Aldehyde and phosphinate analogs of glutathione and glutathionylspermidine: potent, selective binding inhibitors of the *E. coli* bifunctional glutathionylspermidine synthetase/amidase

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Introduction: The tripeptide glutathione is converted to glutathionylspermidine (Gsp) in *Escherichia coli* and in trypanosomatid parasites by an ATP-cleaving Gsp synthetase activity. In parasites, an additional glutathionylation forms bis-(glutathionyl)-spermidine, trypanothione, believed to be the major surveillance thiol involved in oxidant defense mechanisms in trypanosomatid parasites. In *E. coli*, the Gsp synthetase is part of a bifunctional enzyme opposed by the hydrolytic Gsp amidase.

Results: Gsp amidase and Gsp synthetase activities of the bifunctional *E. coli* enzyme can be separately targeted by potent, selective slow-binding inhibitors that induce time-dependent inhibition. The inhibitor γ-Glu–Ala–Gly-CHO most probably captures Cys59 and accumulates as the tetrahedral adduct in the amidase active site. Inhibitory Gsp phosphinate analogs are phosphorylated by ATP to yield phosphinophosphate analogs in the synthetase active site. Binding of phosphinophosphate in the Gsp synthetase active site potentiates the inhibition affinity for the aldehyde at the Gsp amidase active site by two orders of magnitude.

Conclusions: Time-dependent inhibition of the Gsp amidase activity by the aldehyde substrate analog supports previous work that suggests glutathionyl acyl–enzyme intermediate formation in the Gsp amidase reaction mechanism. Use of potent selective inhibitors against Gsp amidase (aldehyde) and Gsp synthetase (phosphinate) activities provides further evidence of interdomain communication in the bifunctional enzyme from *E. coli*.

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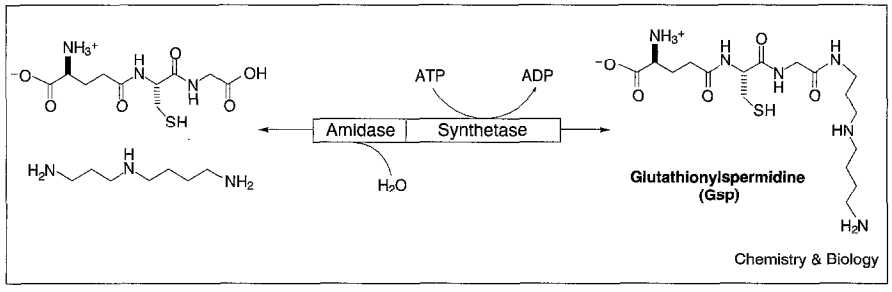
Introduction

In *Escherichia coli* and in trypanosomatid parasites, the tripeptide thiol glutathione (GSH) is enzymatically converted to N^1-glutathionylspermidine (Gsp) by an ATP-cleaving, amide-forming Gsp synthetase [1–4]. In the parasites, a subsequent glutathionylation at the N^8 of Gsp leads to $N^{1,8}$-bis(glutathionyl)-spermidine, known as trypanothione [1,5]. When we purified and characterized the 70 kDa *E. coli* Gsp synthetase, a second activity, hydrolysis of Gsp back to GSH and spermidine (Figure 1), was detected [3]. We confirmed that the *E. coli* enzyme is a bifunctional protein with two separate catalytic domains by expressing an amino-terminal 25 kDa fragment (residues 1–225) as an active Gsp amidase and residues 189 to 619 as a carboxyl-terminal Gsp synthetase fragment [6]. Studies to probe both the regulation of the two opposing activities (to avoid a futile GSH-dependent ATPase cycle) and mechanistic analyses of both the synthetase and amidase catalytic mechanisms have been undertaken.

Interest in the Gsp synthetase/amidase from *E. coli* began with an examination of related enzymes involved in trypanothione biosynthesis, a Gsp synthetase and trypanothione synthetase. As in the *E. coli* Gsp synthetase/amidase, both trypanosomal enzymes catalyze amide bond formation coupled to ATP hydrolysis. Because trypanothione represents the primary oxidant defense mechanism in trypanosomes, the unique trypanothione synthetase represents an attractive drug target for the treatment of trypanosomiasis, which includes the significant medical problems of South American Chagas' disease and African sleeping sickness.

E. coli, however, utilizes a GSH-based system for oxidant defense; thus the physiological role of the enzyme is unclear. Synthesizing Gsp from GSH and spermidine, the Gsp synthetase/amidase represents an intriguing juxtaposition of two important classes of molecule. Whether participating in oxidant defense mechanisms or in complexation with nucleic acids, phospholipids, or proteins via spermidine, the intrinsic balance and regulation of synthetase and amidase activities may be useful in understanding its physiological function in *E. coli*.

