatic reaction. To determine whether the hydroxyl group of Thr380 is indeed critical for hydrolyase activity, a coexpression system was used to produce a T380A mutant in a mature form (T380A-Duet). Although the T380A-Duet mutant formed an αβ-heterotetramer, it completely lacked enzymatic activity when using the substrate analog GNA (Table 2). These observations strongly support the proposed function of Thr380 as the required nucleophile in both the processing and enzymatic reactions.

Acknowledgment—We thank Dr. Melanie Simpson for thoughtful insights and review of the manuscript.

REFERENCES
1. Bjorkholm, B., Falk, P., Engstrand, L., and Nyren, O. (2003) J. Intern. Med. 253, 102–119
2. Sharma, P., and Vakil, N. (2003) Aliment. Pharmacol. Ther. 17, 297–305
3. Chevalier, C., Thiberge, J. M., Ferrero, R. L., and Labigne, A. (1999) Mol. Microbiol. 31, 1359–1372
4. McGovern, K. J., Blanchard, T. G., Gutierrez, J. A., Czinn, S. J., Krakowka, S., and Youngman, P. (2001) Infect. Immun. 69, 4168–4173
5. Busiello, I., Acquaviva, R., Di Popolo, A., Blanchard, T. G., Ricci, V., Romano, M., and Zarrilli, R. (2004) Cell. Microbiol. 6, 255–267
6. Shibayama, K., Kamachi, K., Nagata, N., Yagi, T., Nada, T., Doi, Y., Shibata, N., Yokoyama, K., Yamane, K., Kato, H., Inuma, Y., and Arakawa, Y. (2003) Mol. Microbiol. 47, 443–451
7. Tate, S. S., and Meister, A. (1981) Mol. Cell. Biochem. 39, 357–368
8. Allison, R. D. (1985) Methods Enzymol. 113, 408–425
9. Taniguchi, N., and Ikeda, Y. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 72, 239–278
10. Ikeda, Y., and Taniguchi, N. (2005) Methods Enzymol. 401, 408–425
11. Keillor, J. W., Castonguay, R., and Lherbet, C. (2005) Methods Enzymol. 401, 449–467
12. Suzuki, H., Kumagai, H., Ichigishi, T., and Tochikura, T. (1988) Biochem. Biophys. Res. Commun. 150, 33–38
13. Carter, B. Z., Shi, Z. Z., Barrios, R., and Lieberman, M. W. (1998) J. Biol. Chem. 273, 28277–28285
14. Klinough, C. L., Poland, P. A., Bruns, J. B., and Hughey, R. P. (2005) Meth-
Characterization of H. pylori γ-Glutamyltranspeptidase

