Characterization of recombinant glutathionylspermidine synthetase/amidase from *Crithidia fasciculata*

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Trypanothione [N^1,N^8-bis(glutathionyl)spermidine] is a unique metabolite found only in trypanosomatids, where it subsumes many of the functions of GSH in other organisms. In *Crithidia fasciculata*, two distinct ATP-dependent ligases, glutathionylspermidine synthetase (GspS; EC 6.3.1.8) and trypanothione synthetase (TryS; EC 6.3.1.9), are involved in the synthesis of trypanothione from GSH and spermidine. Both enzymes have been cloned previously, but expression in *Escherichia coli* produced insoluble and inactive protein. Here we report on the successful expression of soluble $(His)_6$-tagged *C. fasciculata* GspS in *E. coli*. Following purification using nickel-chelating affinity chromatography, the tag sequence was removed and the enzyme purified to homogeneity by anion-exchange chromatography. The kinetic parameters of the recombinant enzyme have been determined using a coupled enzyme assay and also by HPLC analysis of end-product formation. Under optimal conditions (0.1 M K^+-Hepes, pH 7.3) GspS has synthetase activity with apparent K_m values for GSH, spermidine and MgATP of 242, 59 and 114 μM respectively, and a k_{cat} of 15.5 s^{-1}. Glutathionylspermidine is formed as end product and the enzyme lacks TryS activity. Like *E. coli* GspS, the recombinant enzyme also possesses amidase activity (EC 3.5.1.78), hydrolysing glutathionylspermidine to GSH and spermidine with a k_{cat} of 0.38 s^{-1} and a K_m of 500 μM. GspS can also hydrolyse trypanothione at about 1.5 % of the rate with glutathionylspermidine. A single amino acid mutation (Cys-79 → Ala) is shown to ablate the amidase activity without affecting the synthetase activity.

Key words: glutathione, Kinetoplastida, ligase, spermidine, trypanothione biosynthesis.

INTRODUCTION

Diseases caused by trypanosomes and *Leishmania* parasites continue to pose a major threat to human health and economic development worldwide [1]. Despite the need for better and safer drugs for these parasitic diseases, only a handful of new drugs has reached the market over the last 25 years [2]. These include difluoromethylornithine (Eflornithine) for human African trypanosomiasis, benznidazole and nifurtimox for the acute stage of Chagas' disease and lipid formulations of amphotericin B for visceral leishmaniasis. Each of these drugs is far from ideal due to several of the following factors: high cost, availability, route of administration, low efficacy and high toxicity. One common and unique biochemical feature of the metabolism of these trypanosomatids that has attracted considerable attention as a potential drug target is trypanothione [N^1,N^8-bis(glutathionyl) spermidine] [3]. Unlike mammalian cells, these organisms lack the GSH/glutathione reductase antioxidant system [4] and instead rely on an analogous system based on trypanothione and trypanothione reductase (TryR; see reviews [5–11]). Reverse genetic studies have validated TryR as a drug target for *Leishmania* spp. [12–14] and *Trypanosoma brucei* [15]. Due to the reciprocally exclusive specificities of TryR for trypanothione disulphide and glutathione reductase for glutathione disulphide, selective inhibition at the disulphide-binding site is feasible [7–10].

To date, the trypanothione-biosynthetic enzymes have received much less attention than TryR, due to difficulties in obtaining sufficient amounts of material in native or recombinant form for detailed molecular studies. In the non-pathogenic insect parasite, *Crithidia fasciculata*, two enzymes are involved in the stepwise biosynthesis of trypanothione from GSH and spermidine (Scheme 1), namely glutathionylspermidine synthetase (GspS; EC 6.3.1.8), and trypanothione synthetase (TryS; EC 6.3.1.9) [16]. Both genes have been cloned and sequenced, but expression in *Escherichia coli* or *Saccharomyces cerevisiae* failed to yield sufficient quantities of active enzyme for detailed study [17]. Thiol analysis of *S. cerevisiae* expressing GspS or TryS from *C. fasciculata*, alone or in combination, confirmed that the correct genes had been isolated. However, the functional assignment of these genes was in conflict with peptide sequence data reported by others [18]. *E. coli* can also synthesize glutathionylspermidine (but not trypanothione [19]), particularly in the stationary phase of growth under anaerobic and acidic conditions [20]. Similarly, *C. fasciculata* accumulates glutathionylspermidine in the stationary phase [21] and one possible function in both of these organisms is to store or regulate polyamine levels, particularly since glutathionylspermidine is broken down rapidly once growth is restored. Significantly, *E. coli* GspS is a bifunctional enzyme with an N-terminal amidase domain (EC 3.5.1.78), responsible for degradation of glutathionylspermidine, and a C-terminal domain, responsible for ATP-dependent synthesis [22,23]. Likewise, GspS from *C. fasciculata* has an N-terminal domain with striking homology to *E. coli* GspS and demonstrable glutathionylspermidine amidase activity [17], although this has been disputed [18]. TryS has a similar N-terminal amidase domain, although this has not been demonstrated to hydrolyse trypanothione or glutathionylspermidine (Scheme 1).

In the present study we report on the successful soluble expression of active GspS from *C. fasciculata*, and examine the physical and kinetic properties of the enzyme. Site-directed mutagenesis has shown that a single cysteine residue is essential for amidase activity in *E. coli* GspS [24]. Accordingly, we have generated the corresponding mutant in GspS and examined amidase activity with glutathionylspermidine and trypanothione as substrates.

