Dissection of Glutathionylspermidine Synthetase/Amidase from *Escherichia coli* into Autonomously Folding and Functional Synthetase and Amidase Domains*

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David S. Kwon‡, Chun-Hung Lin‡, Shoujun Chen§, James K. Coward¶, Christopher T. Walsh‡, and J. Martin Bollinger, Jr.¶

*From the ‡Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802, the §Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 and the ¶Interdepartmental Program in Medicinal Chemistry, College of Pharmacy and Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109

The bifunctional glutathionylspermidine synthetase/amidase from *Escherichia coli* catalyzes both the ATP-dependent formation of an amide bond between N₂ of spermidine (N-(3-amino)propyl-1,4-diaminobutane) and the glycine carboxylate of glutathione (γ-Glu-Cys-Gly) and the opposing hydrolysis of this amide bond ([Bollinger, J. M., Jr., Kwon, D. S., Huisman, G. W., Kolter, R., and Walsh, C. T. (1995) *J. Biol. Chem.* 270, 14031–14041]. In our previous work describing its initial characterization, we proposed that the 61-amino acid (70 kDa) protein might possess separate amidase (N-terminal) and synthetase (C-terminal) domains. In the present study, we have confirmed this hypothesis by expression of independently folding and functional amidase and synthetase modules. A fragment containing the C-terminal 431 amino acids (50 kDa) has synthetase activity only, with steady-state kinetic parameters similar to the full-length protein. A fragment containing the N-terminal 225 amino acids (25 kDa) has amidase activity only and is significantly activated relative to the full-length protein for hydrolysis of glutathionylspermidine analogs. This observation suggests that the amidase activity in the full-length protein is negatively autoregulated. The amidase active site catalyzes hydrolysis of amide and ester derivatives of glutathione (e.g. glutathione ethyl ester and glutathione amide) but lacks activity toward acetylspermidine (N² and N⁶) and acetylspermine (N³), indicating that glutathione provides the primary recognition determinants for glutathionylspermidine amide bond cleavage. No metal ion is required for the amidase activity. A tetrahedral phosphate analogue of glutathionylspermidine, designed as a mimic of the proposed tetrahedral intermediate for either reaction, inhibits the synthetase activity (Kᵢ ~ 10 μM) but does not inhibit the amidase activity.

The polyamine, spermidine (N-(3-amino)propyl-1,4-diaminobutane), and the tripeptide, glutathione (γ-Glu-Cys-Gly, abbreviated GSH), are present at high concentrations (0.1–10 mM) in most cells (see, for example, Refs. 1 and 2 for reviews). With its redox active cysteine thiol, GSH is the primary small-molecule antioxidant in many cells, serving to maintain redox poise and reductively scavenge reactive oxygen species. It is catalytic in these roles by virtue of glutathione reductase, which maintains GSH in active, reduced form. As a polycation, spermidine complexes with nucleic acids, proteins, and phospholipids, thereby influencing their structures and biological properties (3).

An intriguing link between GSH and spermidine metabolism is found in the protozoal parasites of genera *Trypanosoma* and *Leishmania*, including those that cause African sleeping sickness, South American Chagas’ disease, and various afflictions known collectively as leishmaniasis. In these parasites, it appears that the bis(glutathionyl)spermidine conjugate, trypanothione (see Scheme 1 for structure), has appropriated the important antioxidant roles normally played by GSH (4–10). Thus, these parasites lack GSH reductase and GSH peroxidase activities, but have analogous enzymes that use trypanothione (5, 8–10). Because trypanothione appears to have important roles in these pathogens and is not present in their hosts, its metabolism is an obvious target for design of new antiparasitic drugs.

The synthesis of trypanothione from glutathione and spermidine is catalyzed by glutathionylspermidine (GSP) synthetase and trypanothione synthetase (11, 12). Each couples hydrolysis of ATP (to ADP and Pᵢ) with formation of an amide bond (Scheme 1). The intermediate in this pathway, glutathionylspermidine, was first identified in *Escherichia coli* more than 3 decades before the discovery of trypanothione itself, and a GSP synthetase activity was partially purified (13, 14). In spite of its early discovery, the physiological role in *E. coli* of the glutathione–spermidine conjugate is not yet known.

As part of an ongoing investigation of the enzymology and physiology associated with these glutathione–spermidine conjugates, we recently characterized GSP synthetase from *E. coli* (15). We purified the enzyme, isolated and sequenced its gene, overproduced it, and characterized the recombinant protein. We were surprised to discover that the 70-kDa protein possesses a second catalytic activity: hydrolysis of glutathionylspermidine back to glutathione and spermidine. As the net of...
these two activities is hydrolysis of ATP (i.e. futile cycling), we proposed that reciprocal regulation of its activities might be an important feature of the bifunctional enzyme. In addition, because the synthetase activity was selectively abrogated by proteolytic cleavage after Arg-538 of the 619-amino acid protein, we proposed that it might possess separate domains for its two activities (N-terminal amidase and C-terminal synthetase) (15).