Figure 1



The bifunctional Gsp synthetase/amidase in *Escherichia coli*.

In a preliminary communication, we reported that the Gsp phosphinate analog, **1a** (Figure 2), is a potent time-dependent inhibitor that selectively inactivates the synthetase in the presence of ATP but does not inhibit the amidase activity of the bifunctional enzyme [7]. Failure of **1a** to inhibit the Gsp amidase is inconsistent with a mechanism involving direct attack of H_2O, since the phosphinate should be a good mimic of such a tetrahedral adduct. Rather, lack of Gsp amidase inhibition by the phosphinate **1a** suggests initial attack by an enzyme nucleophile. Indeed, pre-steady-state kinetic analysis with the chromogenic γ-Glu–Ala–Gly-O-*p*-nitrophenyl ester substrate gave burst data consistent with rate-determining breakdown of an acyl enzyme intermediate [8]. The amidase is not inhibited by a generic serine protease inhibitor (3,4-dichloroisocoumarin) but is inactivated by iodoacetamide. Furthermore, mutagenesis of Cys59 to Ala inactivates the Gsp amidase [8]. As a further test of the candidacy of Cys59 as a catalytic nucleophile in Gsp amidase activity, we report the synthesis of the γ-Glu–Ala–Gly aldehyde, **2**, and its ability to serve as a selective, slow-binding inhibitor of the Gsp amidase activity, consistent with the ability of aldehyde analogs of cysteine protease substrates to form tight long-lived inhibitor complexes [9,10]. We also report the preparation and characterization of serine (**1b**) and cysteine (**1c**) analogs of the Ala-containing phosphinopeptide, **1a**, (Figure 2) as slow-binding inhibitors of the Gsp synthetase activity.

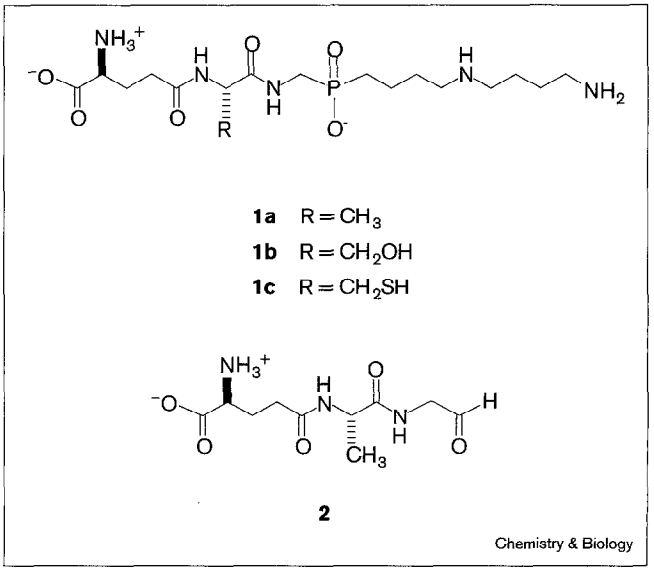
Results

Slow binding inhibition of *E. coli* Gsp amidase by γ-Glu-Ala-Gly-CHO

Synthesis of the aldehyde analog, **2**, of the substrate γ-Glu–Ala–Gly is summarized in Figure 3. As in the phosphinate inhibitor, **1a**, Ala was used in place of Cys to avoid complications of thiol chemistry, in particular, intermolecular thiohemiacetal formation between two molecules of **2**. As for the reduction of the protected methyl ester **3**, excess (four equivalents) diisobutyl aluminum hydride (DIBAL) was used, as the metal hydride would be consumed by amide hydrogens. Furthermore, immediately after the reduction had been accomplished, the final deprotection by trifluoroacetic acid was conducted under argon to prevent oxidation of the aldehyde to the carboxylic acid. After purification by gel filtration and high performance liquid chromatography (HPLC), the target compound **2** was stored at –20°C, and no significant change was observed over a period of a month.

The GSH analog γ-Glu–Ala–Gly is an excellent substrate for the Gsp synthetase activity [6], whereas the corresponding aldehyde, **2**, is incompetent as anticipated. In contrast, when aldehyde, **2**, was incubated with the pure 25 kDa Gsp amidase fragment, time-dependent inhibition was observed (Figure 4a). With aldehyde **2** at 100 μM, all activity was lost in a progressive fashion. This pattern is typical for aldehyde analogs of substrates reacting at the active site of cysteine proteases, in which an initial collisional complex (E·I) isomerizes to a tighter complex (E·I*) according to equation 1. Fitting the observed data to this model allowed determination of K_i and K_i^* (Figure 4b), 1.7 μM and 80 nM, respectively (Table 1). In order to assess the reversibility of inhibition on dilution, 4 μM Gsp amidase fragment was incubated with 1 mM aldehyde **2** for 20 min at 37°C to fully

