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Purification of glutathionylspermidine and trypanothione synthetases from *Crithidia fasciculata*

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Abstract

Two enzymes involved in the biosynthesis of the trypanosomatid-specific dithiol trypanothione–glutathionylspermidine (Gsp) synthetase and trypanothione (TSH) synthetase–have been identified and purified individually from *Crithidia fasciculata*. The Gsp synthetase has been purified 93-fold and the TSH synthetase 52-fold to apparent homogeneity from a single DEAE fraction that contained both activities. This constitutes the first indication that the enzymatic conversion of two glutathione molecules and one spermidine to the N^1, N^8-bis(glutathionyl)spermidine (TSH) occurs in two discrete enzymatic steps. Gsp synthetase, which has a k_{cat} of 600/min, shows no detectable TSH synthetase activity, whereas TSH synthetase does not make any detectable Gsp and has a k_{cat} of 75/min. The 90-kDa Gsp synthetase and 82-kDa TSH synthetase are separable on phenyl Superose and remain separated on gel filtration columns in high salt (0.8 M NaCl). Active complexes can be formed under low to moderate salt conditions (0.0–0.15 M NaCl), consistent with a functional complex in vivo.

Keywords: amide-forming synthetases; glutathione metabolism; glutathionylspermidine synthetase; polyamine metabolism; trypanothione synthetase

The polyamines (putrescine, spermidine, and spermine) and GSH are found in millimolar concentrations in most biological systems. They are thought to be involved in cell growth and differentiation and regulation of important enzymatic processes. GSH plays an important role in the management of oxidative stress and in the maintenance and the regulation of intracellular thiol/disulfide redox balance. Polyamines are required for optimal growth in many cell types, although their precise regulatory functions remain obscure (Tabor & Tabor, 1984). The majority of intracellular GSH of the trypanosomatid parasites, including the pathogens *Leishmania, Trypanosoma cruzi, Trypanosoma congolense*, and *Trypanosoma brucei*, is conjugated to spermidine (Shim & Fairlamb, 1988). In particular, the parasites contain a unique analogue of GSH, in which two GSH tripeptides are covalently linked by a spermidine molecule via their glycine carboxylates, to give $N^{1,8}$-bis(glutathionyl)spermidine (TSH) (Fig. 1). TSH is thought to be an essential redox intermediate in the reduction of intracellular sulfhydryl groups analogous to GSH in other systems. In the trypanosomatid cell, TSH is present almost totally in the dithiol-reduced form because of the action of the unique parasite enzyme, TSH reductase (Shames et al., 1986). TSH may also play an important role in reducing free hydroxyl radicals during oxidative stress in these parasites.

High levels of Gsp have been observed (Docampo, 1990) to accumulate under adverse growth conditions in the trypanosomatid *Crithidia fasciculata* and in the bacterium *Escherichia coli*. In the latter case, all of the intracellular spermidine and a large proportion of the intracellular GSH is converted to N^1-Gsp during stationary phase and anaerobic conditions by Gsp synthetase

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Abbreviations: Bis-Tris-propane, 1,3-bis((tris(hydroxymethyl)methyl)amino) propane; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; GSH, glutathione; Gsp, glutathionylspermidine; $(Gsp)_2$, oxidized glutathionylspermidine; N^1-Gsp, N^1-glutathionylspermidine; HPLC, high performance liquid chromatography; TSH, reduced trypanothione; $T(S)_2$, oxidized trypanothione; Abu, L-alpha-aminobutyryl; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; MW, molecular weight; PAGE, polyacrylamide gel electrophoresis; PK/LDH, pyruvate kinase/lactate dehydrogenase.