In the present study, we have confirmed the hypothesis of separate domains by genetic dissection of the bifunctional protein into independently folding and functional amidase and synthetase fragments, and have characterized the fragments with respect to their steady-state kinetic constants. We have evaluated, as an inhibitor of the synthetase activity, a phosphenone analog of glutathionylspermidine designed to mimic the proposed tetrahedral intermediate. Finally, we have begun to address the question of substrate specificity and mechanism of the amidase reaction by 1) evaluating analogs of GSH, spermidine, and glutathionylspermidine as possible substrates and/or inhibitors of the reaction and 2) testing for the presence of a catalytic metal ion.

EXPERIMENTAL PROCEDURES

Preparation of GSP Synthetase/Amidase and Fragments

Materials—Oligonucleotides were purchased from the Harvard Medical School Biological Chemistry and Molecular Pharmacology departmental biopolymer facility or from Integrated DNA Technologies (Coralville, IA). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). The vector pHET22b and E. coli strain BL21(DE3) were purchased from Novagen (Madison, WI). Construction of the vectors pJMB1 and pGSP has been described previously (15).

Construction of pAMID Vector to Overexpress 25-kDa GSP Amidase Fragment—We had previously (15) subjected the plasmid pJMB1, which contains a 5.8-kilobase pair insert spanning gsp in the vector pBluescript (Stratagene), to the transposon mutagenesis procedure of Berg et al. (16). A mutant plasmid from the resulting set, with the transposon inserted after nucleotide 675, was used as template to amplify a 5’ fragment of gsp (containing the amidase domain) using the polymerase chain reaction (PCR). The primers were 5’-AAGGTAAA-CAATATGAGCAAAGGAACGACCAG-3’ (15), which primes for synthesis of the sense strand beginning just 5’ of gsp and the “Res” sequencing primer of Berg et al. (16), which primes in the transposon for synthesis of the antisense strand of gsp. The resulting 751-base pair PCR fragment was digested with NdeI (which cuts in primer 1) and BamHI (which cuts in the transposon just 3’ of gsp) and ligated into vector pET22b to give pAMID, which encodes a mini fragment of GSP synthetase/amidase consisting of amino acids 1–225 fused to the transposon-encoded dipeptide GV. Sequencing of the plasmid by the Dana Farber Cancer Institute Core Facility confirmed that no mutations were introduced during PCR amplification.

Construction of pSYN Vector to Overexpress 50-kDa GSP Synthetase Fragment—The vector pSYN was constructed to overproduce a C-terminal fragment of GSP synthetase/amidase comprising amino acids 189–619. PCR was used to amplify a 1362-base pair, 3’ fragment of the gsp gene. The template for amplification was the vector pGSP (15), which contains gsp inserted in the plasmid PET22-b (Novagen) via NdeI (5) and EcoRI (3’) restriction sites. Primer 1 (5’-ACCATCTGGGCC- CATATGATCCAGACGGAGGAT-3’) corresponds to nucleotides 550–582 of gsp but introduces an NdeI site (CATATG) for cloning. Primer 2 (5’-ATATGATCTTCGATGTTCCCGGAATTC-3’) primes immediately 3’ of gsp for synthesis of the antisense strand. The amplified fragment was digested with NdeI, which cuts both in primer 1 and at an internal site in gsp after nucleotide 932. The resulting 367-base pair fragment (nucleotides 656–923) was ligated with NdeI-digested pGSP (two sites resulting in excision of nucleotides 1–931 of gsp). Transformants of E. coli strain DH5α containing pSYN (with the NdeI insert in the desired orientation) were identified by restriction analysis of plasmid DNA. Sequencing of the plasmid by the Dana Farber Cancer Institute Core Facility confirmed that no mutations were introduced during PCR amplification.

Growth of Overexpressing Strains—E. coli strain BL21(DE3) transformed with pGSP, pAMID, or pSYN was grown aerobically in LB medium containing 150 µg/ml ampicillin at 37 °C to an A600 of 0.6–0.8, when protein expression was induced by addition of isopropyl-1-thio-β-galactopyranoside to a final concentration of 500 µM for full-length protein and amidase fragment or 100 µM for synthetase fragment. Cultures were incubated an additional 2–4 h and were harvested by centrifugation. A typical yield was 2.5 g of wet cell paste/liter of culture.

Protein Purification—Full-length GSP synthetase/amidase was purified from strain BL21DE3/pGSP as described previously (15).