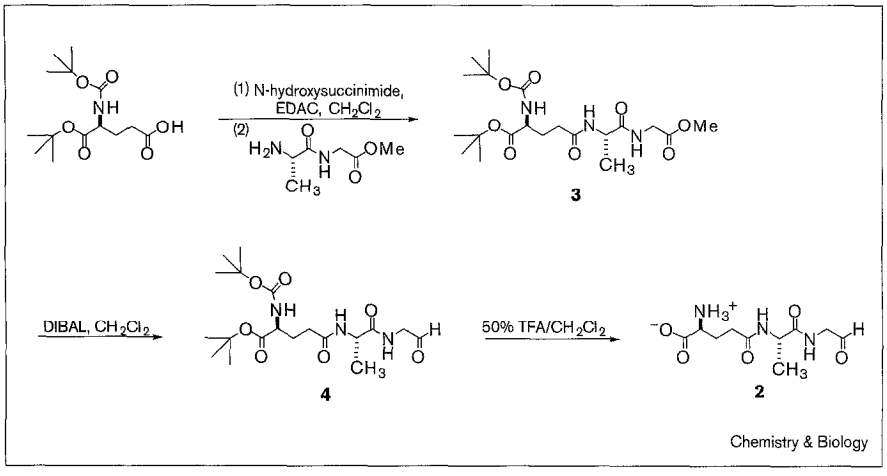
Figure 2



Structure:. **1**, Gsp phosphinate analogs; **2**, γ-Glu–Ala–Gly aldehyde.

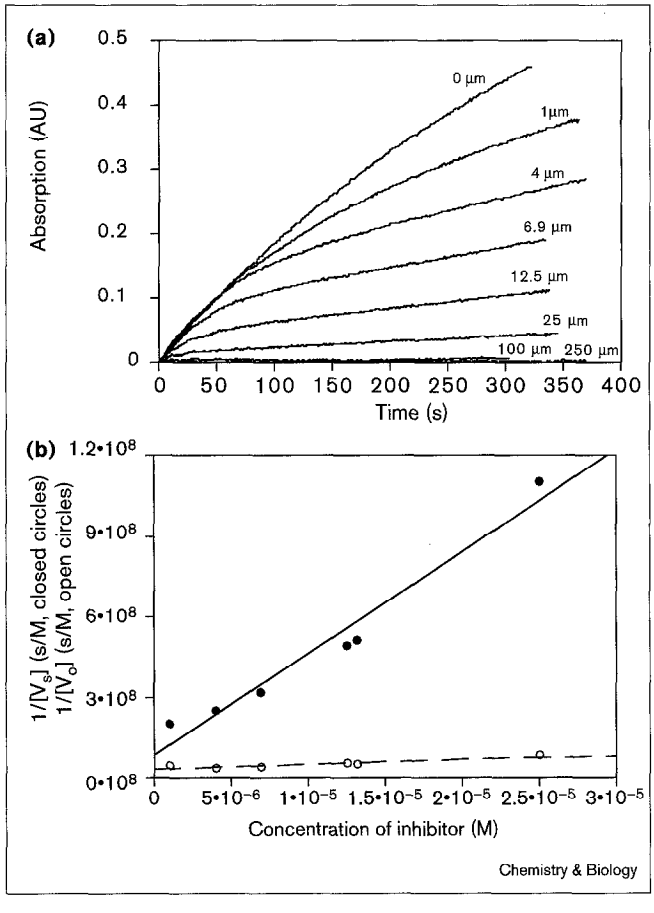
Figure 3

The synthesis of γ-Glu–Ala–Gly aldehyde. EDAC, 1-ethyl-3-3-dimethylaminopropyl)-carbodiamide; TFA, trifluoroacetic acid.



inactivate the enzyme, then diluted 100-fold into a reaction mixture containing 36 mM γ-Glu–Ala–Gly-*p*-nitro-aniline to monitor the recovery of activity. A $t_{1/2}$ of 2 min, corresponding to a k_{regain} (rate of regain of activity from fully inhibited state) of 0.35 min^{-1}, was observed (data not shown).