Abbreviations used: GspS, glutathionylspermidine synthetase; TryS, trypanothione synthetase; TryR, trypanothione reductase.

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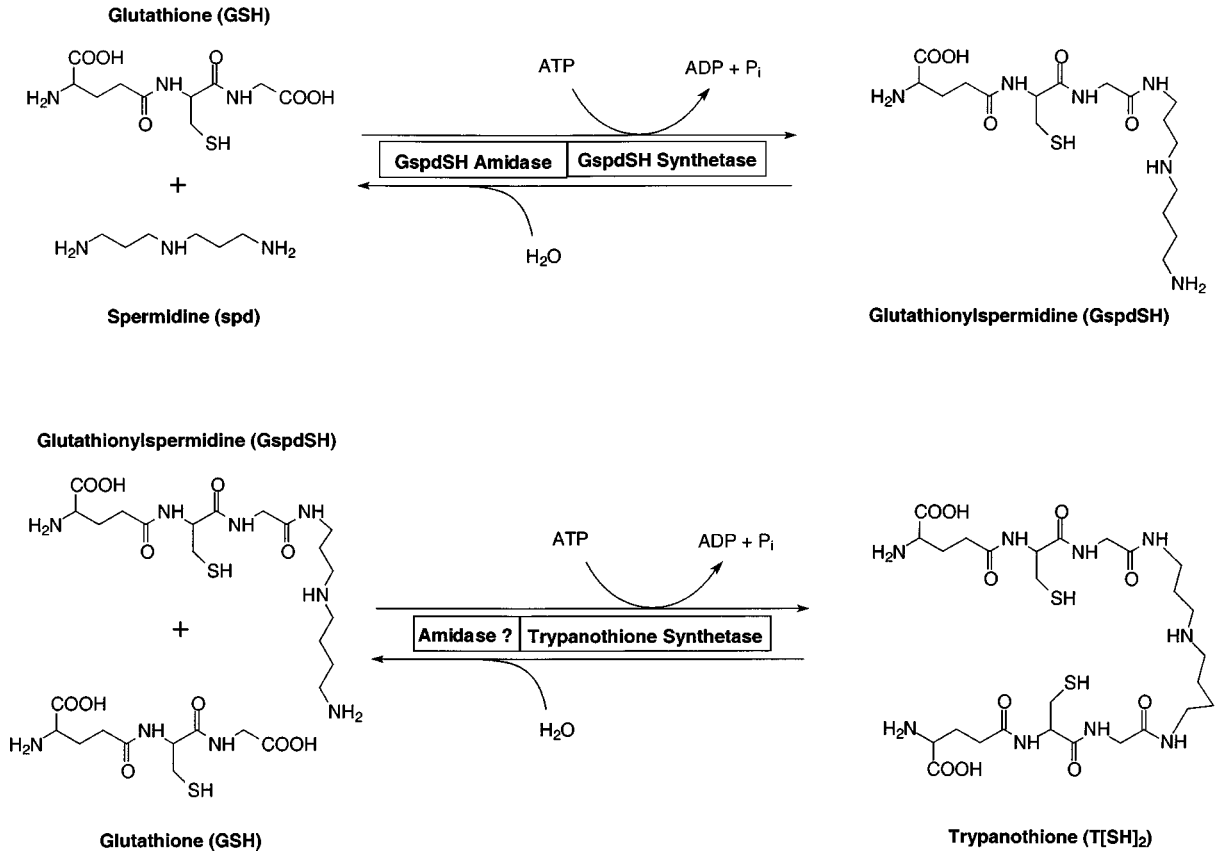

Scheme 1 Enzymic steps of trypanothione biosynthesis and degradation in *C. fasciculata*

EXPERIMENTAL

Organisms and reagents

Routine manipulations were performed in *E. coli* strain JM109 and overexpression in strain BL21(DE3)pLysS. All chemicals were of the highest grade available from Sigma, BDH and Bachem. Restriction enzymes and DNA-modifying enzymes were from Promega or Roche.

Subcloning of GspS and generation of Cys-79→Ala mutant

Isolation of the *GspS* gene (accession number U66520) has been described previously [17]. During the present study the gene was resequenced and some sequencing errors were discovered between nucleotides 922 and 986. This resulted in a double frame shift due to a deletion of nucleotide G^{962} and insertion of another guanine after G^{986}. The corrected open reading frame still encodes a 719-amino acid protein, but the predicted molecular mass is 80321Da rather than the 80336 Da reported in the original publication [17]. The sequence in the original accession number (U66520.2) has been updated.