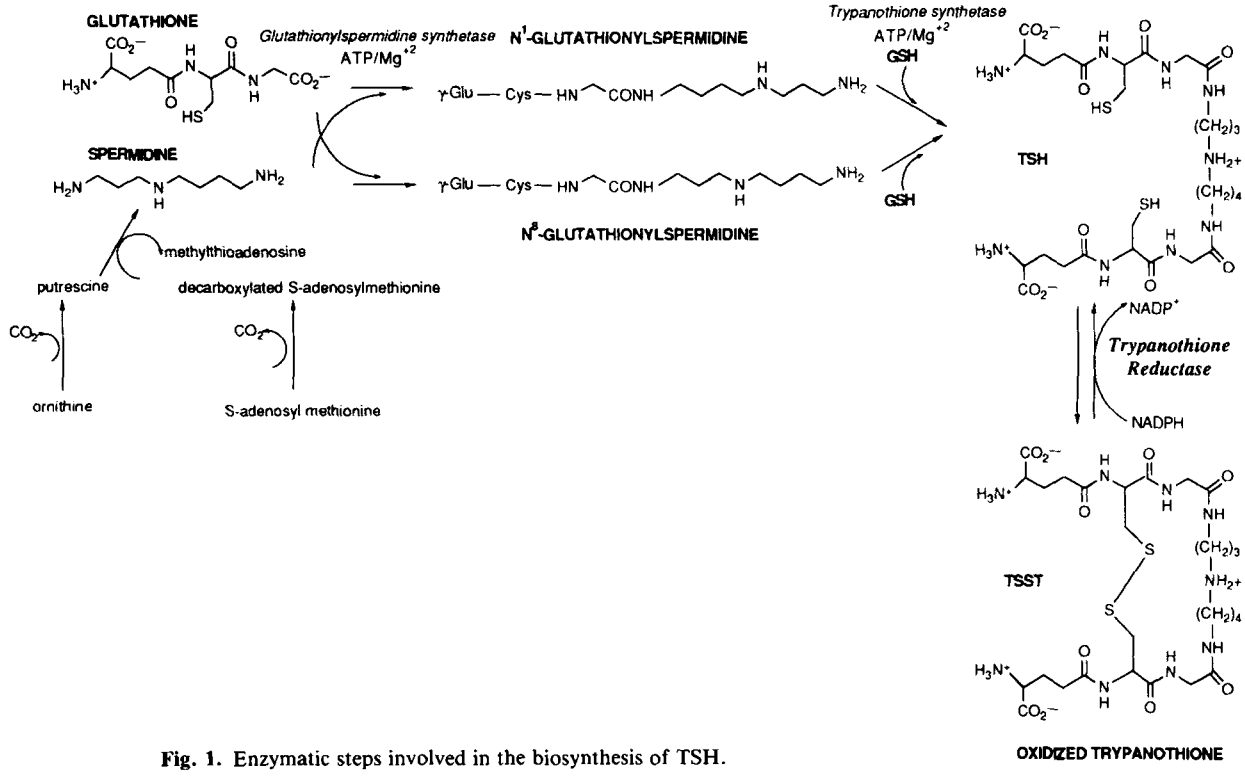


Fig. 1. Enzymatic steps involved in the biosynthesis of TSH.

(Tabor & Tabor, 1975). The functional significance of Gsp (as opposed to TSH) in the trypanosomatid parasites and in *E. coli* is not known, although it has been suggested that Gsp could be a means of regulating cell division and growth by lowering the intracellular levels of free spermidine (Tabor & Tabor, 1975; Shim & Fairlamb, 1988). Zhang and colleagues have recently reported the finding that cationic thiols, like *N*-(2-mercaptoethyl)-1,3-diaminopropane, repair DNA radicals in vitro (Zhang et al., 1988). It may be that Gsp plays a role in repairing DNA damaged during stationary phase of *E. coli*.

Metabolic labeling studies with intact cells and cell-free lysates of *C. fasciculata* have indicated that the biosynthesis of TSH involves ATP-dependent conjugation of intact GSH to the two terminal amino groups of spermidine via Gsp (Fairlamb et al., 1986). Most recently, Henderson and colleagues purified and characterized an enzyme from *C. fasciculata* that was shown to be active in a monomeric form of 83 kDa in a molecular weight in converting GSH to TSH, a TSH synthetase (Henderson et al., 1990). They suggested that catalysis by the ATP-dependent TSH synthetase occurred in two steps, first in the synthesis of either the N^1 or N^8 regioisomer(s) of Gsp and then the synthesis of TSH. As a member of the nonribosomal ATP-dependent amide-forming ligases, TSH synthetase was suggested to be a target for specific amide bond-synthetase inhibitors such as phosphinate and sulfoximine analogues (Duncan & Walsh, 1988).

We now show the resolution of TSH synthetase into two distinct enzymes, a Gsp synthetase and a TSH synthetase, that catalyze the overall two-step reaction. Starting from the previously published purification procedure (Henderson et al., 1990), we have been able to use additional column chromatography steps to separate the two enzymes.

