Evidence for a Glutathionyl-Enzyme Intermediate in the Amidase Activity of the Bifunctional Glutathionylspermidine Synthetase/Amidase from *Escherichia coli*†

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ABSTRACT: Glutathionylspermidine (Gsp) is a metabolite common to *Escherichia coli* and protozoal parasites of the *Trypanosoma* family. Though its role in *E. coli* is unknown, Gsp is known to be an intermediate in the biosynthesis of N^1,N^8-bis(glutathionyl)spermidine (trypanothione), a metabolite unique to trypanosomatids that may allow the parasites to overcome oxidative stresses induced by host defense mechanisms. The bifunctional Gsp-synthetase/amidase from *E. coli* catalyzes both amide bond formation and breakdown between the N^1-amine of spermidine [*N*-(3-aminopropyl)-1,4-diaminobutane] and the glycine carboxylate of glutathione (γ-Glu-Cys-Gly), with net hydrolysis of ATP [Bollinger et al. (1995) *J. Biol. Chem. 270* (23), 14031−14041]. Synthetase and amidase activities reside in separate domains of the protein, and liberation of the amidase domain from the synthetase domain activates the amidase activity as much as 70-fold in k_{cat}/K_m for a chromogenic substrate γ-Glu-Ala-Gly-pNA [Kwon et al., (1997) *J. Biol. Chem. 272* (4), 2429−2436]. When substrates for the Gsp-synthetase activity are present (GSH, ATP-Mg^{2+}), Gsp-amidase is highly activated (15-fold). We provide kinetic and mutagenesis evidence suggesting that the amidase operates by a nucleophilic attack mechanism involving cysteine as the catalytic nucleophile. Stopped-flow studies on the 25 kDa Gsp-amidase fragment and the 70 kDa full-length Gsp-synthetase/amidase with γ-Glu-Ala-Gly-ONp demonstrate burst kinetics characteristic of a covalent acyl-enzyme intermediate. Studies using various group-specific protease inhibitors, such as iodoacetamide, suggest an active-site cysteine or histidine as being relevant to amidase activity, and site-directed mutagenesis indicates that Cys-59 is essential for amidase activity.

The bifunctional glutathionylspermidine (Gsp)[1] synthetase/amidase from *Escherichia coli*, which catalyzes both the synthesis and hydrolysis of Gsp (Scheme 1), has been of recent interest for its implications in human disease and bacterial cell maintenance and metabolism (Bollinger et al., 1995). In protozoal parasites of genera *Trypanosoma* and *Leishmania*, Gsp production is the penultimate step in the synthesis of trypanothione [bis(glutathionyl)spermidine; TSH], a unique metabolite and the critical redox-active, thiol-containing biomolecule in these organisms (Fairlamb et al., 1985, 1986). These parasites lack typical glutathione-(GSH-) dependent redox mechanisms and, instead, rely on an analogous system based on TSH (utilizing a TSH-reductase/peroxidase couple rather than the GSH-reductase/peroxidase commonly found in cells) (Fairlamb & Cerami, 1985; Fairlamb & Henderson, 1987; Henderson et al., 1987; Shames et al., 1986). Believed to employ TSH in oxidant defense mechanisms (Fairlamb et al., 1985), these parasites are known to cause significant human diseases affecting millions of people in South America (Chagas' disease) and Africa (African sleeping sickness) and widespread illnesses known collectively as leishmaniasis. Thus, the Gsp-synthetase in trypanosomatids is an attractive target for drug design.

Gsp synthetase/amidase also represents the unique juxtaposition of two important classes of molecules. Polyamines (spermidine, putrecine, spermine, etc.) are polycationic molecules that have been attributed a variety of roles, including complex formation with nucleic acids, proteins, and phospholipids (Tabor & Tabor, 1984). Little is known about their precise role; however, it is known that their intracellular concentrations are tightly regulated by several feedback, recycling, and export/import mechanisms (Tabor & Tabor, 1984). Interestingly, though polyamines are essential for cell growth, accumulation of spermidine in stationary phase has been observed to result in a loss of cell viability (Fukuchi et al., 1995). GSH is also present in millimolar concentrations in most cells and serves as the primary antioxidant, maintaining redox equilibrium and reductively scavenging reactive oxygen species (Meister, 1988). By virtue of the enzyme glutathione reductase, GSH's antioxidant role is effectively catalytic.

With the ultimate goal of understanding how the enzymology of Gsp-synthetase/amidase relates to its physiological

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[1] Abbreviations: Gsp, glutathionylspermidine; TSH, trypanothione; GSH, glutathione; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EtOAc, ethyl acetate; DMF, *N*,*N*-dimethylformamide; IPTG, isopropyl β-D-thiogalactoside; DTT, DL-dithiothreitol; IAM, iodoacetamide; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Na-PIPES, sodium piperazine-*N*,*N*′-bis(2-ethanesulfonate); Tris, tris(hydroxymethyl)aminomethane.

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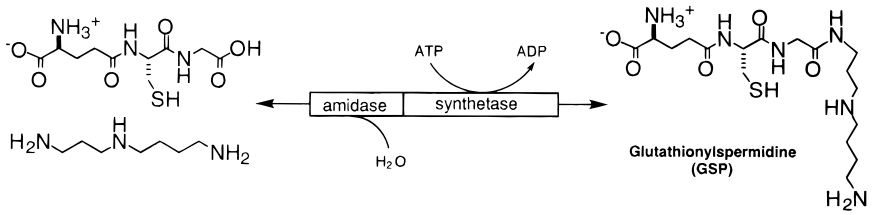
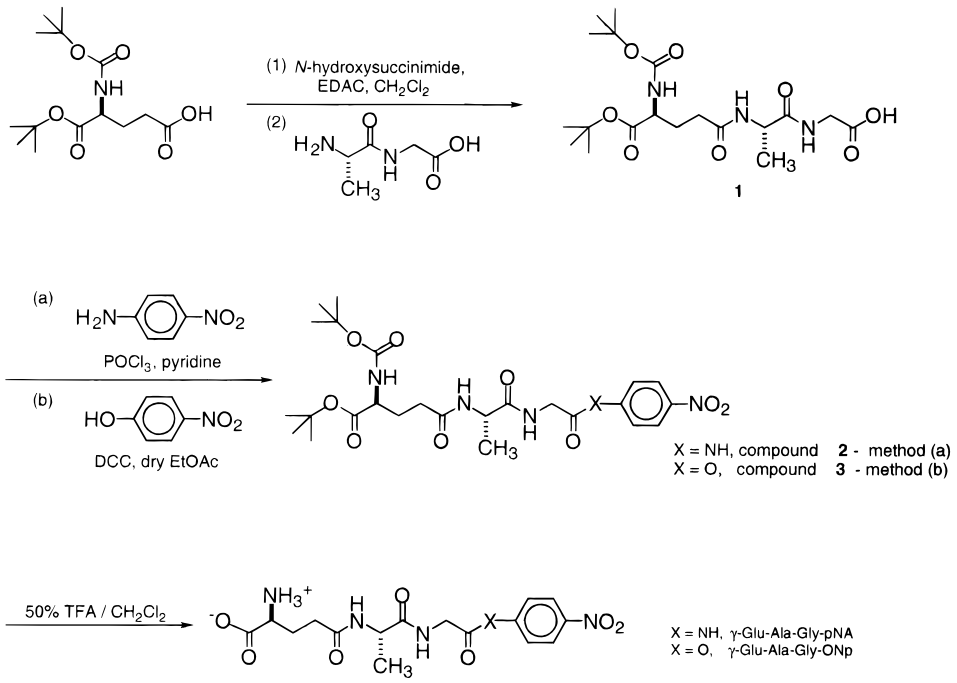
Scheme 1: Bifunctional Glutathionylspermidine Synthetase/Amidase in *E. coli*