In a typical purification of the amidase fragment, cells from 2 liters of culture were resuspended and lysed as described previously (15). Treatment with streptomycin sulfate was as described (15). Ammonium sulfate fractionation steps were as described (15), but the concentrations were 30% of saturation for the first cut and 70% for the second cut. The pellet from the 70% ammonium sulfate step was redissolved in 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1 mM EDTA (buffer A) and dialyzed against buffer A. The desalted solution was loaded on a DEAE-Sepharose (Pharmacia Biotech Inc.) column (2.5 × 25 cm) equilibrated in buffer A. The column was washed with 100 ml of buffer A, and then
developed with a gradient of NaCl in buffer A (50 ml of 0–100 mM, then 450 ml of 100–400 mM). GSP amidase activity of column fractions was determined by a previously described (15) qualitative assay (thin-layer chromatography with detection by ninhydrin staining). Fractions with greatest specific activity were combined (78 ml eluting at 230–280 mM NaCl). The 1 ml aliquot of this pool was made 1 M in (NH₄)₂SO₄ by addition of the solid. This solution was chromatographed in two 5 ml portions on a Phenyl-Superose HR 10/10 column (Pharmacia) equilibrated in 20 mM potassium phosphate (pH 7.25, 5 mM DTT, 1 mM EDTA (buffer B) containing 1 M (NH₄)₂SO₄. After loading, the column was washed with 5 ml of buffer A containing 1 M (NH₄)₂SO₄ and then developed with a gradient of decreasing (NH₄)₂SO₄ concentration (10 ml of 0.1–0.75 M, then 80 ml of 0.75–0.25 M) in buffer B. Fractions comprising the major protein peak were combined (20 ml from two injections, eluting at 550–500 mM (NH₄)₂SO₄). The pool was frozen in liquid N₂ and stored at −80°C. SDS-PAGE analysis of the combined fractions showed the fragment to be >90% pure (Fig. 1, lane 4).

For purification of the synthetase fragment, lysis of cells (17 g of wet cell paste from 8 liters of culture), streptocycin sulfate and ammonium sulfate fractionation steps, and desalting of redisolved ammonium sulfate pellet were carried out as described for the full-length protein (15). The desalted protein was then loaded on a 2.5 × 25-cm DEAE-Sepharose (Pharmacia) column equilibrated in 50 mM Bis-Tris propane-HCl, pH 7.15, 5 mM DTT, 1 mM EDTA (buffer C). The column was developed with a gradient of NaCl in buffer C (70 ml of 0–100 mM, 610 ml of 100–300 mM), and fractions containing synthetase activity were pooled (81 ml eluting at 120–180 mM NaCl). This solution was made 1.2 M in (NH₄)₂SO₄ by addition of the solid and was chromatographed in 10-ml aliquots on a Phenyl-Superose HR 10/10 column (Pharmacia) equilibrated in 20 mM potassium phosphate, pH 6.8, 5 mM DTT, 1 mM EDTA (buffer D) containing 1 M (NH₄)₂SO₄. The column was eluted with a gradient of decreasing (NH₄)₂SO₄ concentration (1–0 M) in buffer D. A fraction of the synthetase activity eluted at the end of this gradient (0 M (NH₄)₂SO₄), and the remainder eluted with HO (free of buffer). The fractions were combined, frozen in liquid N₂, and stored at −80°C. SDS-PAGE analysis of the combined fractions showed the fragment to be >90% pure (Fig. 1, lane 3).

Synthesis of Substrates and Inhibitor

General Synthetic Methods—These methods were as described previously (17). 1H NMR spectra were recorded at 300 and 360 MHz and are reported in the following manner: chemical shift in ppm downfield from internal tetramethyl silane (multiplicity, integrated intensity, coupling constant in hertz).

Materials—Commercially available amino acid and dipeptide precursors were purchased from Bachem or Fisher Scientific. Boc-alanyl glycyl benzyl ester was prepared by a literature procedure (18); melting point (mp) 86–87°C (literature mp 85–86°C; Ref. 18). Trifluoroacetic acid-mediated cleavage of the Boc group afforded Ala-Gly-OBn (mp) 86–87°C (literature mp 85–86°C; Ref. 18). Trifluoroacetic acid-benzylester was prepared by a literature procedure (18); melting point (mp) 174.5–175°C. 1H NMR (DMSO-d₆) δ 6.8–7.2, 7.36 (d, 2H), 7.40 (s, 15H); 13C NMR (CDCl₃) δ 2.15–2.30 (m, 2H), 3.90–4.10 (m, 2H), 4.32–4.51 (m, 2H), 5.10 (s, 2H), 5.15 (s, 2H), 5.20 (s, 2H), 5.70 (d, 1H, J = 6.82), 6.22 (d, 1H, J = 5), 6.78 (br, 1H), 7.40 (s, 15H); 13C NMR (CDCl₃) δ 172.5, 172.2, 172.0, 169.5, 164.8, 129.8, 129.7, 128.3, 128.3, 128.7, 126.7, 67.4, 67.3, 54.0, 49.9, 41.6, 22.3, 29.5, and 17.9.