Results

Separation of TSH and Gsp synthetase

During the course of purification of an apparent TSH synthetase activity, Henderson et al. (1990) reported a 170-fold purification from a DEAE-Sephacel step of a protein of 83 kDa. Using phenyl Superose as a late step in purification, these authors reported a 65% loss of activity in the conversion of N^1 or N^8 Gsp to TSH. The possibility existed that two separate components were being resolved on this hydrophobic column with one component undergoing selective inactivation. Repetition in our hands of the purification steps of ammonium sulfate fractionation, ion-exchange chromatography on DEAE-Sephacel, fast performance liquid chromatography (FPLC) Q fast-flow Sepharose, Sephacryl S-200 gel filtration, and FPLC Mono P confirmed the purification of a single enzyme activity as judged by an ATP-coupled as-

say with spermidine and GSH as substrates. Subsequent chromatography on phenyl Superose led to two resolvable proteins, one with Gsp synthetase activity, the other active as a TSH synthetase using N^1-Gsp as a substrate. The net purification of Gsp synthetase was 93-fold and the TSH synthetase activity (which elutes first on the phenyl Superose column) was 52-fold from the DEAE-Sephacel stage when the two coeluting activities could be detected by the coupling of ATP utilization to NADH consumption in the presence of spermidine and GSH, or of Gsp and GSH as appropriate. The overall yields for Gsp synthetase and TSH synthetase were 10% and 7.5%, respectively (Table 1). Gsp synthetase and TSH synthetase were relatively low abundance proteins in crithidial extract, yielding 0.55 mg and 1.67 mg, respectively, from ca. 500 g of crithidial wet cell mass after the phenyl Superose column. The TSH synthetase component appeared pure at this stage (Fig. 2A), but the Gsp synthetase appeared to contain a contaminant via some SDS-PAGE gels at a MW of 79 kDa, which is not associated with either TSH or Gsp synthetase activity. Both Gsp synthetase and TSH synthetase activities were detected in the gel filtration sample shown in Figure 3 where no 79-kDa contaminant is seen. A contaminant ATPase activity detected by the NADH consumption assay coupled to PK/LDH enzymes was found in DEAE-Sephacel column fractions and was found to be the crithidial homolog of hsp 83 (heat shock protein of 83 kDa) in trypanosomes (Dragon et al., 1987) and will be reported elsewhere. The overall purification as assessed by SDS-PAGE gel analysis (Fig. 2B) reveals contaminants that are mostly eliminated by conservative pooling of fractions at the gel filtration and Mono P steps. During the purification steps, various affinity column chromatographic steps were tested. Although the enzymes bound to N^1-Gsp Sepharose and GSH Agarose and eluted as shown by SDS-PAGE gel analysis with their respective substrates, they were inactivated by these procedures. When methotrexate Agarose was used, the Mono P-purified fraction bound irreversibly.

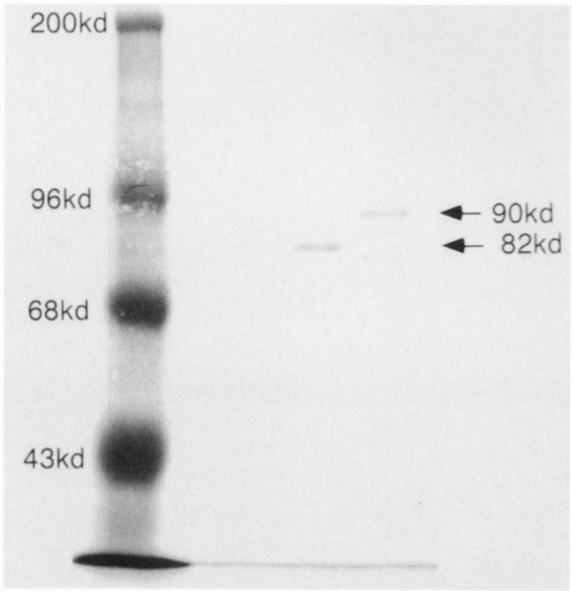
Detection of complexes between TSH synthetase and Gsp synthetase

Resolution of both enzymes was obtained by running a Superose 6 gel filtration column under high salt (800 mM NaCl) and reducing conditions (1 mM DTT), giving 82-kDa and 90-kDa species that were detected by SDS-PAGE analysis. When the same column was run under low salt (150 mM) or no salt and reducing conditions (1 mM DTT), molecular weight species of ca. 400 kDa and 170 kDa were found, perhaps corresponding to a 3:1 or 2:3 ratio of TSH synthetase to Gsp synthetase (the 400-kDa peak) and a 1:1 ratio of either hetero- or homodimers of TSH synthetase and/or Gsp synthetase (the 160–180-kDa peaks) shown in Figure 3. Enzyme activity of both TSH synthetase and Gsp synthetase could not be detected following the dilution effect of the gel filtration run. These data suggest that a complex between the two enzymes forms in vivo and persists during purification.