Scheme 2: Synthesis of γ-Glu-Ala-Gly-pNA and γ-Glu-Ala-Gly-ONp



role in *E. coli*, we have focused on mechanistic studies of the Gsp-amidase reaction. In the work described here, we present evidence from stopped-flow studies that demonstrate burst kinetics and thus strongly implicate formation of a glutathionyl acyl-enzyme intermediate in the reaction. Iodoacetamide inactivation studies suggest that amidase catalysis requires an enzyme thiol group which is protected by substrate, and site-directed mutagenesis identifies cysteine-59 as an essential residue. We also report initial observations of Gsp-synthetase active-site communication with the Gsp-amidase active site in this bifunctional enzyme.

MATERIALS AND METHODS

Synthesis of γ-Glu-Ala-Gly-pNA and γ-Glu-Ala-Gly-ONp Substrate Analogues (*Scheme 2*). All NMR spectra were recorded by NuMega Resonance Labs, Inc. (San Diego, CA) on a Bruker spectrometer at room temperature (298 K). ^{1}H NMR were recorded at 500 MHz and are reported in the following manner: chemical shift in parts per million upfield (integrated intensity, multiplicity, coupling constant in hertz).

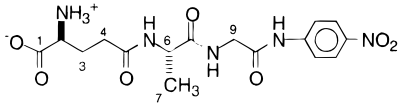
A solution of Boc-Glu(OH)-OtBu (2.00 g, 6.59 mmol), *N*-hydroxysuccinimide (0.800 g, 6.95 mmol), and EDAC (1.33 g, 6.94 mmol) in CH_2Cl_2 was stirred overnight at room temperature. After TLC analysis ($CHCl_3$/MeOH = 5/1) indicated that the starting material disappeared completely and a new nonpolar spot formed, the solvent was evaporated *in vacuo*, and the residue was partitioned between 0.1 N HCl and EtOAc. The organic layer was washed with saturated $NaHCO_3$ (aqueous) and brine, dried over $MgSO_4$, and evaporated to dryness. The resulting product (as the corresponding *N*-hydroxysuccinimide ester) was dissolved in DMF/H_2O (3/1) containing Ala-Gly-CO_2H (9.63 g, 6.59 mmol) and $NaHCO_3$ (1.11 g, 13.2 mmol). After 20 h at room temperature, the reaction solution was evaporated *in vacuo*, neutralized using 1 N HCl, and extracted three times with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated to give compound **1** (2.36 g, 83.1% yield), which was used further without purification.

In order to optimize the synthetic yield of γ-Glu-Ala-Gly-pNA and γ-Glu-Ala-Gly-ONp, two different coupling methods were pursued for preparation of the active amide and ester.

(A) Coupling Method for γ-Glu-Ala-Gly-pNA (Rijkers et al., 1995). Protected γ-Glu-Ala-Gly-CO_2H (1.30 g, 3.02 mmol) and *p*-nitroaniline (0.420 g, 3.02 mmol) were dissolved in dry pyridine (30 mL) and cooled to −15 °C under argon. Phosphorus oxychloride (0.31 mL, 3.32 mmol) was added dropwise with vigorous stirring. The reaction was complete in 30 min, as monitored by TLC ($CHCl_3$/MeOH = 5/1 or EtOAc), and subsequently quenched with crushed ice/H_2O (50 mL). The resulting mixture was extracted three times with EtOAc, and the combined organic layer was washed with saturated $NaHCO_3$ (aqueous) and brine three times each, dried over $MgSO_4$, and evaporated *in vacuo* to give compound **2**.

Treatment of compound **2** with 50% trifluoacetic acid in CH_2Cl_2 under argon for 20 min, evaporation of the solvent, and chromatography on Sephadex LH-20 (EtOH as the

eluent) and Bio-Gel P-2 (H_2O as the eluent) afforded yellowish γ-Glu-Ala-Gly-pNA in 74% yield for the two steps. Further purification by reversed-phase HPLC (preparative C_{18} column of 100 × 20 mm from YMC, Inc., Wilmington, NC) provided the final product with > 99% purity as a white powder. The compound was collected at the retention time of 9.6 min, corresponding to a solvent composition of H_2O (80%) and CH_3CN (20%).



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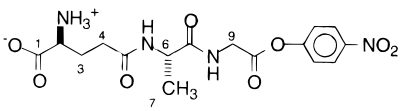
^{1}H NMR (D_2O, HOD = 4.78 Hz) δ 8.157 (2 H, dd, J = 1.85, 7.40 Hz, aromatic), 7.619 (2 H, dd, J = 1.85, 7.40 Hz, aromatic), 4.341 (1 H, quartet, J = 7.22 Hz, H6), 4.081 (2 H, s, H9), 3.765 (1 H, t, J = 6.22 Hz, H2), 2.493 (2 H, dd, J = 6.92, 14.9 Hz, H4), 2.141 (2 H, m, H3), and 1.422 (3 H, d, J = 7.22 Hz, H7). ^{13}C NMR δ 175.28 (carbonyl), 174.02 (carbonyl), 173.13 (carbonyl), 169.01 (carbonyl), 142.71 (aromatic), 142.49 (aromatic), 124.23 (aromatic), 119.48 (aromatic), 53.297, 49.230, 42.329, 30.274, 25.251, and 15.673. Positive ion-spray mass calcd for $C_{16}H_{22}N_5O_7$ ($M + H^+$), 396.1; found, 396.1.

(B) Coupling Method for γ-Glu-Ala-Gly-ONp (Bodanszky & Bodanszky, 1994). A volume of 3.5 mL of dicyclohexylcarbodiimide (3.5 mmol, 1 M in CH_2Cl_2 from Aldrich Co., Milwaukee, WI) was added dropwise into the solution containing compound **1** (1.50 g, 3.48 mmol) and *p*-nitrophenol (0.484 g, 3.48 mmol) in EtOAc (50 mL, anhydrous) under argon. The reaction gradually became cloudy, and formation of *N*,*N*′-dicyclohexylurea was indicated by a massive white precipitate. After TLC analysis ($CHCl_3$/MeOH = 5/1) indicated that the starting material had disappeared completely and a new nonpolar spot had formed (EtOAc/hexane = 3/1), the precipitate was removed by filtration and washed thoroughly with EtOAc. The filtrate and washings were combined and evaporated *in vacuo* to give compound **3**.