GspS was excised from pET16b-GspS by digestion with *Bam*HI/*Nde*I and subcloned into the *Bam*HI/*Nde*I site of pET15b creating plasmid pET15b-GspS. Using plasmid pET15b-GspS as a template, the Cys-79 → Ala mutant was obtained using the 'megaprimer' method [25] as described in [25a]. The primers used in the first reaction were 5′-GCGCACAAACTCG-ACGGCTTGCCACTTCACACC-3′ (named CfG_C79A*, with the underlined portion containing the point mutation) and 5′-CGCCGCCCACATATGTCGTCGCTG-3′ (named pET5′E). This generated a megaprimer of 263 bp that was used in the subsequent reaction with the addition of primer pET3′E, 5′-TCCGCTGCAGTTATGTTTCCCCCTC-3′, to generate the full-length sequence. The 2.18 kb fragment was cloned via pCR-Blunt II-TOPO (Invitrogen), into the *Nde*I/*Bam*HI sites of pET15b to generate plasmid pET15b-GspS*. Sequencing revealed that the desired Cys-79→Ala mutation had been introduced, but that a frame shift had occurred during PCR at the 5′ start site, requiring that an additional PCR be carried out. Using plasmid pET15b-GspS* as a template, the primers used were 5′-GGAATTCGCCCTTCGCCGCCCACATATGTCGTCGC-3′ (pET5′-GspS) and pET3′E. The PCR was performed in a volume of 50 μl containing 0.4 mM of each dNTP, 100 ng of each primer, 50 ng of pET15b-GspS* DNA and 3 units of *Pfu* DNA polymerase with buffer (Promega). The reaction mixture was then subjected to the following for 30 cycles: denaturation for 30 s at 93 °C, annealing for 60 s at 30 °C and elongation for 4 min at 72 °C. A final 10 min extension at 72 °C was included. The resulting 2.18 kb fragment was cloned via pCR-Blunt II-TOPO into pET15b to generate plasmid pET15b-C79A.

Soluble expression of GspS

A 4 litre culture of BL21(DE3)pLysS/pET15b-GspS was grown at 37 °C with vigorous agitation in Terrific Broth containing 50 $\mu g \cdot ml^{-1}$ carbenicillin and 12.5 $\mu g \cdot ml^{-1}$ chloramphenicol. When the culture reached a D_{600} of ≈ 1.4, the culture was cooled to 25 °C and agitation reduced to 100 rev./min. Isopropyl β-D-thiogalactoside was added to a final concentration of

0.5 mM. Cultures were grown for an additional 16 h and then harvested by centrifugation. Cells were washed with 500 ml of 20 mM Tris (pH 8.0) and lysed in 50 ml of breaking buffer either by flash freezing in an ethanol/dry-ice bath followed by rapid thawing or by French press. Breaking buffer comprises 20 mM Tris/HCl, pH 8.0, 0.5 M NaCl, 5 mM $MgCl_2$, 100 $\mu g \cdot ml^{-1}$ DNAse I and protease-inhibitor cocktail (Roche). Cell debris was separated and discarded after centrifugation at 30000 ***g*** for 30 min at 4 °C.

Purification and properties of GspS

The supernatant containing soluble protein was diluted 2-fold with 20 mM Bis-Tris propane {1,3-bis[tris(hydroxylmethyl) methylamino]propane}, 20 mM Tris (pH 7.4) and 0.5 M NaCl, passed through a 0.2 μm Steriflip filter and loaded on to a nickel-chelating Sepharose high-performance column (Pharmacia) pre-equilibrated with the same buffer. Protein was eluted using a linear gradient from 0 to 1 M imidazole, active fractions were pooled, dialysed against PBS (10 mM phosphate buffer/2.7 mM KCl/137 mM NaCl, pH 7.4) and digested with human thrombin (50 $\mu g \cdot ml^{-1}$; 2 h at 25 °C) to remove the $(His)_6$ tag. After overnight dialysis at 4 °C against 20 mM Bis-Tris propane/20 mM Tris (pH 7.4)/1 mM EDTA/1 mM dithiothreitol, the sample was loaded on to a 6 ml ResourceQ column (Pharmacia). Protein was eluted with a linear gradient from 0 to 0.5 M NaCl and active fractions pooled, concentrated and dialysed against 20 mM Tris, pH 8.0, containing 1 mM dithiothreitol, 1 mM EDTA and 0.01 % sodium azide. Aliquots of 100 μl were dispensed, frozen rapidly and stored at −80 °C. Under these conditions, the enzyme loses less than 10 % of its activity over four months.

Native molecular mass was determined by Superdex 200 column chromatography (Pharmacia) against gel-filtration standards (Bio-Rad). Molecular mass was determined by matrix-assisted laser-desorption ionization–time-of-flight MS in linear mode using sinapinic acid as a matrix on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems). N-terminal sequence was determined by pulsed-liquid Edman sequencing on a Procise 494 protein sequencer (Applied Biosystems) after SDS/PAGE and transfer on to a PVDF membrane.

Kinetic analysis of GspS synthetase activity

Kinetic analysis (K_m and k_{cat} values) and pH optimization studies of synthetase activity were performed using a continuous spectrophotometric assay at 340 nm and 25 °C, in which ATP hydrolysis is coupled through pyruvate kinase and lactate dehydrogenase to oxidation of NADH. Each 1 ml assay contained 100 mM K^+-Hepes, pH 7.3, 0.2 mM NADH, 1 mM phosphoenolpyruvate, 5 mM dithiothreitol, 0.5 mM EDTA, 10 mM $MgSO_4$, 2 units $\cdot$ ml^{-1} L-lactate dehydrogenase, 2 units $\cdot$ ml^{-1} pyruvate kinase, 40 nM GspS (3.2 $\mu g \cdot ml^{-1}$) and various amounts of ATP, GSH and spermidine. One unit of activity was defined as the amount of enzyme required to oxidize 1 μmol of NADH to NAD^+ in 1 min. The apparent K_m value for each substrate was determined under saturating concentrations of the other substrates involved (2 mM ATP, 10 mM GSH or 10 mM spermidine).