Table 1. *Purification table*

Step	Volume (mL)	Protein (mg)	Units (μmol NADH/min)	Specific activity (units/mg)	Fold purification[a]
30–55% $(NH_4)_2SO_4$	630	11,500	Both (58)[b]	(0.005)	(1)
			Gsp (37)[c]	(0.003)	(1)
			TSH (20)[c]	(0.002)	(1)
DEAE-Sephacel	180	630	Both 56[d]	0.089	1 (16)
			Gsp 45[e]	0.071	1 (22)
			TSH 11[f]	0.017	1 (10)
Mono Q	30	30	Both 36	1.2	13 (208)
Gel filtration – Sephacryl S-200	20	28	Both 31	1.1	12 (192)
Mono P	10	3.8	Both 18	4.8	54 (864)
Phenyl Superose					
Peak II Gsp	1.5	0.55	Gsp 3.9	6.7	93 (2,084)
Peak I TSH	1.5	1.67	TSH 1.5	0.91	52 (535)

[a] Fold purification calculations are based on the DEAE-Sephacel fraction, but estimations based on the ammonium sulfate cut are in parentheses.
[b] As estimated from the radiolabeled assay described by Fairlamb et al. (1986), which was used in Henderson et al. (1990).
[c] In the coupled assay using spermidine and GSH as substrates, 35% of the NADH oxidized is due to TSH (Fairlamb et al., 1986).
[d] Both enzyme activities were estimated with spermidine and GSH as substrates.
[e] Calculated from [d] minus [f].
[f] TSH synthetase activity was determined using N^1-Gsp and GSH as substrates.



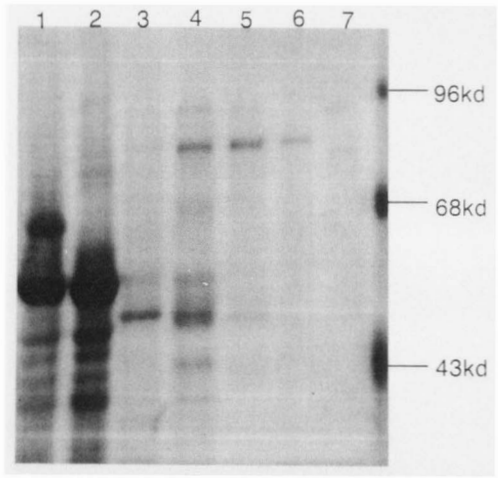


Fig. 2. Analysis of Gsp synthetase and TSH synthetase purification by 7.5% SDS-PAGE. **A**: Lane (left) molecular weight markers, (middle) TSH synthetase (82 kDa) after phenyl Superose purification, and (right) Gsp synthetase (90 kDa) after phenyl Superose purification. **B**: Lane 1, cell-free extract; 2, ammonium sulfate cut; 3, DEAE-ion exchange column; 4, Q fast-flow Sepharose-purified material; 5, Mono P-purified material; 6, TSH synthetase after phenyl Superose column; 7, Gsp synthetase after phenyl Superose column.

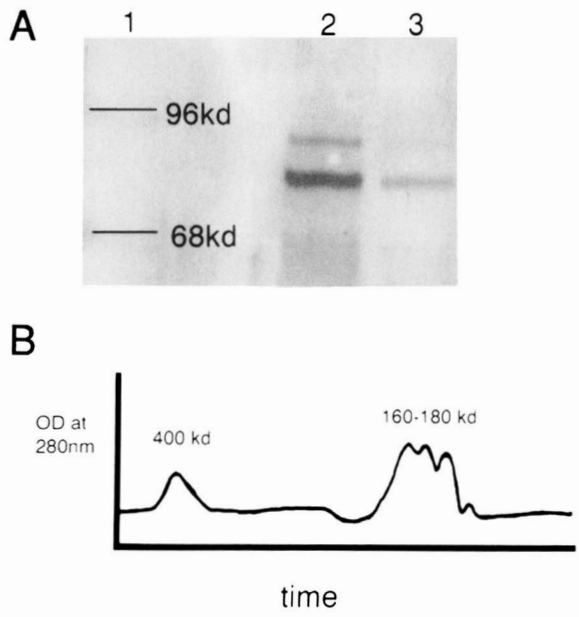


Fig. 3. Analysis of complex formation between Gsp synthetase and TSH synthetase by Superose 6 column chromatography. Twenty micrograms of the Mono P-purified proteins were mixed in 150 mM NaCl and 1 mM DTT in 20 mM Bis-Tris-propane buffer at pH 7.4, and the column was developed in the same buffer. **A**: A 7.5% SDS-polyacrylamide gel with silver staining used for detection of protein fractions. Lane 1, molecular weight markers; 2, protein components of the 400-kDa MW fraction (4 μg in 20 μL); and 3, protein components of the pooled 160–180-kDa fractions (1 μg in 20 μL). **B**: Absorbance trace at 280 nm of the protein eluate from the Superose 6 column (HR 10/30).