Treatment of compound **3** with 50% trifluoroacetic acid in CH_2Cl_2 under argon for 20 min, evaporation of the solvent, and chromatography on Sephadex LH-20 (EtOH as the eluent) and Bio-Gel P-2 (H_2O as the eluent) afforded yellowish γ-Glu-Ala-Gly-ONp in 67.5% yield for the two steps. Further purification by reversed-phase HPLC (preparative C_{18} column, 100 × 20 mm from YMC, Inc.) was required to give a white powder with > 95% purity. The compound was collected at the retention time of 10.7 min, corresponding to a solvent composition of H_2O (78%) and CH_3CN (22%).



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^{1}H NMR (D_2O, HOD = 4.78 Hz) δ 8.320 (1 H, d, J = 8.97 Hz, aromatic), 8.153 (1 H, d, J = 9.04 Hz, aromatic), 7.382 (1 H, d, J = 8.97 Hz, aromatic), 6.955 (1 H, d, J = 9.04 Hz, aromatic); 4.234 (1 H, q, J = 7.36 Hz, H6), 3.936 (2 H, s, H9), 3.788 (1 H, dd, J = 6.26, 12.7 Hz, H2), 2.492 (2 H, m, H4), 2.132 (2 H, m, H3), and 1.390 (3 H, d, J = 7.36 Hz, H7). ^{13}C NMR δ 175.45 (carbonyl), 173.78 (carbonyl), 172.91 (carbonyl), 168.82 (carbonyl), 144.84 (aromatic), 139.78 (aromatic), 125.71 (aromatic), 124.83 (aromatic), 121.82 (aromatic), 114.88 (aromatic), 53.099, 48.966, 40.665, 30.303, 25.206, and 15.730. Positive ion-spray mass calculated for $C_{16}H_{21}N_4O_8$ ($M + H^+$), 397.1; found, 397.1.

Protein Expression and Purification. pET22b-based expression plasmids containing wild-type or mutant genes (a) pGSP (Bollinger et al., 1995), 619 aa full-length Gsp-synthetase/amidase, (b) pAMID (Kwon et al., 1997), wild-type Gsp-amidase fragment (residues 1−225 of full-length protein), (c) pAmC59A or pAmC173A, Gsp-amidase fragment mutants (C59A and C173A), or (d) pFLC59A, full-length Gsp-synthetase/amidase C59A mutant (FLC59A) behind the T7 promoter were used to transform the *E. coli* expression strain BL21(DE3) (Novagen; Madison, WI), according to previously reported methods (Bollinger et al., 1995; Kwon et al., 1997). Luria broth (LB) liquid cultures were grown aerobically with shaking, and protein expression was induced by the addition of IPTG (final 500 μM), as described (Bollinger et al., 1995; Kwon et al., 1997). Protein purification was as previously reported (Bollinger et al., 1995; Kwon et al., 1997)—mutant proteins were purified identically to their wild-type counterparts. In a typical preparation, 30−40 mg of purified protein were obtained per liter of cell culture. Preparations were judged to be 90−95% pure by SDS−PAGE, and concentrations were determined by amino acid analysis (HHMI Biopolymer Facility, Harvard Medical School, Boston, MA), UV absorbance using 6 M guanidine hydrochloride in 0.02 M sodium phosphate buffer (Gill & von Hippel, 1989), and Bradford assay (Bradford, 1976) with bovine serum albumin (Pierce, Rockford, IL) as standard.

Preparation of Enzymes for Stopped-Flow Studies. Gsp-synthetase/amidase and the Gsp-amidase fragment were prepared for stopped-flow studies by dialyzing protein samples into the stopped-flow buffer (60 mM MES, pH 5.7); concentrations for Gsp-synthetase/amidase and the Gsp-amidase fragment were 30 and 16 mg/mL, respectively. Though 5 mM DTT was used in the purification buffers, it was not included in the stopped-flow buffer because it induced nonenzymatic substrate (γ-Glu-Ala-Gly-ONp and γ-Glu-Ala-Gly-pNA) decomposition.

Rapid Kinetic Analysis by Stopped-Flow Spectroscopy. Stopped-flow studies were conducted using a KinTek Stopped-Flow Spectrophotometer SF-2001 (KinTek Instruments Co.; University Park, PA). The light path of the flow cell is 5 mm, and all experiments were conducted at 4 °C. Three drive syringes were available for buffer, substrate, or enzyme. The final concentration of the γ-Glu-Ala-Gly-ONp substrate, prepared in H_2O to prevent buffer-catalyzed color production, was 4 mM for the Gsp-amidase fragment and 8 mM for the full-length Gsp-synthetase/amidase. Protein was prepared in the stopped-flow buffer (60 mM MES, pH 5.7) and was varied from 2.7 to 10.8 mg/mL (Gsp-amidase fragment) and from 5.0 to 20 mg/mL (full-length Gsp-synthetase/amidase).

The released chromophore, *p*-nitrophenol, has a λ_{max} of 340 nm at pH 5.7; however, in order to avoid high background absorbance, *p*-nitrophenol production was measured at 360 nm (ϵ = 3081 AU·cm^{-1}·M^{-1}). A background

Table 1: Primers Used for Site-Directed Mutagenesis[a]

mutant	product A	product B
Gsp-amidase fragment C59A	(S) GAATATATGGGCCACAAGTGGCAA-GCCGTTGAATTTGCTCGC	(S) CATATGAGCAAAGGAACGACCAGCCAGGATGC
	(AS) CACGTTCTGTTCCGCAATACG	(AS) CGGCTTGCCACTTGTGGCCCATATATTCG
Gsp-amidase fragment C173A	(S) CGCGAGCTGGAGATGGTGGTCGAAAA-CGGCGCCTATACCCTG	(S) AGGCGCCGTTTTCGACCACCATCTCCAGCTCG
	(AS) CGAATTCGGATCCTTAACGTACG-TTTTCGTTCCATTTGCCCT	(AS) GAATATATGGGCCACAAGTGGCAAGCCGTT-GAATTTGCTCGC
full-length C59A	(S) CACCATACCCACGCCGAAACAAGC	(S) GAATATATGGGCCACAAGTGGCAAGCCGTTG-AATTTGCTCGC
	(AS) CGGCTTGCCACTTGTGGCCCATATATTCG	(AS) TGCGTGAAGATACATCAGGTGCAGC

[a] Mutations were introduced by the method of gene splicing by overlap extension, as described in Materials and Methods (S, sense; AS, antisense). All primers are written from 5′ to 3′.

hydrolysis rate was measured with buffer and substrate in the absence of enzyme. For a typical experiment, a light source slit width of 1.5 mm and a total shot volume of 130 μL were used. Raw data were transferred into KaleidaGraph (Synergy Software, Reading, PA) for graphical analysis.

Steady-State Analysis with Gsp-Synthetase/Amidase and Gsp-Amidase Fragment. Amidase activity was assayed using γ-Glu-Ala-Gly-pNA or γ-Glu-Ala-Gly-ONp as substrate in either 100 mM Na-PIPES (pH 6.8) at 37 °C or 60 mM MES (pH 5.7) at 4 °C. Due to high background absorbance from nonenzymatic decomposition, γ-Glu-Ala-Gly-ONp was not investigated in 100 mM Na-PIPES (pH 6.8). In a final volume of 400 μL, the assay contained 100 mM Na-PIPES at pH 6.8 (or 60 mM MES at pH 5.7), 39 ng of Gsp-amidase fragment or 14 μg of full-length Gsp-synthetase/amidase, and varying concentrations of substrate. The reaction was initiated by addition of the enzyme, and increasing absorbance was monitored at 405 nm (for *p*-nitroaniline) or 360 nm (for *p*-nitrophenol).