γ-Glu-Ala-Gly-OL—A suspension of 6 (0.48 g, 0.81 mmol) and 10% Pd/C (100 mg) in a mixture of EtOH (80 ml) and MeOH (25 ml) was shaken on a Parr hydrogenator at 40 psi for 16 h. The catalyst was removed by filtration. The filtrate was concentrated, and the resultant hygroscopic solid material was purified by ion-exchange chromatography on DEAE-cellulose using a gradient of NaHCO₃ (pH 7.8) as eluant. A white crystalline product (hygroscopic) was obtained in quantitative yield; mp 195–197°C (literature mp: 193–195°C; Ref. 18); 1H NMR (DMSO-d₆) δ 1.35 (d, 3H, J = 7.2), 2.05–2.15 (m, 2H), 2.24–2.55 (m, 2H), 3.62 (t, 1H), 3.71–3.84 (m, 2H), 4.30–4.40 (m, 1H); 13C NMR (DMSO-d₆) δ 175.7, 174.4, 172.9, 171.6, 53.8, 50.7, 41.9, 32.6, 27.1, and 18.5.

γ-O-Benzyl-Z-γ-Glutamylanalylglycylamide (7)—7 was synthesized from Z-Glu-OBn and Ala-Gly-NH₂-HCl by both the MCCA and DCC methods with yields of 22% and 78%, respectively. The resultant syrropy residue was triturated with EtOH. A yellow solid was obtained, which was triturated successively with 5% citric acid, H₂O, 5% NaHCO₃, and acetone·Et₂O (1:1 v/v). A white powder was obtained; a portion of this product was recrystallized from MeOH, mp 194–195°C; 1H NMR (CDCl₃) δ 1.45 (d, 3H, J = 7.1), 1.70–2.05 (dm, 2H), 2.25 (t, 2H), 3.42–3.68 (m, 2H), 4.05–4.25 (m, 2H), 5.0–5.1 (m, 2H), 5.12 (s, 2H), 7.06 (s, 1H), 7.16 (s, 1H), 7.21–7.49 (m, 10H), 7.79 (d, 1H, J = 7, 8), 8.05–8.15 (m, 2H); 13C NMR (CDCl₃) δ 176.10, 175.1, 174.6, 174.0, 154.2, 129.7, 129.3, 129.0, 68.1, 67.9, 55.2, 51.2, 43.4, 32.8, 28.2, and 17.6.

γ-Glu-Ala-Gly-NH₂—2 was prepared by two methods. In method 1, a mixture of 7 (0.1 g, 0.2 mmol) and 10% Pd/C (50 mg) in MeOH (25 ml) was shaken on a Parr hydrogenator (40 psi) for 20 h. The catalyst was removed by filtration and the filtrate was concentrated to afford a syrupy material, which was triturated with acetone and a minimum amount of MeOH. The resultant white solid product was recrystallized from MeOH·Et₂O to afford a white crystalline material (0.05 g, 87%); mp 194–195°C; 1H NMR (CDCl₃) δ 1.41 (d, 3H, J = 6.8), 2.10–2.25 (m, 2H), 2.52–2.61 (m, 2H), 3.62–3.73 (m, 1H), 3.87 (s, 2H), 4.25–4.35 (q, 1H); 13C NMR (CDCl₃) δ 176.2, 175.8, 175.1, 174.0, 55.4, 51.4, 43.4, 32.7, 27.6, 17.5; mass spectroscopy (fast atom bombardment) m/z 275 (MH⁺, 100), 177 (20.3), 155 (23), 119 (18), 85 (38), high resolution mass spectrum (fast atom bombardment) calculated for C₁₁H₁₇O₇N₂ (MH⁺) 275.355, found 275.355. For method 2, a solution of H-Ala-Gly-NH₂ (1.01 g, 5.6 mmol), Boc-Glu(Osu)-OBu (2.24 g, 5.6 mmol), and N-methylmorpholine (1.3 ml) in DMF (80 ml) was stirred for 10 h at room temperature. The solvent was evaporated in vacuo, and the residue was partitioned between 0.1 M HCl and EtOAc. The organic layer was washed with saturated NaHCO₃(aq) and brine, dried over MgSO₄, and evaporated to