Figure 4



(a) Time-dependent inhibition of Gsp amidase fragment by γ-Glu–Ala–Gly aldehyde. **(b)** Secondary plot of (a). V_s = steady-state rate and V_o = initial rate of curves in (a).

$$E + I \underset{K_i}{\rightleftharpoons} E \cdot I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E \cdot I^* \qquad (1)$$

K_i^*

Slow-binding inhibition of *E. coli* Gsp synthetase by phosphinate analogs

Synthesis of **1a** along with its precursor **5a** was carried out as previously described (Figure 5) (S.C. and J.K.C., unpublished data). Compounds **5b** and **5c** were prepared using similar procedures. However, attempts to convert **5b** to **1b**, as described for the conversion of **5a** to **1a** (treatment of **5a** with 30% HBr in AcOH), resulted in the simultaneous acetylation of the serine-hydroxyl group. Consequently, conversion of **5b** to **1b** was achieved by using trimethylsilyl bromide (TMSBr) as the deprotective reagent. Thus, treatment of **5b** with excess TMSBr followed by hydrogenolysis gave **1b** in 52% yield after ion-exchange chromatography purification. The conversion of **5c** to **1c** proved to be more problematic. Treatment of **5c** with either HBr/AcOH or TMSBr resulted in a mixture of products as monitored by ^{31}P nuclear magnetic resonance (NMR), and none of the desired product **1c** could be isolated. Therefore, **5c** was treated with I_2/MeOH [11] followed by HBr/AcOH to give the corresponding cystine derivative **6** in satisfactory yield. The cysteine-phosphinate **1c** could then be generated *in situ* by the treatment of **6** with dithiothreitol (DTT) prior to enzyme assay. In order to evaluate the polyamine binding requirement during the inhibitor–enzyme interaction, a non-polyamine containing phosphinopeptide was also synthesized in five steps from N-Z-phosphinoglycine [12].

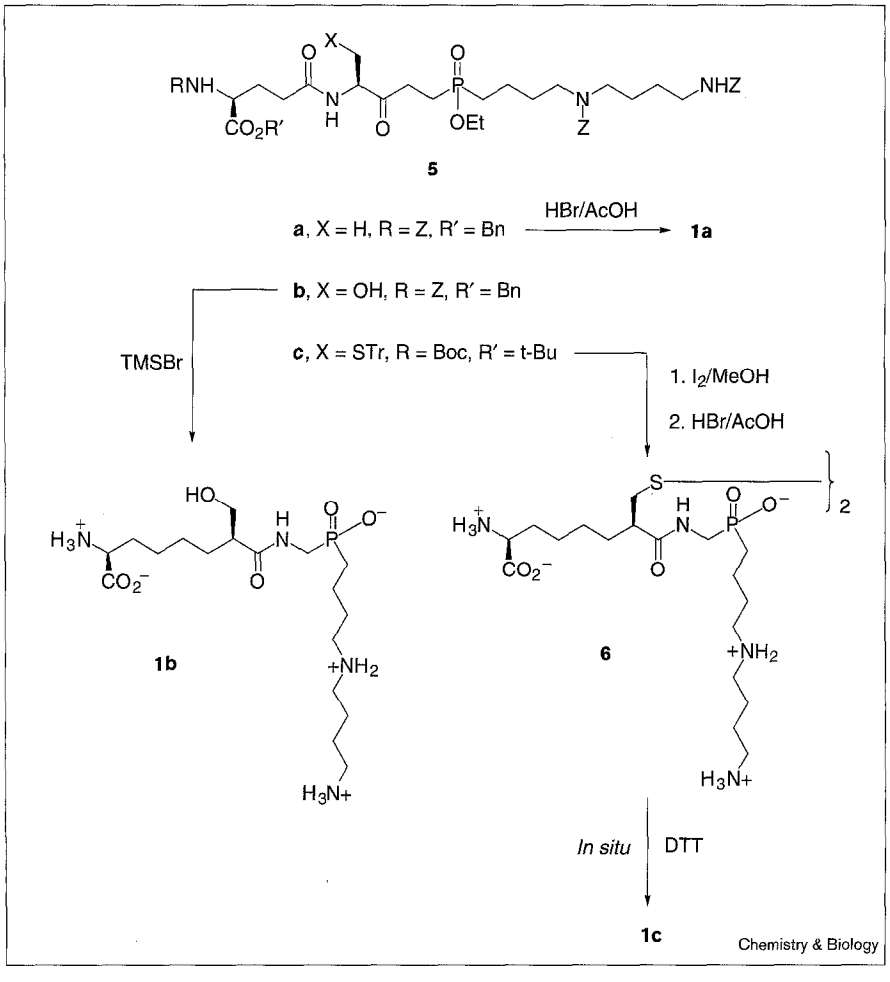
Table 1

K_i and K_i^* values for aldehyde and phosphinate analogs in selective time-dependent inhibition of Gsp amidase and Gsp synthetase activities.