Data were fitted using non-linear regression with the program GraFit. The pH optimum of the enzyme was determined in a mixed buffer system containing 16.7 mM each of Mes, Ches [2-(*N*-cyclohexylamino)ethanesulphonic acid] and Hepps (adjusted to a range of pH values using KOH). In addition, 20 units $\cdot$ ml^{-1} of each coupling enzyme (in 50 % glycerol stock; Roche) were included to ensure that they were not rate limiting at the extremes of pH tested. The pH of the mixture was determined at the end of each assay. The effect of ionic strength was determined as above, in 50 mM Hepes buffer, pH 7.3, plus 0–500 mM KCl or $(NH_4)_2SO_4$.

Demonstration of GspS amidase activity and kinetics

Amidase activity was assayed in 100 mM K^+-Hepes, pH 7.3, in a final volume of 100 μl in the presence of 0.2 mM glutathionylspermidine disulphide or 0.4 mM trypanothione disulphide (pre-reduced for 5 min with 0.05 units of recombinant *T. cruzi* TryR [26] and 0.5 mM NADPH) before the addition of GspS. After incubation for 1 h at 37 °C, aliquots of 10 μl were taken and added to 40 μl of 40 mM Li^+-Hepps/4 mM diethylenetriamine-penta-acetic acid, pH 8.0, derivatized with 2 mM monobromobimane and analysed by HPLC [21]. For the time course, disulphides were reduced by incubation with 1 mM TCEP [Tris(2-carboxyethyl)phosphine] rather than TryR/NADPH, prior to addition of enzyme.

Kinetic analysis of the GspS amidase function was performed with the same assay and derivatization system but with either 15 mM TCEP (for assays with 0.5–2.5 mM glutathionylspermidine disulphide) or 1.5 mM TCEP (for assays with < 0.5 mM glutathionylspermidine disulphide) and 44.4 $\mu g \cdot ml^{-1}$ GspS enzyme. Glutathionylspermidine disulphide was added (0.016–2.5 mM) to start the reaction. A higher stock concentration of monobromobimane (10 mM) was used to ensure complete derivatization of all thiol groups.

CD of wild-type and mutant GspS

Wild-type and mutant GspS were dialysed into 20 mM sodium phosphate buffer, pH 7.0. Protein concentrations were determined by theoretical extinction coefficient and UV absorbance. The far-UV CD spectra of both wild-type and mutant were analysed at 0.4 mg $\cdot$ ml^{-1} in a 0.02 cm pathlength quartz cell at 20 °C using a JASCO J-600 spectropolarimeter (Jasco UK) by Dr S. M. Kelly at the University of Glasgow (Glasgow, Scotland, U.K.).

RESULTS AND DISCUSSION

Overexpression and purification of GspS from *E. coli*

Using the conditions described previously, we confirmed that induction and expression of *GspS* in pET16b in *E. coli* BL21 (DE3) produced large amounts of insoluble and inactive protein [17]. After subcloning *GspS* into the *Nde*I/*Bam*HI sites of pET-15b, expression in *E. coli* BL21(DE3)pLysS yielded enzyme activity in the soluble fraction. The most important condition for optimal expression over the previous method was growth to a higher density in rich medium at 37 °C followed by cooling to 25 °C and induction for 16 h. Purification of GspS to apparent homogeneity by SDS/PAGE (Figure 1) was accomplished with two chromatographic steps (Ni^{2+} affinity and anion exchange) and thrombin cleavage to remove the $(His)_6$ tag. This current method far surpasses the existing time-consuming methods for the isolation of the native protein from *C. fasciculata* [16,18], with typical yields of 5–10 mg/l of induced culture. The Cys-79 → Ala mutant was purified in exactly the same manner as the wild-type enzyme with similar yields.

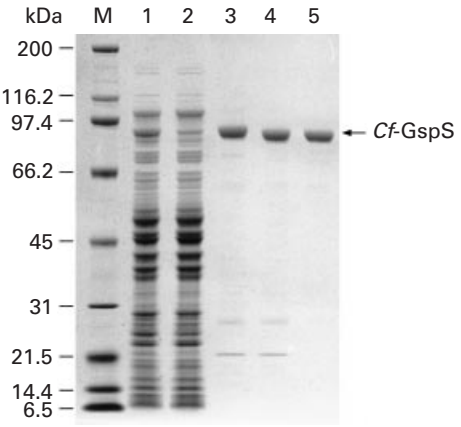

Figure 1 SDS/PAGE analysis of the purification profile of recombinant GspS in *E. coli*

Lane 1, soluble fraction of BL21(DE3)pLysS/pET15b-GspS; lane 2, flow-through from nickel-chelating Sepharose high-performance column; lane 3, pooled fractions after imidazole elution; lane 4, after removal of the $(His)_6$-tag with thrombin protease; lane 5, pooled fractions from chromatography on Resource-Q. Approx. 2 μg of protein was loaded into each lane. *Cf*-GspS indicates recombinant GspS. M, molecular-mass standards.

Table 1 Physical and kinetic parameters of GspS/amidase

Physico-chemical properties were determined on recombinant $(His)_6$-tagged protein after cleavage with thrombin. The kinetic parameters for the synthetase and amidase were measured in 100 mM K^+-Hepes, pH 7.3. Values in parentheses were measured in 50 mM Bis-Tris propane/50 mM Tris buffer, pH 7.5, with recombinant $(His)_6$-tagged protein. Apparent K_m values were not significantly different from those of the cleaved enzyme when assayed in 100 mM K^+-Hepes, pH 7.3. Amidase kinetics were determined using the HPLC method on recombinant $(His)_6$-tagged protein. MALDI-TOF MS, matrix-assisted laser-desorption ionization–time-of-flight MS; IEF, isoelectric focusing; GspdSH, glutathionylspermidine.