\[ \text{ZNH-BOC 3} \]
\[ \text{H-COOMe 4} \]
\[ \text{H-NH₂ 5} \]
\[ \text{H-NH₂ 5} \]

\[ \text{H-NH₂ 5} \]
\[ \text{H-NH₂ 5} \]

\[ \text{H-NH₂ 5} \]
dryness. The residue was chromatographed on silica gel with CHCl3-
MeOH (12:1) to give Boc-γ-Glu(α-OtBu)-Ala-Gly-NH2 (2.04 g) in 85 %
yield. Treatment of the protected tripeptide with 50 % trifluoroacetic acid in 
CH2Cl2 under N2 for 30 min, evaporation of the solvent, and 
chromatography on Sephadex G-10 afforded γ-Glu(Ala)-Gly-NH2 in 85 %
yield. TLC analysis of the product (n-ProOH/ACOH/H2O:O/H2O:O) on silica 
gave one spot which comigrated with the material synthesized and 
characterized by Method 1 above, and the 1H NMR spectra of the 
products from the two methods were identical.

γ-Glu-Cys-Gly-NH2 (GSH Amide)–GSH amide was prepared by am-
omolysis of glutathione ethyl ester. In a typical reaction, 10 ml of 2 M 
NH4OH (Alrich) was cannulated under N2 into a sealed, 
N2-purged vessel containing 0.10 g of glutathione ethylester, 0.052 g of 1
1

reaction, and the solvent was evaporated 
to dryness. The residue was chromatographed on silica with 
CHCl3:MeOH (12:1) to give a 0.2-ml aliquot of 1 M dithiothreitol in methanol was added to the 
reaction, and the solvent was evaporated in vacuo. The solid was redissolved 
in 5 ml of 0.1 % trifluoroacetic acid in H2O. The solution was 
filtered through a column of 1.5 ml of Dowex AG-50 (Na+ form, Bio-Rad) 
to remove remaining NH4+. Fractions containing product (flow-through) 
were then filtered through a C18 space cartridge (Rainin) to remove oxidized 
and reduced dithiothreitol (product elutes in the void volume, while dithiothreitol is retarded). Fractions containing product were 
evaporated to dryness in vacuo, and product was redissolved in 10 mM 
dithiothreitol in H2O for use. The product with Rf = 0.2 was quantita-
tively converted to a species that comigrated with GSH by treatment 
with GSP synthetase/amidase. In this conversion, ammonia was pro-
duced, as detected by the glutamate dehydrogenase spectrophotometric 
assay described below. The quantity of NH3 released following exhaust-
ive hydrolysis was assumed to be equal to the quantity of glutathione 
amide originally present.

Phosphonate Analog (8) of Glutathionylspermidine—The hydrox-
ypermidine-containing phosphopeptide, 8 (see “Results” for structure), 
was prepared in a convergent synthesis based on the retrosynthetic 
pathway outlined previously (17). The detailed syntheses of 8 and 
related phosphopeptides, together with more extensive inhibi-
tion studies, will be published elsewhere.2

Characterization of GSP Synthetase/Amidase and Fragments

Materials—35S-Glutathionylspermidine was prepared enzymatically from 
[35S]glutathione (New England Nuclear) as described previously 
(15). N2-glutathionylspermidine disulfide was purchased from Bachem 
Bioscience Inc. (Switzerland). ATP, glutathione, spermidine, NADH, 
NAD, NADP, NADPH, phospho(enol)pyruvate, 2-oxoglutarate, dithiothreitol, 
glutamate dehydrogenase from Proteus species (product no. G-4387), and 
 alcohol dehydrogenase from Bakers’ Yeast (product no. A-3263) were 
purchased from Sigma. Aldehyde dehydrogenase from yeast (prod-
uct no. 171832), and pyruvate kinase and lactate dehydrogenase (the 
 latter two in an equi-unit suspension in 3.2 mM ammonium sulfate) were 
purchased from Boehringer Mannheim.

Protein Assays—Protein concentrations were determined by the 
method of Bradford as supplied by Bio-Rad. Bovine serum albumin 
(Pierce) was used as standard.

Enzyme Activity Assays—GSP synthetase activity was measured by a 
previously described coupled assay (15). Amidase activity toward 
 glutathionylspermidine was assayed radiometrically by monitoring 
conversion of [35S]GSP to 35S-Glutathionylspermidine spectrophotometrically.