Compound	Amidase inhibition		Synthetase inhibition		Enzyme form
	K_i	K_i^*	K_i	K_i^*	
2	1.7 μM	80 nM	–	–	Gsp amidase fragment
2	98 μM	20 μM	–	–	Gsp synthetase/amidase
2	6.8 μM	250 nM	–	–	Gsp synthetase/amidase + **1a** + ATP
1a	–	–	3.2 μM	7.8 nM	Gsp synthetase/amidase
1b	–	–	4.8 μM	9.2 nM	Gsp synthetase/amidase
1c	–	–	2.1 μM	3.1 nM	Gsp synthetase/amidase

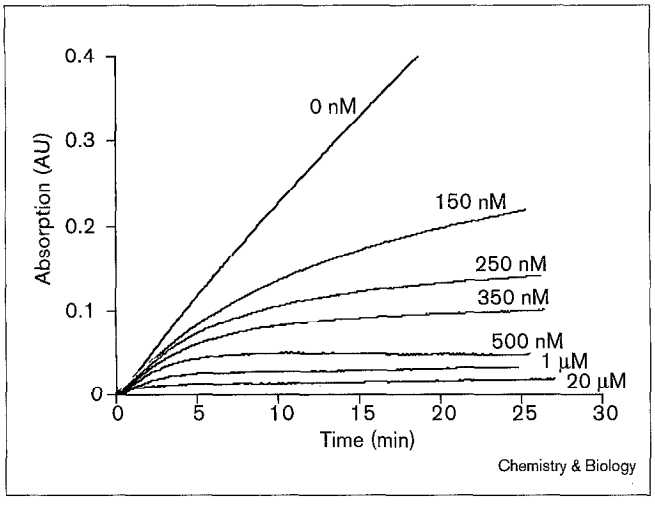
In a previous preliminary report we noted that a series of Gsp phosphapeptide analogs required both a spermidine moiety and the P-CH_2-phosphinate linkage in order to observe time-dependent slow-binding inhibition of the Gsp synthetase activity in full-length bifunctional enzyme. Inhibition of the full-length Gsp synthetase activity by **1a** yielded a K_i of 3.2 μM and a K_i^* of 7.8 nM (Table 1), a 410-fold gain in potency by K_i/K_i^* ratio, in accord with equation 1. No inhibition of the amidase was detected [7]. Here, we report the evaluation of Ser (**1b**) and Cys (**1c**)

Figure 5



The synthesis of the Gsp phosphinate inhibitors **1a**–**c**. Z, carbobenzyloxy; Str, S-trityl.

Figure 6



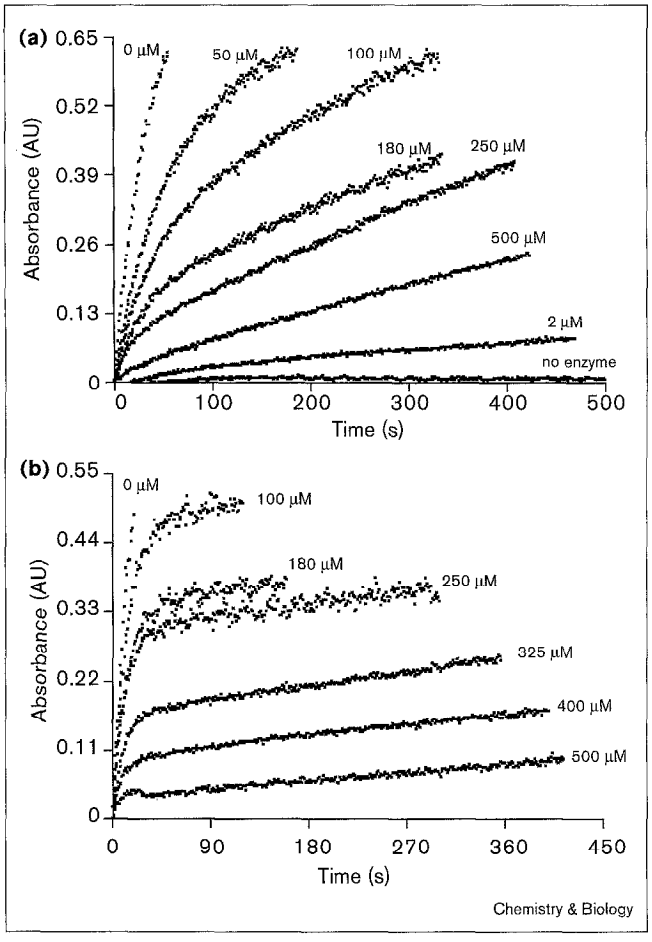
The time-dependent inhibition of full-length Gsp synthetase/amidase by cysteine-containing Gsp phosphinate **1c**.

analogs of the Gsp-phosphinate inhibitor, the latter most closely approximating the γ-Glu–Cys dipeptidyl moiety of the physiological substrate. Both **1b** (data not shown) and **1c** (Figure 6) exhibit time-dependent progressive tightening of inhibition, as observed with **1a**. Phosphinates **1b** and **1c** show a 500-fold and 670-fold tightening (Table 1), respectively, from low micromolar K_i to low nanomolar K_i^* values upon phosphorylation in the synthetase active site. The Cys-phosphinate **1c** (K_i^* = 3.1 nM) forms a threefold tighter complex than either the Ala or Ser analogs, **1a** and **1b**. The $t_{1/2}$ for regain of catalytic activity by dissociation of **1a**, **b**, or **c** from the enzyme following dilution of the E·I* complex into assay solutions free of inhibitor was 25, 27 and 28 min, respectively.