Inhibition of Amidase Activity by Iodoacetamide. A final concentration of 700 nM of Gsp-amidase fragment or the full-length Gsp-synthetase/amidase was incubated with various concentrations of IAM at 0 °C. Periodically, an aliquot was withdrawn and frozen immediately in liquid nitrogen for subsequent inhibition assays. The inhibition effect of IAM on the Gsp-amidase activity was observed when the frozen samples were added (50-fold dilution) to an assay mixture containing 100 mM Na-PIPES (pH 6.8) and 15 mM γ-Glu-Ala-Gly-pNA at 37 °C. The increasing absorbance at 405 nm was monitored, and the resulting initial rate of hydrolysis was recorded as V_t (where t is time of incubation with IAM) or V_0 (rate of control—incubation in the absence of IAM).

Substrate Protection against Iodoacetamide Inactivation. To eliminate the background due to the assay substrate, γ-Glu-Ala-Gly-pNA, saturating concentrations of γ-Glu-Ala-Gly-NH_2 (Kwon et al., 1997) were used to protect against IAM inactivation. The procedure was similar to the inhibition experiments except that γ-Glu-Ala-Gly-NH_2 was incubated with the enzyme and IAM at 0 °C before activity measurements. The concentration of γ-Glu-Ala-Gly-NH_2 was 28 mM ($4K_m$) for the Gsp-amidase fragment and 240 mM ($10K_m$) for the full-length Gsp-synthetase/amidase.

Site-Directed Mutagenesis of Amidase Domain Residues. Cysteine to alanine point mutations were constructed in the Gsp-amidase fragment (C59A and C173A) and the full-length Gsp-synthetase/amidase (C59A) by the method of gene splicing by overlap extension (Ho et al., 1989). In the first round of PCR for each mutant, two products (A and B) were obtained using corresponding sense and antisense primers (Table 1), both products containing the desired mutation (approximately 25 bp sequence overlap spanning the mutation site). In each case, the PCR template used was the corresponding wild-type plasmid, pGSP or pAMID (Bollinger et al., 1995; Kwon et al., 1997). In the second round of PCR, round I products, purified from agarose gel by the QiaexII gel extraction kit (Qiagen, Santa Clarita, CA), were used in an equimolar ratio as templates, and corresponding sense and antisense primers were used to amplify the entire combined product.

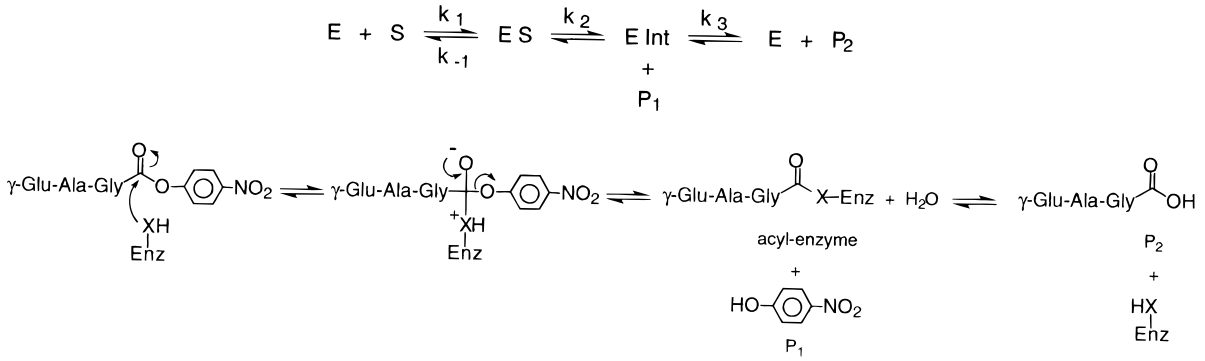
The PCR product from round II was purified from agarose gel and digested with restriction endonucleases in parallel with the corresponding wild-type expression vector. After complete digestion of both the PCR product and vector, each was purified from agarose gel, and the products were subsequently ligated using T4 DNA ligase (New England Biolabs, Beverly, MA). The ligation reaction was used to transform *E. coli* DH5α (Gibco-BRL, Grand Island, NY). Plasmid from one successful transformant was used to transform the *E. coli* expression strain BL21(DE3), in which the enzymes were overproduced. All mutant constructs were sequenced to confirm the desired mutation and that no other mutations had been introduced by PCR (Dana Farber Cancer Institute—Molecular Biology Core Facility, Boston, MA).

Circular Dichroism of Wild-Type and Mutant Gsp-Amidase Fragments. Wild-type Gsp-amidase fragment and both cysteine to alanine mutant proteins were prepared for CD analysis by dialyzing samples into 1 mM Tris-HCl, pH 7.6, and protein concentrations were determined by amino acid analyis and UV absorbance. Final enzyme concentrations of 10 μM were used for CD analysis. CD spectra were acquired on an Aviv Model 62DS CD spectrophotometer (Lakewood, NJ) at 25 °C over a wavelength range of 200−300 nm. A total of four scans was averaged. Sample buffer was used as a blank, and data are reported as mean residue ellipticity: $(\theta) = A/(10bcn)$ [A = millidegrees, b = path length, c = concentration, n = number of residues; (θ) reported in degrees centimeter^2 per decimole per residue]

RESULTS

Evidence for Acyl-Enzyme Intermediate by Stopped Flow. In previous work, we reported a lack of a metal cation in the 70 kDa full-length Gsp-synthetase/amidase and the 25 kDa Gsp-amidase fragment as well as unimpaired catalysis in the presence of EDTA (Kwon et al., 1997; Bollinger et al., 1995). Also, phosphonate and phosphinate substrate analogues, which are often potent inhibitors of proteases

Scheme 3: Mechanism of Glutathionylspermidine Amidase



catalyzing the direct addition of water to yield tetrahedral adducts mimicked by the tetrahedral phosphorus inhibitors (Bartlett & Giangiordano, 1996; Morgan et al., 1991; Radzicka & Wolfenden, 1995), have no effect on amidase activity, although they inhibit the synthetase activity with $K_i = 10\ \mu$M (phosphonate; Kwon et al., 1997) and $K_i^* = 8$ nM (phosphinate; Chen et al., 1997a). Thus, nucleophilic attack mechanisms, involving enzyme residues such as cysteine and serine, were implicated and have been investigated.

Previous studies on the amidase activity demonstrated that substitution for the spermidine group of Gsp is well tolerated, and we have demonstrated that the analogue γ-Glu-Ala-Gly-pNA (Ala substituted for Cys to minimize side reactions from the thiol side chain and also to minimize the number of synthetic steps) is a reasonable substrate for the amidase activity, with $K_m = 7$ mM and $k_{cat} = 300\ s^{-1}$ for the Gsp-amidase fragment (Kwon et al., 1997). Consequently, we hypothesized that the corresponding chromogenic ester, γ-Glu-Ala-Gly-ONp, would be a suitable substrate analogue to detect an acyl-enzyme intermediate (Scheme 3)—a covalent glutathionyl-enzyme.