ods Enzymol. 401, 426–449
15. Suzuki, H., and Kumagai, H. (2002) J. Biol. Chem. 277, 43536–43543
16. Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C., Smith, J. L., Tomchick, D. R., and Murzin, A. G. (1995) Nature 378, 416–419
17. Oinonen, C., and Rouvinen, J. (2000) Protein Sci. 9, 2329–2337
18. Alm, R. A., Ling, L. S., Moir, D. T., King, B. L., Brown, E. D., Doig, P. C., Smith, D. R., Noonan, B., Guild, B. C., deJonge, B. L., Carmel, G., Guminino, P. I., Caruso, A., Uri-Nickelsen, M., Mills, D. M., Ives, C., Gibson, R., Merberg, D., Mills, S. D., Jiang, Q., Taylor, D. E., Vovis, G. F., and Trust, T. J. (1999) Nature 397, 176–180
19. Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., Mckenney, K., Fitzegerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weidman, J. M., Fuji, C., Bowman, C., Watthey, L. Wallin, E., Hayes, W. S., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and Venter, J. C., (1995) Nature 388, 539–547
20. Pace, C. N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) Protein Sci. 4, 2411–2423
21. Elving, P. J., Markowitz, J. M., and Rosenthal, I. (1956) Anal. Biochem. 28, 1179–1180
22. Tate, S. S., and Meister, A. (1985) Methods Enzymol. 113, 400–419
23. Kellor, J. W., Menard, A., Castonguay, R., Lherbet, C., and Rivard, C. (2004) J. Phys. Org. Chem. 17, 529–536
24. Ikeda, Y., Fujii, J., Anderson, M. E., Taniguchi, N., and Meister, A. (1995) J. Biol. Chem. 270, 22223–22228
25. Thompson, G. A., and Meister, A. (1977) J. Biol. Chem. 252, 6792–6798
26. Comtois, S. L., Gidley, M. D., and Kelly, D. J. (2003) Microbiology (Read.) 149, 121–129
27. Shi, Z. Z., Habib, G. M., Lebovitz, R. M., and Lieberman, M. W. (1995) Gene (Amst.) 167, 233–237
28. Lieberman, M. W., Wiseman, A. L., Shi, Z. Z., Carter, B. Z., Barrios, R., Ou, C. N., Chevez-Barrillos, P., Wang, Y., Habib, G. M., Goodman, J. C., Huang, S. L., Lebovitz, R. M., and Matzuk, M. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7923–7926
29. Chevez-Barrios, P., Wiseman, A. L., Rojas, E., Ou, C. N., and Lieberman, M. W. (2000) Exp. Eye Res. 71, 575–582
30. Habib, G. M., Shi, Z. Z., Ou, C. N., Kala, G., Kala, S. V., and Lieberman, M. W. (2000) Hepatol. 32, 556–562
31. Kumar, T. R., Wiseman, A. L., Kala, G., Kala, S. V., Matzuk, M. M., and Lieberman, M. W. (2000) Endocrinology 141, 4270–4277
32. Rojas, E., Valverde, M., Kala, S. V., Kala, G., and Lieberman, M. W. (2000) Mutat. Res. 447, 305–316
33. Will, Y., Fischer, K. A., Horton, R. A., Kaetzel, R. S., Brown, M. K., Hedstrom, O., Lieberman, M. W., and Reed, D. J. (2000) Hepatology 32, 740–749
34. Hanigan, M. H., Lykissa, E. D., Townsend, D. M., Ou, C. N., Barrios, R., and Lieberman, M. W. (2001) Am. J. Pathol. 159, 1889–1894
35. Hanigan, M. H., Frierson, H. F., Jr., Swanson, P. E., and De Young, B. R. (1999) Hum. Pathol. 30, 300–305
36. Hanigan, M. H., Gallagher, B. C., Townsend, D. M., and Gabarra, V. (1999) Carcinogenesis 20, 553–559
37. Gallagher, B. C., Rudolph, D. B., Hinton, B. T., and Hanigan, M. H. (1998) Carcinogenesis 19, 1251–1255
38. Suzuki, H., Kumagai, H., and Tochikura, T. (1986) J. Bacteriol. 168, 1325–1331
39. Minami, H., Suzuki, H., and Kumagai, H. (2003) FEMS Microbiol. Lett. 224, 169–173
40. Abbott, W. A., and Meister, A. (1983) J. Biol. Chem. 258, 6193–6197
41. Castonguay, R., Lherbet, C., and Kellor, J. W. (2003) Biochemistry 42, 11504–11513
42. Ikeda, Y., Fujii, J., Taniguchi, N., and Meister, A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 126–130
43. Brunscher, H., and Schumacher, G. (1992) Eur. J. Biochem. 205, 77–83
44. Li, Y., Chen, J., Jiang, W., Mao, X., Zhao, G., and Wang, E. (1999) Eur. J. Biochem. 262, 713–719
45. Hashimoto, W., Suzuki, H., Nohara, S., Tachi, H., Yamamoto, K., and Kumagai, H. (1995) J. Biochem. (Tokyo) 118, 1216–1223
46. Hashimoto, W., Suzuki, H., Yamamoto, K., and Kumagai, H. (1995) J. Biochem. 118, 75–80
47. Inoue, M., Hira, I., Suzuki, H., Kumagai, H., and Sakata, K. (2000) Biochemistry 39, 7764–7771