Altered potency and equilibrium of γ-Glu-Ala-Gly·CHO in full-length Gsp synthetase/amidase – modulation by Gsp phosphinate

The susceptibility of the amidase active site to inhibition by **2** was then examined in the full-length bifunctional enzyme. Time-dependent progressive inhibition was again observed (Figure 7a) with elevated inhibition constants, K_i = 98 μM and K_i^* = 20 μM (Table 1), reflecting a diminished potency of 50-fold in K_i and 240-fold in K_i^* for the amidase active site when contained in full-length bifunctional enzyme. We have demonstrated that the k_{cat} for amidase activity is suppressed 20-fold in the full-length Gsp synthetase/amidase compared to the Gsp amidase fragment alone [6] and that the phosphinate analog **1a**, binding at the synthetase site, accelerates amidase turnover (k_{cat}) by 20-fold [8]. Consequently, we have examined inhibition of Gsp amidase activity in the full-length enzyme by **2** in the presence of 50 μM **1a** and 2 mM ATP, preincubated for 5 min to saturate the synthetase active site. Comparison of Figure 7b with Figure 7a, shows more potent inhibition by

Figure 7



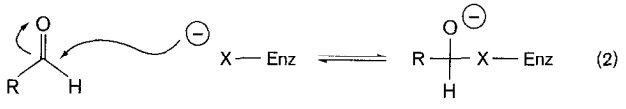
(a) The time-dependent inhibition of full-length Gsp synthetase/amidase by γ-Glu–Ala–Gly aldehyde. High signal-to-noise ratios due to high background absorbance of the assay substrate, γ-Glu–Ala–Gly-*p*-nitro-aniline, at 36 mM. **(b)** The time-dependent inhibition of full-length Gsp synthetase/amidase by γ-Glu–Ala–Gly aldehyde in the presence of Gsp phosphinate **1a** and ATP.

2 when the synthetase active site was saturated by **1a** + ATP. A K_i of 6.8 μM and K_i^* of 0.25 μM were calculated (Table 1). Thus, K_i tightens by 16-fold and K_i^* by 80-fold in the amidase active site when the synthetase active site is occupied by its slow-binding inhibitor.

Discussion

The tripeptide aldehyde **2** was prepared to test as a slow-binding, time-dependent inactivator of the Gsp amidase activity. Validation of such behavior further supports a reaction mechanism involving nucleophilic catalysis by the amidase, with Cys 59 as leading candidate for the nucleophile, given earlier results from C59A mutagenesis, iodoacetamide alkylative inactivation, and burst kinetics supporting acyl–enzyme intermediate formation. Other peptide aldehydes have been potent active-site titrants of proteases using cysteine nucleophiles in catalysis, notably Ac·DVED·CHO and Ac·YVAD·CHO for the caspases

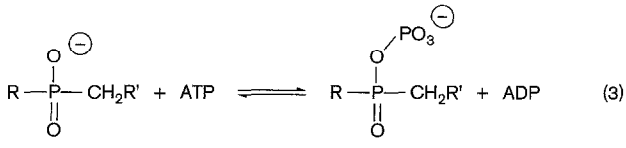
interleukin-converting enzyme (ICE) and CPP-32, which are involved in programmed cell death pathways [13–15]. In those cases, the time-dependent inhibition has been correlated with covalent tethering of the aldehyde as the tetrahedral thiohemiacetal adduct to the active cysteine by X-ray structures for both ICE and CPP-32 [15]. An analogous accumulating tetrahedral adduct would be consistent for the Gsp amidase mechanism (equation 2). While Cys 59 is the likely nucleophile, until structural studies prove the point, there is the formal possibility that Enz-X is an oxygen nucleophile, given the precedent that amino-terminal threonine in the proteasome β subunits is trapped as a stabilized hemiacetal [16–18].



(2)

There is a clear difference in both absolute affinity and partition between E·I and E·I* of the Gsp amidase active site when in the bifunctional full-length enzyme or when present as the autonomous 25 kDa domain. The 50-fold weaker K_i for the aldehyde **2** in full-length Gsp synthetase/amidase compared to the Gsp amidase fragment suggests an altered ground state geometry. Additionally, the isomerization of initial E·I to tightened E·I* complex has a 1:4 partition E·I/E·I* for full-length enzyme, but a 1:20 partition favoring E·I* accumulation in the Gsp amidase fragment. If indeed E·I* is the tetrahedral (thiohemiacetal) adduct of equation 2, the differential stability could reflect subtle differences in microenvironment, such as the pK_a of the attacking enzyme nucleophile or the strength of a hydrogen bond that may stablize the tetrahedral oxyanion.