The direct detection of acyl-enzyme intermediates that arise during nucleophilic catalysis is possible if acyl-enzyme breakdown is slower than release of the leaving group. As has been classically demonstrated by Hartley and Kilby (1954) for the serine protease chymotrypsin, we hypothesized that in going from the amide to the ester substrate, the rate-determining step might change from acyl-enzyme formation (amide substrate) to breakdown of the acyl-enzyme intermediate (ester substrate). With hydrolysis of the intermediate being rate-limiting in the case of the ester substrate, we proposed to monitor accumulation of the acyl-enzyme by rapid kinetic analysis using stopped-flow spectroscopy to detect a burst of P_1 production.

Conditions for stopped-flow analysis were determined by substrate stability and enzyme activity. γ-Glu-Ala-Gly-ONp (as described in Materials and Methods) was prepared in H_2O to minimize observed background nonenzymatic hydrolysis. In addition, 60 mM MES buffer at pH 5.7 was used for maximum substrate stability during the reaction. Though the amidase activity has an alkaline pH optimum (>pH 8.5) (Bollinger et al., 1995), substrate (γ-Glu-Ala-Gly-ONp) hydrolysis in base was observed to be rapid, such that steady-state kinetic constants were unattainable even at pH 6.8. Amidase activity at pH 5.7 is approximately 5% that observed at pH 6.8 but sufficient to measure activity by stopped-flow, as described below. For the hypothetical mechanism (Scheme 3), rapid equilibrium interaction of enzyme and substrate is followed by tripeptidyl acyl-enzyme formation and release of the first product (P_1; *p*-nitrophenol). The final step of the reaction is hydrolysis of the acyl-enzyme intermediate to release P_2 (γ-Glu-Ala-Gly), thus regenerating free enzyme.

For both the Gsp-amidase fragment and the full-length Gsp-synthetase/amidase using γ-Glu-Ala-Gly-ONp as substrate, formation of the *p*-nitrophenol product was sufficiently rapid that the burst phase was complete within the 3 ms dead time of the instrument. In the absence of direct kinetic observation of a burst phase, we relied on the fact that the magnitude of the burst phase should be—and was observed to be—directly proportional to enzyme concentration. The steady-state rate, also proportional to enzyme concentration, may be fit as a straight line and extrapolated back to $t = 0$ (Figure 1, dashed lines) in order to obtain the absorbance corresponding to the amplitude of the burst phase.

Table 2 presents the results of stopped-flow analyses, comparing enzyme concentrations with observed burst amplitudes and observed steady-state rates. For both the Gsp-amidase fragment and the full-length Gsp-synthetase/amidase (Table 2) with the γ-Glu-Ala-Gly-ONp substrate, observed burst amplitudes were directly proportional to enzyme concentration (plotted in Figure 1 insets). Furthermore, steady-state rates observed by stopped-flow (Table 2) were also proportional to enzyme concentration (plotted in Figure 1 insets) and were equivalent to rates measured by steady-state methods (Table 3). Comparison of the observed and theoretical results for burst amplitude indicates a high fractional burst stoichiometry (Table 2).

For comparison, we have studied both amide and ester substrate analogues with the full-length Gsp-synthetase/amidase and the Gsp-amidase fragment under conditions of stopped-flow and standard assay conditions (Table 3). From this comparison, we note that the amidase activity is significantly activated as the amidase domain is liberated from the synthetase domain. A 5-fold increase in k_{cat} and over 400-fold increase in k_{cat}/K_m for the γ-Glu-Ala-Gly-ONp substrate analogue confirm the activation phenomenon we have previously reported (Kwon et al., 1997).

Attempts to demonstrate the expected lack of burst kinetics with the γ-Glu-Ala-Gly-pNA substrate proved challenging due to poor K_m values (36 mM for the full-length Gsp-synthetase/amidase; 9.6 mM for the Gsp-amidase fragment) and complications of substrate and product solubility. Active-site saturation could not be achieved due to insolubility of the substrate in either H_2O or buffer, and the insolubility of the nitroaniline product, which precipitated and obstructed the flow cell, prevented accurate data collection.

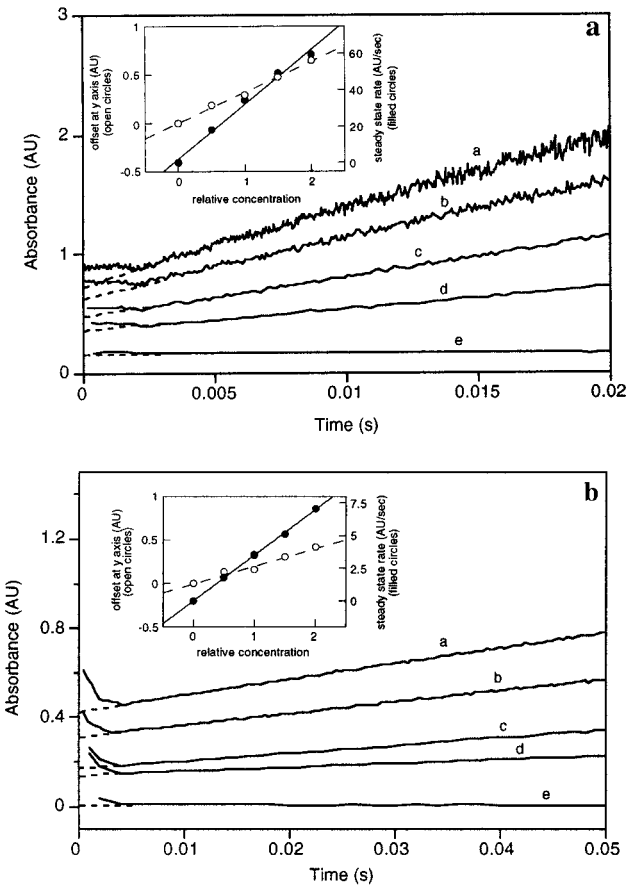


FIGURE 1: (a) Stopped-flow analysis of Gsp-amidase fragment with γ-Glu-Ala-Gly-ONp. Stopped-flow studies were conducted at 4 °C in a final buffer of 40 mM MES buffer, pH 5.7. Dashed lines indicate the extrapolation of steady-state rates back to the *y*-axis, representing the burst magnitude. The inset plots burst magnitudes (open circles) and steady-state rates (closed circles) versus relative enzyme concentrations. Enzyme concentrations (milligrams per milliliter) used were as follows: (a) 10.8, (b) 8.1, (c) 5.4, (d) 2.7, and (e) 0. (b) Stopped-flow analysis of the full-length Gsp-amidase with γ-Glu-Ala-Gly-ONp. Stopped-flow studies were conducted at 4 °C in a final buffer of 40 mM MES buffer, pH 5.7. Dashed lines indicate the extrapolation of steady-state rates back to the *y*-axis, representing the burst magnitude. The inset plots burst magnitudes (open circles) and steady-state rates (closed circles) versus relative enzyme concentrations. Enzyme concentrations (milligrams per milliliter) used were as follows: (a) 20, (b) 15, (c) 10, (d) 5, and (e) 0.