Substrate	Units	Recombinant GspS
Molecular mass		
Predicted	Da	80602
MALDI-TOF MS	Da	80524
SDS/PAGE	–	86000
Gel filtration	–	85200
pH optimum	–	7.31 ± 0.09
Isoelectric point		
Predicted	–	5.1
Determined by IEF	–	4.6
Synthetase		
K_m for GSH	μM	242 ± 14 (694 ± 74)
K_m for spermidine	μM	59 ± 12 (142 ± 14)
K_m for MgATP	μM	114 ± 8 (230 ± 28)
k_{cat}	s^{-1}	15.5 ± 0.5 (10.2 ± 0.5)
Amidase		
K_m for GspdSH	μM	500 ± 175
k_{cat}	s^{-1}	0.38 ± 0.02

Enzyme characterization

N-terminal analysis indicated the amino acid sequence GSHM SSLPHN (the initiating methionine in the native protein is underlined) as predicted for recombinant GspS expressed in pET15b after cleavage at the thrombin site. Tryptic digestion and peptide mass fingerprinting also identified the expressed protein to be GspS from *C. fasciculata* by BLAST search. Matrix-assisted laser-desorption ionization–time-of-flight MS analysis of the recombinant GspS revealed a nominal molecular mass of 80524 Da that correlates well with the predicted nominal molecular mass (80602 Da) after thrombin cleavage (Table 1). Migration on SDS/PAGE showed a high anomalous apparent molecular mass of ≈ 86000, as reported previously [16,17]. By gel filtration, the recombinant enzyme behaves as a monomer and we did not detect any dimer formation, in contrast to [18].

Kinetic characterization of GspS synthetase activity

Using the coupled assay system, the specific activity of GspS was determined to be 7.9 units $\cdot$ mg^{-1}, which is comparable with the published values for the native enzyme of 8.4 and 6.7 units $\cdot$ mg^{-1} [16,27]. HPLC analysis showed that trypanothione was not formed when GspS was incubated with MgATP and either spermidine plus GSH or glutathionylspermidine plus GSH (results not shown), confirming that GspS only catalyses the first step in the biosynthesis of trypanothione (Scheme 1). The pH profile was determined in a mixed buffer system as described in the Experimental section. Addition of 2 mM MgADP to control incubation mixtures confirmed that the coupling enzymes were not rate limiting. The enzyme is active over a broad pH range from 6.8 to 8.0 (Figure 2A), with a pH optimum of 7.3 and apparent pK_a values of 5.9 ± 0.1 and 8.7 ± 0.1. Activity at pH 7.3 is dependent on buffer concentration, with an optimum in the range 0.05–0.1 M K^+-Hepes buffer (Figure 2B, inset). Higher concentrations of Hepes are inhibitory, as are KCl or $(NH_4)_2SO_4$ (Figure 2B, inset). When plotted as a function of total ionic strength of the medium, the data for Hepes, KCl and $(NH_4)_2SO_4$ fit a single curve with optimal activity in the range 0.02–0.07 mol $\cdot$ l^{-1} (Figure 2B). Accordingly, 0.1 M K^+-Hepes, pH 7.3 ($I = 0.04$ mol $\cdot$ l^{-1}) was used for subsequent kinetic studies. Under conditions where the other two substrates were saturating, apparent K_m values for GSH, spermidine and MgATP were determined to be 242, 59 and 114 μM respectively, with a mean k_{cat} of 15.5 ± 0.5 s^{-1} (Table 1). Similar K_m values were obtained with the $(His)_6$-tagged enzyme (287 ± 17, 77 ± 13 and 160 ± 16 respectively). As these apparent K_m values for GSH and spermidine were respectively 10- and 8-fold lower than those reported previously by Koenig et al. [18], the kinetic analyses were repeated under these authors' conditions (50 mM Bis-Tris propane/50 mM Tris, pH 7.5). Although the apparent K_m values increased by $\approx$ 2-fold, significant discrepancies remain for GSH and spermidine (Table 1). The reason for this is not clear, but could be due to other differences in assay conditions [e.g. ionic-strength effects due to $(NH_4)_2SO_4$ in the coupling enzymes], assay method (HPLC versus spectrophotometric) or purity of enzyme preparation. Native GspS and TryS co-purify as a complex [16] and thus TryS might also modify the kinetic behaviour of GspS. It is worth noting that the studies by Koenig et al. [18] employed partially purified enzyme and, as discussed previously [17], it is likely that this enzyme preparation contained functionally inactive TryS, since the peptide sequence data reported by them for GspS correspond exactly with our TryS sequence and not GspS. Expression of soluble recombinant TryS and reconstitution with GspS could test this possibility. Clearly the fact that our recombinant protein shows GspS activity and no TryS activity confirms our initial findings [17] and raises doubts about earlier reports.