RESULTS

Dissection of Bifunctional Protein into Separate Domains

Preparation of Amidase Fragment and Comparison to Full-
length Protein—Our previous finding that trypsin cleavage of 
GSP synthetase/amidase after Arg-538 gives a 61.6-kDa N-
terminal fragment with only amidase activity suggested that the 
protein might possess independent amidase and synthetase 
domains (15), and led us to test whether a smaller N-terminal 
fragment might independently fold into a functional amidase 
domain. We used a previously constructed set of transposon 
insertional mutants of gsp in the high copy number plasmid 
pBluescript (15). Several plasmids with the transposon 
fragment fused to the transposon-encoded dipeptide GV. A plasmid with the transposon inserted after nucleotide 675 encoded the 
smallest N-terminal fragment, amino acids 1–225 of GSP syn-
thetase/amidase fused to the transposon-encoded dipeptide GV. A plasmid with the transposon inserted after nucleotide 207 did not give rise to increased activity, suggesting that the minimal N-terminal fragment needed for amidase activity is 
between 69 and 225 amino acids. The 225-amino acid N-termi-
nal fragment, was overproduced, and was purified to > 95 % 

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J. K. Coward, manuscript in preparation.
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**gsp-E coli**
225-KWLD--------KDFLOAIVQGQQVQINQDPY--------HYTTI-FESASE
**yfc-E coli**
MLRNH-VPVRRLDLQIAADNGDFPDHTI-------DNIEYMDERSARYFPILQTHE
**ygic-E coli**
MRKVS-ITRTPDREKAEHGFPMH--------YGEFOXEDAYKLIAQVE
**ygic-HinfIenzae**
MKRVTGEPTRPDVMQQQINQDFYNNPLPSGGYSHYNDVAYFETLAED

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**gsp-E coli**
QELIAKACNEHLMYHATDKVLKGDMLALPDKILWPRRLLSQKRRRH
**yfc-E coli**
EQIHEFPLEGNCVLEQVRQVEDEILQTLAFLPYDVIASWRARGP
**ygic-E coli**
QKSTRMQEKVCNVEKASDELMTQEREPITVPQHAYTSPSGLTHQP

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**gsp-E coli**
HMITQGRMDPCDREM--LKVBYYNADSACHTEAGLIERWEAGQ---
**yfc-E coli**
SLY-GRMDFAWCNGNPVFKLYNSNATFPTSSLYESAFQQLWLEKDRSGI
**ygic-E coli**
SLY-SRLYQGOGPTGEKPLRNNATFPTSLYEAFFQQLWLEQNLGN
**ygic-HinfIenzae**
erylQDFDQVQGNL-NKMFYENADTFPTSSLNEEAVQQLWLEQNLGN

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**gsp-E coli**
------NGNPY-AGELINELAGWIAHSMARPF--VHIOMOKDIINEHYAQFME
**yfc-E coli**
PRDADQNYAERGLISRPSL---YSREFPYFCCOCR-EDSTEVLYQ
**ygic-E coli**
PQGSDQFNSLSQKELIFDFREVLRQFGQLLHCTCDDTV-EDGRTIQLYQ
**ygic-HinfIenzae**
---KHRODQNYWIEHLIKHQFPLQKQGSKTPFPLHSMQADGRSDKWNYLDA

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**gsp-E coli**
QALHODQQGFTRRILGRLDGEQDAAGQ-LIDGRSLNVWVNKVTWANSTFQ--
**yfc-E coli**
DCAOQAQQQERSFY--IDILQJLGQV-ITLTDNVLQRFAQFLYPLEMV--
**ygic-E coli**
DCATERIAITFSLY-DIDQGKEGQFTDLQVQLSINLFRLFWFPM-
**ygic-HinfIenzae**
DVAYRAGNHIIQLA-VEDIGYNSETKFVDLNPQITFPMLFPLNL---

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**gsp-E coli**
QIZEVSDREFAVPQRTGPHQNNBRLLDVEFLVEPLFLAVTVIQPKNA
**yfc-E coli**
-MSRDNQPLIRRERQW-------VPKFLWLSNKG
**ygic-E coli**
-LRBMPSTKLEDQAVRF-
**ygic-HinfIenzae**
-SHAEFARNHTTAEFPR-------IKPAWMLKNSKA

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**gsp-E coli**
ILPFLWGLLPFHHYLLDDTFTYUNEDE-LLVKTGYAVKPIAGRCGNSIDLV
**yfc-E coli**
ILPAFLWGLLPFHHYLLDDTFTYUNEDE-LLVKTGYAVKPIAGRCGNSIDLV
**ygic-E coli**
LKLPMWGMATPHEHNLASSNP-DGKDPQAARQEGYVKPKIPYSRGPSVTRIF
**ygic-HinfIenzae**
LLAKIMVYPHNCHPLPAYPTPLFPRKDL--MWKKPLGLGERANFY