The second class of Gsp synthetase/amidase inhibitors that show progressive time-dependent inhibition is the series of phosphinate analogs (**1a–c**), that are selective for the Gsp synthetase active site. Tight-binding properties of these Gsp phosphinate analogs, K_i/K_i^* ratios of 410–670, permitted mechanism-based titration of the neighboring Gsp synthetase active site in the bifunctional Gsp synthetase/amidase and allowed a test of intersite communication. Preincubation was conducted with **1a** + ATP to inactivate the Gsp synthetase active site and to load it with the slowly dissociating phosphinophosphate analog (equation 3) of the tetrahedral reaction intermediate for Gsp synthetase catalysis. Consequently, the Gsp amidase active site could then be probed for K_i and K_i^* for aldehyde **2** when the synthetase site was thus occupied.



(3)

Indeed, a 16-fold tightening in K_i and, more impressively, an 80-fold tightening in K_i^* were obtained. This result is consistent with the hypothesis of interacting active sites, as we have reported a 20-fold increase in Gsp amidase k_{cat} in the full-length enzyme via the same occupancy of **1a** + ATP at the Gsp synthetase active site or by liberation of the amidase as a separate catalytic fragment [6]. The net K_i^* of 250 nM for the aldehyde **2** when the synthetase active site is occupied by **1a** is only threefold higher than the K_i^* of 80 nM of aldehyde **2** for the separate Gsp amidase fragment. Thus, both by analysis of catalytic flux (amidase k_{cat}) or by inhibition potency and K_i/K_i^* equilibria for the aldehyde **2**, there is parallel evidence for negative regulation of the upstream Gsp amidase by the downstream Gsp synthetase domain when the bifunctional enzyme is at rest. Furthermore, when the Gsp synthetase domain is in mid-catalytic cycle, its architecture must reversibly alter not only to convert glutathione, ATP and spermidine substrates to produce Gsp, ADP and P_i, but also to transiently release the autoinhibition of the hydrolytic Gsp amidase domain. It remains to be seen whether the k_{cat} effects and K_i/K_i^* changes monitor a common set of microscopic steps in amidase catalytic efficiency, for example, by lowering the pK_a of Cys 59 to increase its nucleophilicity in order to facilitate acyl-S-enzyme formation (catalysis) or by stabilizing thiohemiacetal formation/accumulation and, thereby, affect K_i^*.

At this juncture time-dependent, slow binding inhibitors, phosphinates **1a–c** and aldehyde **2** have been developed that selectively block either the Gsp synthetase (**1a–c**) domain or the Gsp amidase (**2**) domain of the bifunctional *E. coli* Gsp synthetase/amidase enzyme. Each takes advantage of mechanistic pathway analysis to have each active site catalyze isomerization to the tightened complex.

Significance

The *Escherichia coli* glutathionylspermidine (Gsp) synthetase/amidase is an intriguing catalyst for at least two reasons. First, the physiological function of Gsp formation in *E. coli* from the antioxidant thiol glutathione and the polyamine spermidine is not yet understood. Although Gsp is a precursor to bis(glutathionyl)-spermidine (trypanothione) in trypanosomatid parasites, trypanothione has not been detected in *E. coli*. Second, the juxtaposition of an ATP-dependent amide synthetase and an opposing amidase in a bifunctional protein is unusual and requires regulation at the enzyme level to avoid futile glutathione- and spermidine-dependent ATPase action. The demonstration that γ-Glu–Ala–Gly aldehyde is a potent time-dependent specific inhibitor of the amidase activity supports an *S*-acyl-enzyme mechanism for amidase catalysis. Furthermore, the 100–250-fold modulation of amidase K_i^* down to a 80–250 nM range either by occupancy of the synthetase domain, by its own specific slow-binding inhibitor, or by removal of

the synthetase domain entirely (amidase fragment), corroborate interdomain communication and negative regulation of the amidase by the synthetase domain at rest. With the γ-Glu–Ala–Gly aldehyde for the amidase and the three Gsp phosphinate analogs (Ala, Ser, Cys) for the synthetase, potent mechanism-based slow-binding inhibitors specific for each active site of the bifunctional *E. coli* Gsp synthetase/amidase are available for dissection of mechanism and physiological role.

Materials and methods

Synthesis of phosphinate and aldehyde inhibitors and synthetase substrates

Experimental procedures for the synthesis of **1b**, **1c** and **2** may be found in the Supplementary material. Synthesis of the tripeptides γ-Glu–Ala–Gly [6] and γ-Glu–Ser–Gly for use as alternative synthetase substrates was carried out using conventional solution-phase techniques. Experimental details for synthesis of the Ser-containing peptide are available from the authors (S.C. & J.K.C.).