Demonstration of GspS amidase activity

GspS from *E. coli* can hydrolyse glutathionylspermidine to form GSH plus spermidine due to an amidase activity in the

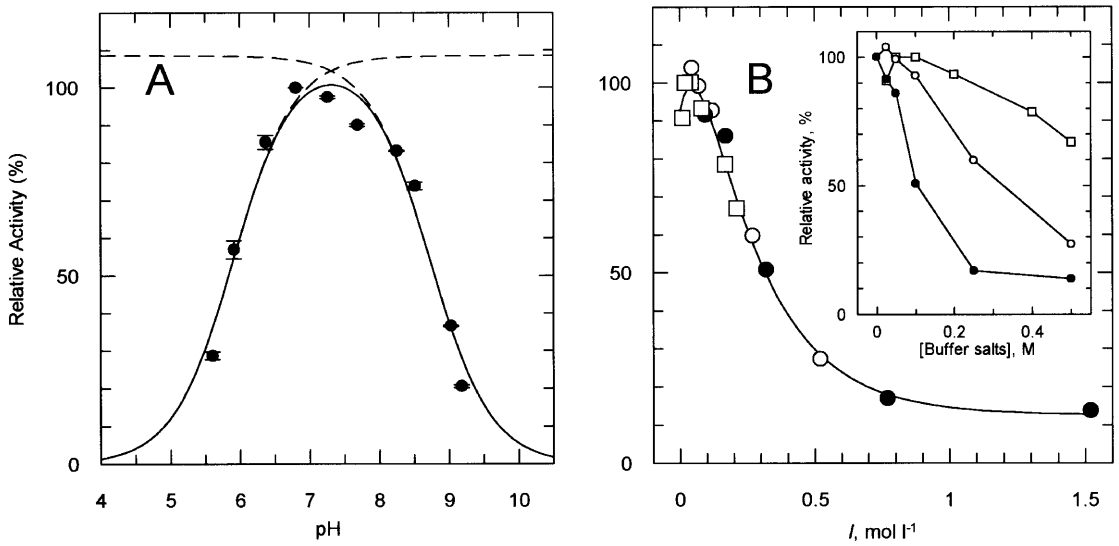

Figure 2 Effect of pH and ionic strength on GspS synthetase activity

(**A**) pH-dependence. Coupled assays were carried out at the indicated pH as described in the Experimental section. Activity is expressed relative to the maximum activity obtained. Bars indicate S.D. from three measurements. (**B**) Effect of buffer concentration and ionic strength. The assay mixtures contained either 50 mM K^+-Hepes, pH 7.3, plus salt [○, KCl; ●, $(NH_4)_2SO_4$] or various amounts of Hepes buffer (□) and saturating substrates, as described in the Experimental section. Activity is expressed as a percentage of the activity determined in 50 mM Hepes buffer. The inset shows the effect on activity as a function of buffer or salt concentration.

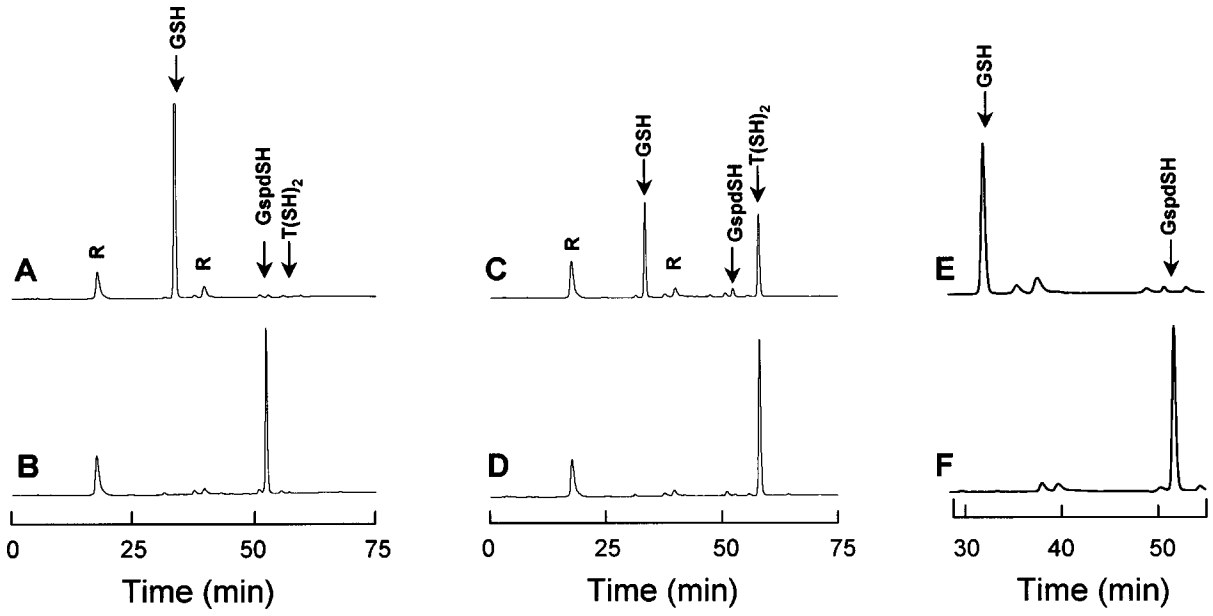

Figure 3 Demonstration of the amidase function of GspS by HPLC

Aliquots were removed from the assay mixture, derivatized with monobromobimane and analysed for thiol content by HPLC as described in the Experimental section. Native enzyme (**A**, **C**) or heat-inactivated enzyme (**B**, **D**) was incubated for 1 h at 37 °C in the presence of either glutathionylspermidine (GspdSH; **A**, **B**) or trypanothione [$T(SH)_2$; **C**, **D**]. Glutathionylspermidine was incubated with 0.125 mg · ml^{-1} of either wild-type (**E**) or Cys-79 → Ala mutant (**F**) GspS in a total volume of 0.1 ml. Elution times of authentic GSH, glutathionylspermidine and trypanothione are indicated by arrows; R indicates peaks derived from monobromobimane reagent.