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**gsp-E coli**
SRHEEVLTKSTSG-------FAEQQNYQQLCMKVDKIYQVFTPTVGN
**yfc-E coli**
DGKNVVRDHAADD-------YAEPHYQAFQPLLPRGSDTLLGISVIVDE
**ygic-E coli**
E-NKXRTEABBOP-------YOEQNYQQLCMKVDKIYQVFTPTVGN
**ygic-HinfIenzae**
EKNYQERAAKQSEHSTFYCVGGYQVYQKIPSHILPSTPISWQGSAVOD

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**gsp-E coli**
YGTTCLRQDGSLVIKBSDE1PPLLTVK
**yfc-E coli**
ACQMGIRENLTALITDTSRFPVPHYAG
**ygic-E coli**
PAIG2RERDALITQDMRFPYHPHTVE
**ygic-HinfIenzae**
ACQMGIRENLTALITDTSRFPVPHYAG

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**Preparation of Synthetase Fragment and Comparison to Full-length Protein**—The recent discovery that the tertiary folds of d-alanine:d-alanine ligase and glutathione synthetase from *E. coli* are closely related despite minimal similarity in their primary structures (20) suggested that this fold might be characteristic of a family of bacterial ATP-cleaving (ADP-forming), amide bond-forming enzymes. d-alanyl:d-Ala ligase and GSH synthetase are composed of ~310 amino acids each, and we considered this as an estimate for the extent of the synthetase domain of GSP synthetase/amidase. A second consideration in designing a synthetase-only construct was the sequence similarity among the C-terminal ~370 amino acids of GSP synthetase/amidase and the hypothetical protein products of three other bacterial open reading frames, ygiC and yjcF from *E. coli* and ygiC from *Haemophilus influenzae* (Fig. 2). These hypothetical proteins may be related ATP-cleaving, amide bond-forming enzymes, and the region of homology may therefore delimit the synthetase domain. On the basis of these considerations, two potential synthetase constructs were prepared. A fragment containing the C-terminal 318 amino acids (beginning with Met-312), which does not span the entire homology region, was found to be insoluble upon overexpression and to lack detectable synthetase activity. In contrast, a fragment of 431 amino acids (beginning at Met-189) is soluble and active for ATP-dependent glutathionylspermidine synthesis. This fragment was purified to ~90% homogeneity (Fig. 1, lane 3) by the
same two-column procedure employed for the recombinant, full-length protein. The protein eluted from the hydrophobic interaction matrix, Phenyl-Superose, in two fractions, the first eluting with column buffer containing no (NH₄)₂SO₄ and the second eluting with H₂O (requiring removal of even the buffer salt). This strong interaction suggests that the synthetase fragment has surface-exposed hydrophobic residues not displayed in the full-length protein.

Steady-state kinetic parameters for the synthetase activities of full-length GSP synthetase/amidase and the C-terminal synthetase fragment were determined (Table I). The fragment has only slightly greater affinity (nanomolar composition of the resulting tetrahedral adduct (Scheme 3, 9)) due to their extremely slow dissociation kinetics, to produce species akin to 13 that (presumably) closely mimic the normal tetrahedral intermediates (Scheme 3, A) by elimination of phosphate (21–23). There is extensive precedent for potent inhibition of several of these, including D-alanine:D-alanine ligase (24–26), glutamine synthetase (27–29), and glutathione synthetase (30), by phosphonate (12) and phosphinate (11) analogs of the corresponding substrates. Some of these analogs can undergo enzyme-mediated phosphoryl transfer from ATP to the phosphon(phin)ate O (a step that is typically slow, leading to slow-binding inhibition kinetics), to produce species akin to 12-14 that are capable of binding to the active site.

**Inhibitor Design and Testing**

**Phosphonate Analog of GSP as Inhibitor of Synthetase Activity**—Those ATP-cleaving (ADP-forming), amide bond-forming enzymes that have been well characterized are believed to employ a mechanism (Scheme 3, A) involving phosphoryl transfer from ATP to the carbonyl oxygen to form an acyl phosphate (9), attack on this intermediate by the amine, and decomposition of the resulting tetrahedral adduct (10) by elimination of phosphate (21–23). There is extensive precedent for potent inhibition of several of these, including D-alanine:D-alanine ligase (24–26), glutamine synthetase (27–29), and glutathione synthetase (30), by phosphonate (11) and phosphinate (12) analogs of the corresponding substrates. Some of these analogs can undergo enzyme-mediated phosphoryl transfer from ATP to the phosphon(phin)ate O (a step that is typically slow, leading to slow-binding inhibition kinetics), to produce species akin to 13 that (presumably) closely mimic the normal tetrahedral intermediates (Scheme 3, A) and inhibit with high affinity (nanomolar Kᵢ) due to their extremely slow dissociation (24, 27, 28, 31). On the basis of these precedents, we designed the hydroxyspermidine-containing phosphopeptide, 8, as a potential inhibitor of GSP synthetase, with the afore-
mentioned Ala → Cys substitution incorporated for synthetic convenience (see Scheme 3).