Inhibition of Amidase Activity by Iodoacetamide. A variety of group-specific enzyme inhibitors were tested for inhibition of the amidase activity. Among these, iodoacetamide (IAM) was observed to inactivate the amidase activity in a time-dependent manner. Figure 2a demonstrates time-dependent inactivation of the Gsp-amidase fragment by IAM. In the absence of saturation, an apparent second order rate constant of 0.0017 $mM^{-1}\cdot s^{-1}$ was obtained from a plot of k_{app} against inhibitor concentration (Figure 2b).

In order to assess the mechanistic relevance of IAM modification, substrate protection experiments were performed with amide substrates. Two different substrate analogues were used. The γ-Glu-Ala-Gly-NH_2 (nonchromogenic) (Kwon et al., 1997) substrate was used to protect the active site, and the chromogenic γ-Glu-Ala-Gly-pNA was subsequently used to assay for remaining amidase activity. Aliquots from the inhibition reaction, which contained enzyme, IAM, and (when appropriate) γ-Glu-Ala-Gly-NH_2, were diluted 50-fold into the amidase assay containing the chromogenic substrate, reducing inhibitor concentrations to negligible levels. Figure 2c demonstrates protection against amidase inactivation, when the γ-Glu-Ala-Gly-NH_2 substrate at $4K_m$ was added to the inhibition reaction, indicating that IAM modification occurs in or near the amidase active site. Together, these data suggest that cysteine, or possibly histidine, is an important residue for amidase activity and that the relevant residue is in or near the amidase active site. In the full-length Gsp-synthetase/amidase, amidase activity was also inhibited by IAM in a time-dependent manner with an apparent second-order rate constant of 0.0012 $mM^{-1}\cdot s^{-1}$ (data not shown), but surprisingly, substrate (γ-Glu-Ala-Gly-NH_2) at $10K_m$ was unable to provide significant protection against IAM modification.

Mutagenesis of Gsp-Amidase Fragment. Inactivation of amidase activity by IAM and protection by substrate against inhibition suggested that a cysteine residue might play a critical role. The Gsp-amidase fragment contains only two cysteine residues, Cys-59 and Cys-173. Therefore, each cysteine in the Gsp-amidase fragment was individually mutated to alanine. The mutant enzymes purified identically to the wild-type Gsp-amidase fragment and were each characterized for activity. With the γ-Glu-Ala-Gly-pNA assay, the C173A mutant retained amidase activity comparable to wild-type Gsp-amidase fragment levels (Table 4). The catalytic efficiency of the C173A mutant fragment (k_{cat}/K_m = 47 $mM^{-1}\cdot s^{-1}$; K_m = 5.5 mM and k_{cat} = 260 s^{-1}) is consistent with that of the wild-type Gsp-amidase fragment (k_{cat}/K_m = 43 $mM^{-1}\cdot s^{-1}$). However, C59A showed 30 000-fold reduced amidase activity. CD studies comparing the wild-type Gsp-amidase fragment and both cysteine mutant proteins indicated a lack of major conformational alteration caused by the Cys to Ala mutations (Figure 3). These data indicate an essential role for Cys-59 in amidase catalysis.

The C59A mutation was also constructed in the full-length Gsp-synthetase/amidase (FLC59A), and amidase activity was undetectable (data not shown). In addition, FLC59A was examined to verify that synthetase activity was intact. The K_m values for GSH and spermidine were measured to be 670 μM and 75 μM, respectively, values consistent with previously reported constants for the full-length Gsp-synthetase activity: K_m(GSH) = 700 μM, K_m(spermidine) = 60 μM (Bollinger et al., 1995). Though K_m values were similar, the k_{cat} for the Gsp-synthetase in FLC59A was observed to be 2 s^{-1}, a 3.5-fold reduction from the wild-type full-length synthetase activity turnover number of 7 s^{-1}. The interpretation of this reduction in k_{cat} is unclear; however, we have previously reported a turnover of 2 s^{-1} for an N-terminal truncation protein, Gsp-synthetase fragment, that catalyzes only Gsp synthesis (no detectable Gsp-amidase activity) (Kwon et al., 1997).

Amidase Activation and Communication between Synthetase and Amidase Active Sites. Recent work with Gsp substrate analogues (GSH-NH_2, GSH-OEt, γ-Glu-Ala-Gly-pNA) (Kwon et al., 1997) has demonstrated that the amidase activity is significantly activated in the recombinant 25 kDa fragment. This phenomenon has now been examined in the full-length enzyme. Figure 4 demonstrates that the amidase activity in the full-length protein may be activated by the addition of various combinations of Gsp-synthetase substrates. Significant activation occurs with the addition of ATP-Mg^{2+} and GSH to the γ-Glu-Ala-Gly-pNA assay (Gsp-

Table 2: Stopped-Flow Study Burst Magnitudes and Steady-State Rates Using Gsp-Amidase Fragment and Full-Length Gsp-Amidase

(a) Gsp-amidase fragment[a]				(b) full-length Gsp-amidase[b]			
enzyme concentration (μM)	observed burst magnitude (AU)	theoretical burst magnitude[c] (AU)	observed burst stoichiometry[d] (%)	enzyme concentration (μM)	observed burst magnitude (AU)	theoretical burst magnitude[e] (AU)	observed burst stoichiometry[d] (%)
107	0.187	0.151	124	71.0	0.133	0.0942	141
214	0.292	0.303	96.4	142	0.160	0.188	85.1
321	0.480	0.455	105	213	0.307	0.283	108
428	0.630	0.607	104	284	0.422	0.377	112

(a) Gsp-amidase fragment[a]			(b) full-length Gsp-amidase[b]		
enzyme concentration (μM)	observed steady-state rate (AU/s)	k_{cat} (s^{-1}; derived from observed rate)[f]	enzyme concentration (μM)	observed steady-state rate (AU/s)	k_{cat} (s^{-1}; derived from observed rate)[g]
107	16.9	102	71.0	1.78	16.2
214	33.9	103	142	3.53	16.1
321	49.2	99.5	213	5.12	15.6
428	63.3	96.1	284	7.07	16.1

[a] By steady-state kinetic study, k_{cat} was determined to be 103.3 s^{-1}. [b] By steady-state kinetic study, k_{cat} was determined to be 19.5 s^{-1}. [c] Theoretical burst magnitude can be obtained according to Beer's law (λ_{max} = 360 nm, ϵ = 3081 AU·cm^{-1}·M^{-1}, light path = 0.5 cm) when the enzyme is highly saturated (91.9%) by excess of substrates (11.4K_m). [d] Observed burst magnitude divided by theoretical burst magnitude and converted to percentage. [e] Theoretical burst magnitude can be obtained according to Beer's law (λ_{max} = 360 nm, ϵ = 3081 AU·cm^{-1}·M^{-1}, light path = 0.5 cm) when the enzyme is highly saturated (86.1%) by excess of substrates (6.2K_m). [f] When the enzyme is highly saturated (91.9%) by excess of substrates (11.4K_m), k_{cat} values can be obtained according to the equation V_{max} = [E]k_{cat} and Beer's law (λ_{max} = 360 nm, ϵ = 3081 AU·cm^{-1}·M^{-1}, light path = 0.5 cm). [g] When the enzyme is highly saturated (86.1%) by excess of substrates (6.2K_m), k_{cat} values can be obtained according to the equation V_{max} = [E]k_{cat} and Beer's law (λ_{max} = 360 nm, ϵ = 3081 AU·cm^{-1}·M^{-1}, light path = 0.5 cm).