C-terminal domain. Sequence alignment of the GspS with *E. coli* GspS showed 21.4 % identity in 246 amino acids in this region. However, Koenig et al. [18] were unable to detect GspS amidase activity in native GspS from *C. fasciculata*, in contrast to Tetaud et al. [17]. We therefore examined recombinant GspS for amidase activity by incubation with either glutathionylspermidine or trypanothione, in the absence of other substrates, and subsequent analysis by HPLC (Figures 3A–3D). After 1 h of incubation with enzyme, glutathionylspermidine was hydrolysed completely to GSH (Figure 3A). Glutathionylspermidine was recovered

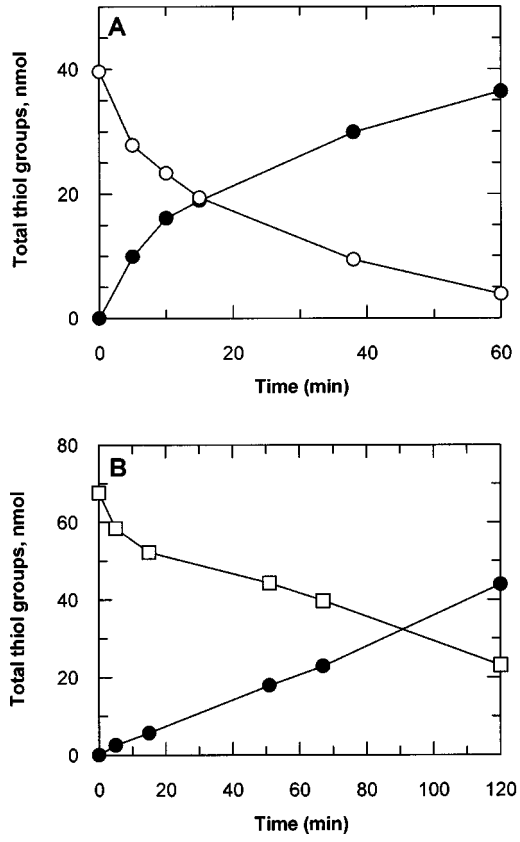

Figure 4 Time course of amidase activity of GspS

Aliquots (0.01 ml) were removed from the assay mixture (0.1 ml) at specified time points, derivatized with monobromobimane and analysed for thiol content by HPLC as described in the Experimental section. (**A**) Glutathionylspermidine incubated with 0.125 mg · ml^{-1} GspS (○, glutathionylspermidine remaining; ●, GSH formed). (**B**) Trypanothione incubated with 2.5 mg · ml^{-1} GspS (□, trypanothione remaining; ●, GSH formed). Thiols are expressed as total thiol groups and were calculated using known amounts of synthetic standards.

unchanged in the heat-inactivated sample, indicating that the reaction is enzyme dependent (Figure 3B). Under identical conditions, trypanothione was converted partially into GSH and glutathionylspermidine (96 and 4% of the end products, respectively; Figure 3C). No GSH or glutathionylspermidine peaks were detected in the heat-inactivated sample (Figure 3D). Thus GspS can hydrolyse trypanothione to GSH and spermidine via the intermediate glutathionylspermidine. However, glutathionylspermidine appears to be the preferred substrate of the amidase domain of the enzyme, since most of the intermediate formed is rapidly hydrolysed further to GSH and spermidine.

A time course of the amidase activity revealed stoichiometric conversion of either substrate to GSH and spermidine (Figure 4). Hydrolysis of trypanothione was much slower; more than 20-fold more enzyme and a longer time course were used in the experiment shown in Figure 4(B). Based on the initial linear rates of GSH formation from either substrate, glutathionylspermidine is hydrolysed about 60 times faster than trypanothione (130 ± 29 and 2.0 ± 0.3 nmol of GSH formed · min^{-1} · mg^{-1}, respectively). In this experiment, glutathionylspermidine was below the limits of detection with trypanothione as a substrate, which is consistent with the marked preference of the amidase component of the enzyme for glutathionylspermidine over trypanothione.

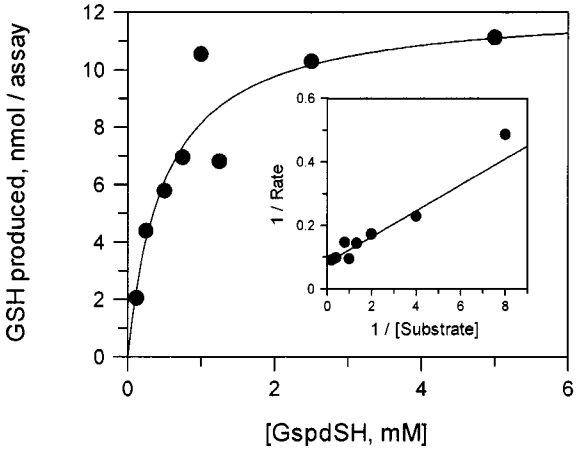

Figure 5 Kinetics of glutathionylspermidine hydrolysis by GspS amidase

Formation of GSH was determined by HPLC as described in the Experimental section. The activity is expressed as the amount of GSH formed in 10 min in each 0.1 ml assay. GspdSH, glutathionylspermidine.