Phosphonate 8 is a potent inhibitor of GSP synthetase activity with respect to the substrate GSH (Fig. 3). Analysis of Fig. 3 according to non-competitive or mixed-type inhibition (Scheme 4) gave inhibition constants of 6 μM for inhibitor binding to free enzyme (Kᵢ) and 14 μM for binding to the enzyme-GSH complex (Kᵢᵣ). No time-dependent (slow-binding) inhibition was observed (Fig. 4), nor was 8 found to stimulate uncoupled ATP hydrolysis by the synthetase. These results suggest that phosphorylation of 8 is not occurring, and, therefore, that the inhibitor may be acting as a simple bisubstrate analog rather than as an intermediate or transition state mimic.

Phosphonate Analog of GSP as Inhibitor of Amidase Activity—In addition to their use as slow-binding inhibitors of amide bond-forming enzymes, phosphapeptides have also been used as amidase inhibitors (32, 33). Thus, for amidases that facilitate direct attack of H₂O (e.g. zinc or aspartic proteases), a tetrahedral species akin to 14 is an intermediate (Scheme 5), and phosphapeptides analogous to 8 have been observed to bind tightly as mimics (33, 34). Phosphonate 8 was therefore tested for inhibition of amidase-catalyzed γ-Glu-Cys-Gly-NH₂ hydrolysis. In the presence of 100 μM substrate, 1.5 mM 8 had no discernible effect on the amidase activity of either the full-length protein or the amidase fragment. Thus, 8 is at best a poor (high mM Kᵢ) inhibitor of GSP amidase.

Analysis for Metal Ions in GSP Synthetase/Amidase and the Amidase Fragment—In order to test for the presence of a catalytic metal ion in the amidase active site, the metal ion contents of GSP synthetase/amidase and amidase fragment were determined. Neither contains significant quantities of zinc, iron, manganese, cobalt, or nickel, though stoichiometric Ca²⁺ was found in both. As Ca²⁺ is not known to act as a cofactor for amide hydrolysis, these observations suggest that a metal ion is not required for GSP amidase catalysis.

Discussion

The above results demonstrate that the two activities of glutathionylspermidine synthetase/amidase reside in independently folding and functional domains, suggesting that the protein evolved by fusion of amidase and synthetase fragments. By their fusion, any possibility for differential regulation of the component activities of this potential futile cycle at the level of transcription or translation is seemingly eliminated (although production of alternative mRNAs is formally possible). Assuming that the physiological function of the protein does not derive from futile ATP consumption, the two activities are probably differentially regulated post-translationally, either by an allosteric mechanism or by covalent modification. The observation by Tabor and Tabor (35) that glutathionylspermidine accumulates in saturated, anaerobic cultures of E. coli B grown in glucose-rich medium and is rapidly (in less than 5 min) and completely hydrolyzed following dilution into fresh medium lends credence to the suggestion of physiological regulation. The increased amidase activity of the N-terminal fragment relative to the full-length protein suggests one possible mechanism for differential regulation; if this activity is inhibited in the context of the full-length protein, relief of inhibition either
Dissection of Glutathionylspermidine Synthetase/Amidase

by proteolysis or by a conformational change upon ligand binding could selectively enhance amidase activity.

The selective advantage (if any) conferred to E. coli by this unique bifunctional enzyme remains an enigma. We previously proposed that the enzyme might serve to modulate levels of free spermidine or glutathione (15). If so, the presence of synthetase and amidase activities would allow for a bidirectional response (increase or decrease in concentration) without requirement for new protein synthesis or for synthesis or degradation of the relevant metabolite. In addition, in regulating levels of free spermidine, the specificity of the amidase for glutathione-containing amides would render this system orthogonal with the spermidine acetyltransferase system (36).

The amino acid sequence of the amidase domain provides no clue as to catalytic mechanism, as it lacks similarity with any known protein sequence. The amidase may therefore function by acid/base-assisted direct attack of $\text{H}_2\text{O}$ (as do the aspartic and zinc proteases) or by covalent catalysis (as do the serine and cysteine proteases). Two observations suggest that the latter is more likely. First, the domain lacks a catalytic metal ion. Second, the phosphonate $8$, which should closely mimic the tetrahedral intermediate for direct attack by $\text{H}_2\text{O}$, does not inhibit the amidase. We are currently using chromogenic ester derivatives of glutathione and rapid kinetic methods to search for a glutathionyl enzyme intermediate.

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