Table 3: Steady-State Kinetic Parameters for Gsp-Amidase Activity in the Full-Length Gsp-Synthetase/Amidase and Gsp-Amidase Fragment[a]

	full-length protein			Gsp-amidase fragment		
substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1}·s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1}·s^{-1})
γ-Glu-Ala-Gly-pNA[b]	24	14	0.58	7.2	310	43
γ-Glu-Ala-Gly-pNA[c]	36	13	0.37	9.6	20	2.08
γ-Glu-Ala-Gly-ONp[c]	1.3	19.5	0.68	0.35	103.3	295

[a] Due to the instability of γ-Glu-Ala-Gly-ONp, the compound was not studied in 100 mM Na-PIPES (pH 6.8). [b] The assay was studied in 100 mM Na-PIPES (pH 6.8) at 37 °C. [c] The assay was studied in 60 mM MES (pH 5.7) at 4 °C.

synthetase substrates were both saturating); the full-length amidase activity may be activated as much as 15-fold when γ-Glu-Ala-Gly-pNA is saturating. Also, significant but reduced activation was observed with a nonhydrolyzable ATP analogue, AMP-PNP, and also with a slowly hydrolyzable ATP analogue, ATP-γ-S. EC_{50} values (50% of effective concentration) were determined for ATP-γS and GSH to be 0.28 and 0.45 mM, respectively (Gsp-synthetase K_m for ATP-γ-S and GSH are 0.3 and 0.7 mM, respectively). When the Gsp-phosphinate analogue (Chen et al., 1997a) was coincubated with ATP and GSH to achieve potent inhibition at the Gsp-synthetase active site, the velocity of the full-length Gsp-amidase activity increased as much as 20-fold (data not shown).

DISCUSSION

In this work, we have provided evidence which indicates that in the *E. coli* bifunctional enzyme, which makes and cleaves the amide bond between glutathione and spermidine, Gsp hydrolysis to GSH and spermidine proceeds with formation of a glutathionyl acyl-enzyme intermediate. We have addressed this question by the synthesis and analysis of amide and ester derivatives of the substrate (γ-Glu-Ala-Gly-pNA and γ-Glu-Ala-Gly-ONp) in which spermidine was replaced by a chromogenic nitrophenyl substituent. The *p*-nitroanilide was, in the end, not useful for initial burst assessment; however, the *p*-nitrophenyl ester was useful, with the constraint that its susceptibility to base hydrolysis required us to operate off the pH optimum and measure turnover at pH 5.7. Given that the Gsp-amidase fragment with the γ-Glu-Ala-Gly-pNA (amide) substrate exhibits a 5-fold reduced k_{cat}, compared to the γ-Glu-Ala-Gly-ONp (ester) substrate (Table 3), it is likely that a change in rate-determining step has occurred in going from the ester to the thermodynamically less activated amide substrate. The hypothetical glutathionyl acyl-enzyme intermediate for both the amide and ester substrates is identical, and it is therefore assumed that the rates of hydrolysis (k_3; Scheme 3) of this common acyl-enzyme species are equivalent. The plausible explanation for the 5-fold reduction in turnover is that acyl-enzyme formation is rate-limiting in the case of the amide substrate at pH 5.7.

Analysis by stopped-flow spectroscopy is consistent with the hypothesis that the nucleophile is provided by the enzyme. We have demonstrated burst amplitudes for *p*-nitrophenol production that suggest greater than 95% of the 25 kDa Gsp-amidase fragment and the 70 kDa full-length Gsp-synthetase/amidase accumulates as a tripeptidyl acyl-enzyme intermediate during single-turnover measurements with γ-Glu-Ala-Gly-ONp. Subsequent turnover rates correspond well with the values obtained from steady-state kinetics. From the high burst stoichiometry, it is likely that the rate constant for acyl-enzyme formation (k_2; Scheme 3) is much greater than the rate of acyl-enzyme hydrolysis (k_3). If the ratio of k_2:k_3 were as low as 20:1, the burst amplitude would be 90% of enzyme concentration, a difference that

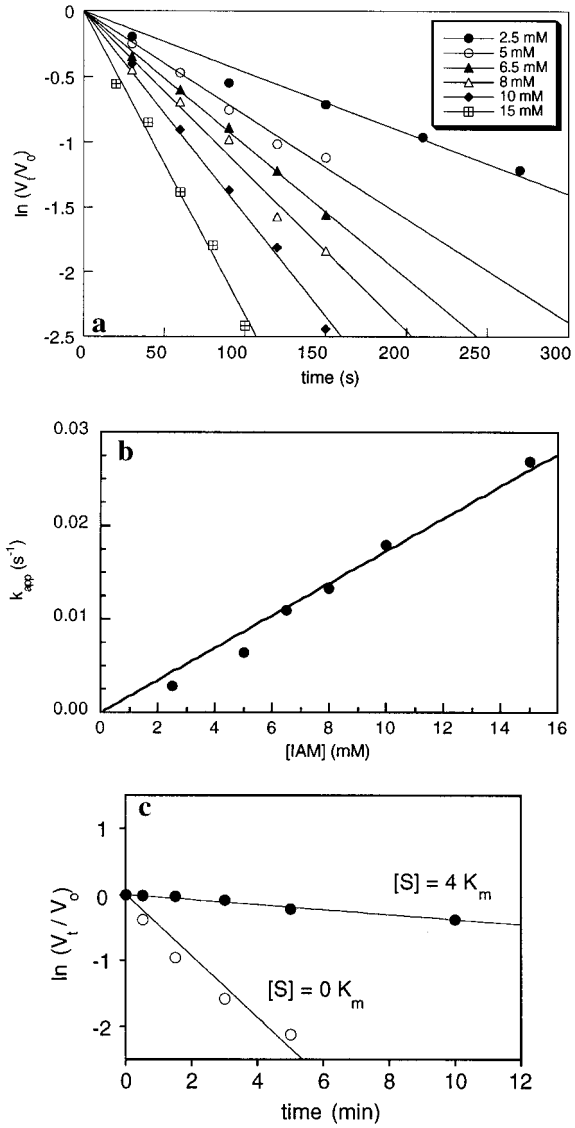


FIGURE 2: (a) Time-dependent inactivation of Gsp-amidase fragment by iodoacetamide. Inactivation of Gsp-amidase fragment activity was performed as described in Materials and Methods. Iodoacetamide concentrations were as indicated, and aliquots of the inactivation reaction were diluted 50-fold into the γ-Glu-Ala-Gly-pNA chromogenic assay. (b) Secondary plot of inactivation of Gsp-amidase fragment by iodoacetamide from panel a. (c) Substrate protection of Gsp-amidase fragment by γ-Glu-Ala-Gly-NH_2 against iodoacetamide inactivation. Saturating amounts of γ-Glu-Ala-Gly-NH_2 (28 mM = $4K_m$) were used to protect the Gsp-amidase from inactivation by iodoacetamide. Experiments were performed similarly to normal inactivation studies, except that γ-Glu-Ala-Gly-NH_2 was added to the inactivation reaction.