Kinetic characterization of GspS amidase activity

Conditions were established so that GSH formation from glutathionylspermidine was linear for up to 20 min. Under these conditions, similar rates of hydrolysis of glutathionylspermidine were observed with either $(His)_6$-tagged or fully purified recombinant enzymes [92.2 ± 7.1 and 68.3 ± 12.1 nmol of GSH formed · min^{-1} · (mg of protein)$^{-1}$ respectively]. For convenience, subsequent kinetic characterization was carried out with the $(His)_6$-tagged protein. At pH 7.3, the amidase function of GspS obeys simple Michaelis–Menten kinetics (Figure 5 and Table 1). Within the limits of experimental error, the K_m for glutathionylspermidine is comparable with the value obtained for *E. coli* amidase at pH 7.5 (500 ± 175 versus 900 ± 200 μM, respectively), although turnover (k_{cat}) is 5-fold lower (0.38 versus 2.1 s^{-1} respectively; Table 1). The amidase activity of GspS is likely to play a physiological role in the cell, since glutathionylspermidine concentrations vary between 500 μM in logarithmic-phase cells and 2500 μM in stationary-phase cells [21,28]. Under physiological conditions the concentrations of GSH are 0.6 and 0.4 mM and free spermidine are 2.8 and 1.8 mM for logarithmic-and stationary-phase cells, respectively, so some futile cycling and hydrolysis of ATP would be expected to occur due to the opposing activities of the synthetase and amidase domains of GspS. In the absence of any regulation, the amidase would be predicted to be rate limiting, because the k_{cat} value for the synthetase is 40-fold higher than the amidase activity (Table 1). A kinetic study of trypanothione hydrolysis by GspS was not done due to the high cost of substrate.

Mutagenesis studies on amidase activity

Previous studies of the *E. coli* GspS [24] have highlighted the absolute requirement of a single specific cysteine residue (Cys-59) for amidase activity. To determine if the corresponding residue was also essential in GspS, Cys-79 was mutated to Ala by PCR site-directed mutagenesis [25]. The resulting Cys-79 → Ala mutant was found to retain synthetase activity with K_m values for

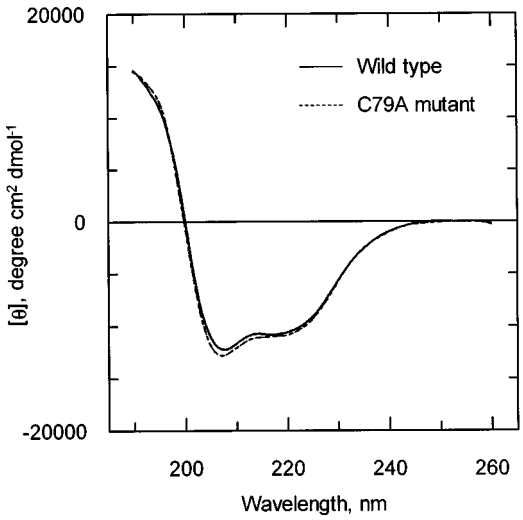

Figure 6 Far-UV CD spectra of wild-type and Cys-79 → Ala mutant proteins

Wild-type, solid line; Cys-79 → Ala mutant, dotted line. The secondary structure estimations using the SELCON procedure [30] are very similar (α-helix, 27.5% versus 28.3%; anti-parallel β-sheet, 17.1% versus 28.6%, and parallel β-sheet, 4.1% versus 3.8%, for wild-type and the Cys-79 → Ala mutant respectively).

GSH, spermidine and MgATP of 171 ± 22, 78 ± 8 and 96 ± 6 μM respectively, and a k_{cat} value of 12.5 ± 0.5 s^{-1}, which are similar to those for the wild-type enzyme (Table 1). Fluorescence spectra of both proteins were identical (results not shown). Furthermore, CD spectra of the wild-type and mutant are virtually superimposable (Figure 6), indicating that the Cys-79 → Ala mutation has not affected secondary structure. Despite the fact that the Cys-79 → Ala mutant appeared to be folded correctly and possess synthetase activity, the amidase activity was no longer evident when compared with the wild-type (Figures 3E and 3F). This suggests that Cys-79 may function as the catalytic nucleophile for hydrolysis of the amide bond between the glycine carboxylate of GSH and spermidine, as proposed for Cys-59 in the *E. coli* enzyme [24].

Conclusions

The data presented here confirm our previous contention [17] that *GspS* encodes a protein with GspS and glutathionylspermidine amidase activity, which contradicts some of the conclusions of Koenig et al. [18]. For reasons that are not clear, the kinetic properties of the ligase domain of the recombinant protein are broadly similar, but not identical, to earlier reports on the native enzyme [18,29]. The possibility that co-operative interactions between the native complex of GspS and TryS affect the kinetic behaviour merits further investigation. The kinetic properties of the amidase domain of GspS are similar to those of the *E. coli* enzyme [22,24] and a cysteine residue (Cys-79) is essential for catalysis. Thus GspS is responsible for both synthesis and degradation of glutathionylspermidine in *C. fasciculata*. How these opposing catalytic functions are regulated to avoid futile cycling with net hydrolysis of ATP is an interesting problem for further study. A fully detailed kinetic and structural analysis of this potentially important drug target is underway.

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