Preparation of Gsp amidase fragment and full-length Gsp synthetase/amidase

Proteins were overproduced in *E. coli* and purified according to [3,6].

Gsp amidase inhibition assay by γ-Glu–Ala–Gly·CHO

The γ-Glu–Ala–Gly-*p*-nitroanilide was used as substrate for inhibition of the Gsp amidase activity [8]. Assay mixtures of 390 µl contained 100 mM NaPIPES (pH 6.8) and 11.4 mM γ-Glu–Ala–Gly-*p*-nitroanilide (24 mM used for the full-length Gsp synthetase/amidase). Gsp amidase fragment (10 µl of 256 nM) (1.2 µM of the full-length protein) were added to initiate inhibition assays. The absorption at 405 nm ($\varepsilon = 9940\ cm^{-1}\cdot M^{-1}$) and 37°C was monitored to measure the release of *p*-nitroaniline.

The resulting data were analyzed and determined to follow a slow-binding inhibition pattern with an isomerization from the initial enzyme–inhibitor complex E·I to the final tight-binding complex E·I* (equation 1). The kinetics can be described on the basis of an integrated equation (equation 4), in which V_o and V_s are the initial and the steady-state rates, k is the apparent rate constant for establishing the steady-state equilibrium, and P is amount of product accumulated during a period of time t.

$$p = V_s \cdot t + (V_o - V_s)\frac{(1-e^{-kt})}{k} \qquad (4)$$

V_o and V_s are defined as in equations 5 and 6: where K_i and K_i^* are the initial and final inhibition constants, respectively.

$$V_o = \frac{V_{max}\cdot[S]}{K_m(1+\frac{[I]}{K_i})+[S]} \qquad (5)$$

$$V_s = \frac{V_{max}\cdot[S]}{K_m(1+\frac{[I]}{K_i^*})+[S]} \qquad (6)$$

*Gsp synthetase inhibition assay by phosphinates **1a–c***

Inhibition of the Gsp synthetase activity was observed spectrophotometrically by coupling the hydrolysis of ATP to oxidation of NADH via pyruvate kinase/lactate dehydrogenase reactions. The assay was initiated by adding purified Gsp synthetase/amidase (12.8 nM) to an assay mixture which contained the following components (final concentration): 1.56 mM glutathione, 10 mM spermidine, 2 mM ATP, 2.7 mM $MgCl_2$, 1 mM phospho(enol)pyruvate, 0.2 mM NADH, 50 µg ml^{-1} lactate dehydrogenase, 100 µg ml^{-1} pyruvate kinase, and various concentrations of inhibitor all in 50 mM NaPIPES (pH 6.8) at 37°C. Slow binding inhibition with E·I⇌E·I* was also observed for the synthetase inhibition, and equations 3–5 were also used for analysis to calculate K_i and K_i^*.

Dissociation of E·I complex*

The E·I* complex of Gsp amidase fragment and aldehyde **2** was prepared by incubation of 120 nM Gsp amidase fragment with 200 µM γ-Glu–Ala–Gly·CHO at room temperature for 20 min. A sample (3 µl) of the incubation mixture was withdrawn and diluted 100-fold in 300 µl of 100 mM Na-PIPES (pH 6.8) containing 36 mM γ-Glu–Ala–Gly-pNA as the assay substrate. Recovery of Gsp amidase activity was assayed at 37°C by monitoring the release of *p*-nitroanilide at 405 nm (light path = 2 mm). The resulting data were transferred into KaleidaGraph and fitted by equation 7 (p: product formation; V_s: steady state rate) to determine the dissociation constant k_4 (see equation 1 for definition).

$$p = V_s\left(t - \frac{1}{k4}\left(1-e^{-k4t}\right)\right) \qquad (7)$$

The E·I* complex of Gsp synthetase and the phosphinate (**1a**, **1b** or **1c**) was prepared by incubation of the full-length Gsp synthetase/amidase enzyme (100 nM) with 2 µM phosphinate inhibitor, 2 mM ATP and 2 mM $MgCl_2$ at room temperature. After 30 min, 5 µl of the incubation mixture was withdrawn and diluted 100-fold in 500 µl of 100 mM Na-PIPES (pH 6.8) containing 8 mM glutathione, 10 mM spermidine, 2 mM ATP, 2.7 mM $MgCl_2$, 1 mM phospho(enol)pyruvate, 0.2 mM NADH, 50 µg ml^{-1} lactate dehydrogenase and 100 µg ml^{-1} pyruvate kinase. Recovery of Gsp synthetase activity was assayed at 37°C. Equation 7 was used to determine the dissociation constant and the half-life of E·I* complex.

Supplementary material

Experimental details for the synthesis of compounds **1b**, **1c** and **2** are published with this paper on the internet.

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