Table 4: Specific Activity of Wild-Type and Mutant Gsp-Amidase Fragment Proteins

amidase fragment	specific activity (units/mg)[a]	relative ratio
wild-type	880	34000
C173A	830	32000
C59A	0.026	1.0

[a] One unit = 1 μmol of γ-Glu-Ala-Gly-pNA hydrolyzed per minute.

would be easily detectable. With a k_2:k_3 ratio of at least 20:1 and the fact that steady-state rates from stopped-flow and steady-state methods are equivalent, acyl-enzyme hydrolysis (k_3) appears to be distinguishably rate-limiting. Furthermore, Cys-59 is a leading candidate as the enzyme

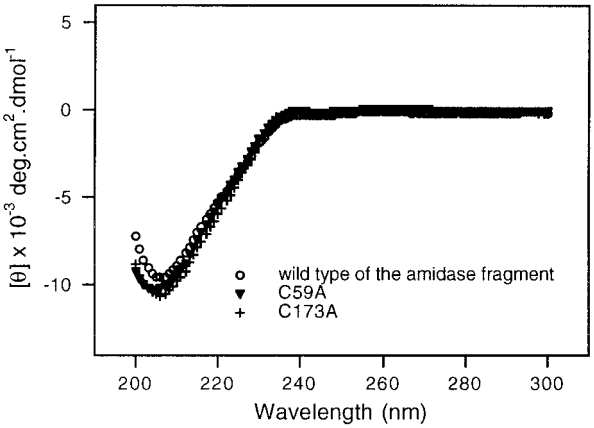


FIGURE 3: CD analysis of amidase fragment proteins. CD analysis was performed as described in Materials and Methods using wild-type amidase fragment (open circles), amidase fragment mutant C59A (closed triangles), and amidase fragment mutant C173A (+).

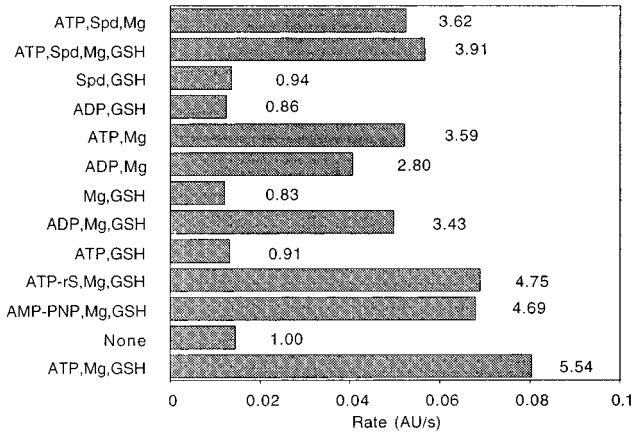


FIGURE 4: Activation of full-length Gsp-amidase activity by Gsp-synthetase substrates. The hydrolysis of γ-Glu-Ala-Gly-pNA (15 mM) was used to assay Gsp-amidase activity. *y*-Axis labels indicate Gsp-synthetase substrate combinations added to the reaction. Rates are normalized to the observed rate in the absence of added substrates.

nucleophile, as we demonstrate (1) inactivation by IAM and substrate protection against inactivation and (2) mutagenesis of Cys-59 to Ala that reduces amidase activity 30 000-fold relative to wild-type levels. Proof of a glutathionyl acyl-enzyme will ultimately require direct structural studies of the covalent intermediate.

We have proposed that the homologous 80 kDa Gsp-synthetase enzyme in trypanosomatids (Koenig et al., 1997) contains an analogous amidase activity (Bollinger et al., 1995; Kwon et al., 1997). In fact, an alignment of the Gsp-synthetase from *Crithidia fasciculata* exhibits a high degree of identity in the region of the essential Cys-59 of the *E. coli* enzyme, whereas no homology exists in the region of Cys-173, the only other cysteine residue in the Gsp-amidase fragment. It is possible, therefore, that a potential trypanosomal Gsp-amidase may also utilize a cysteine residue as a catalytic nucleophile, although amidase activity has not yet been demonstrated for either of the similar parasite enzymes, Gsp-synthetase or trypanothione-synthetase, which catalyzes subsequent amide bond formation between the N^8-amine of glutathionylspermidine and the glycine carboxylate of a second molecule of GSH. We note that the Gsp-amidase domain shows no significant primary sequence homology to any other known protein. Current work focuses on

overproduction of the Gsp-synthetase from *C. fasciculata* in *E. coli*.

In addition, we have explored further the activation phenomenon of the amidase activity. In previous work, we demonstrated that the amidase domain is significantly activated in the separate Gsp-amidase fragment. In this work, we have examined amidase activation in the context of the full-length Gsp-synthetase/amidase and find that the synthetase and amidase active sites communicate with each other, as demonstrated by the ability to activate amidase activity as much as 15-fold by addition of synthetase substrates. Occupancy of the synthetase active site may initiate communication through the protein as manifest by the release of inhibition of the amidase activity. Furthermore, the observations (Figure 4) are reminiscent of the protection of the full-length Gsp-synthetase/amidase against trypsin proteolysis by the specific combination of synthetase substrates ATP-Mg^{2+} and GSH (Bollinger et al., 1995). The explanation for the lack of substrate protection by γ-Glu-Ala-Gly-NH_2 against IAM inhibition of the full-length Gsp-amidase is unclear; however, it may be due to the context of the amidase active site in the full-length enzyme. An understanding of the observed amidase activation phenomenon may eventually suggest an explanation for the lack of protection. Gsp-synthetase activity analysis in the full-length C59A mutant demonstrated that K_m values for GSH and spermidine were equivalent to those of wild-type enzyme. However, k_{cat} was observed to be slightly reduced, from 7 to 2 s^{-1}. The reason for this decrease in k_{cat} is presently unclear, though it may be another indication of communication between the active sites.

In this regard, we raise the question of the role of Gsp-synthetase/amidase *in vivo*, for activation studies indicate that as Gsp-synthetase is operating (Gsp synthesis), Gsp-amidase is activated (Gsp hydrolysis). The observed regulation of the amidase activity may be due to a conformational change, such as removal of an occluding domain or repositioning of the catalytic nucleophile. As previously suggested (Bollinger et al., 1995), the communication observed here may be the manifestation of a regulation mechanism *in vivo*, in which the balance of opposing synthetic and hydrolytic activities in *E. coli* is altered depending on culture growth stage or conditions. Regulation may be designed to maintain the pools of either GSH, spermidine, or even Gsp. Gsp-synthetase/amidase may, in fact, be analogous to another bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, in which opposing activities are intrinsically unbalanced, though reaction products are able to modulate one or the other activity. With such precedent, it is unlikely that the purpose of Gsp-synthetase/amidase in *E. coli* is to maintain an ATP futile cycle. Insight into these issues awaits bacterial physiology studies.

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