Physical and kinetic characterization

TSH synthetase and Gsp synthetase, purified as described in Table 1, were found by SDS-PAGE analysis to have molecular weights of 82 kDa and 90 kDa, respectively. The pure TSH synthetase was found to be highly specific for N^1-Gsp and GSH (N^8-Gsp was not available to be tested) and was found to be dependent on ATP hydrolysis in the presence of Mg^{2+}. Similarly, the pure Gsp synthetase was found to catalyze the first step toward TSH synthesis by converting GSH and spermidine to N^1-Gsp with ATP hydrolysis but had no activity with Gsp as a substrate.

The K_m and k_{cat} values were obtained (Table 2) for GSH, spermidine, and N^1-Gsp at each of the enzymes' respective pH optima. Under conditions where all substrates were present at saturating conditions, the specific activity of Gsp synthetase at pH 6.5 was determined to be 6.7 units/mg (Table 1), which corresponds to a k_{cat} of 600/min; the specific activity of TSH synthetase at pH 7.5 was found to be 0.91 units/mg (Table 1) corresponding to a k_{cat} of 75/min. Confirming findings on partial reactions published by Henderson et al. (1990), there was negligible ATPase activity in the presence of spermidine or N^1-Gsp but, at saturating GSH, both enzymes catalyzed polyamine-independent ATP hydrolysis at ca. 6% of the rate supported by both substrates.

Confirmation of product formation by each enzyme

To corroborate the kinetic specificity of substrate utilization each enzyme was tested for its ability to catalyze the

Table 2. *Catalytic parameters of spermidine (tested with GSP synthetase) and GSH (tested with both enzymes) analogs*

	Relative K_{cat} (spermidine = 1.0)	Relative K_{cat} for TSH synthetase	Relative K_{cat} for GSP synthetase
Spermidine analogs			
N^1-acetyl spermine	0.7		
N^1-acetyl spermidine	0		
Putrescine	0		
Spermine	0.8		
1,4-Bis-3-amino propyl piperazine	0		
1,3-Diamino propane	0		
3-Dimethylaminopropylamine	0		
1,7-Diamino heptane	0		
N-propyl-1,3-propanediamine	0.7		
3-Phenyl-1-propylamine	0		
N-methylpropanediamine	0.25		
N-cyclohexyl-1,3-propanediamine	0.25		
3,3-Iminobispropylamine	0.5		
GSH analogs			
Cys-Gly		0.041	0.041
H-Glu-Abu-Gly-OH		0.8	0.6
H-Ala-Gly-OH		0	0
Glu-Cys		0	0
H-Abu-Gly-OH		0	0.2

synthesis of either TSH or Gsp by direct product analysis. Gsp, TSH, and $T(S)_2$ were separable with good resolution (Fig. 4C). After various times of incubation at room temperature of Gsp synthetase with ATP, DTT, GSH, spermidine, or N^1-Gsp at saturating concentrations, product formation was assessed by reverse-phase HPLC. Analysis of the time course of product formation by UV detection (Fig. 4A) indicated synthesis of Gsp only at the 3-h time point. At 6.5 h, a further increase in Gsp was accompanied by a small shoulder in the TSH region of the gradient. By 20.5 h the minor peak was resolvable with a retention time corresponding closely to that of TSH. It is likely that this represents slow nonenzymatic autooxidation of Gsp to the disulfide form of $(Gsp)_2$ (data not shown) although the possibility of TSH formation remains. From the 6.5-h time point, the specificity of Gsp formation is at least 100:1. Incubations of the same amount of Gsp synthetase with Gsp, GSH, ATP, DTT, and Mg^{2+} gave no detectable production of TSH or $T(S)_2$ in a 3-h period (data not shown). Similarly, saturating solutions of ATP, DTT, GSH, spermidine, or N^1-Gsp were incubated with TSH synthetase at different times at room temperature as shown in Figure 4D. No product was detected when spermidine replaced N^1-Gsp in the reaction mixture (data not shown). There was a linear correlation of Gsp decrease (0.3 μmol initially) and TSH increase (0.21 μmol at the last time point) over the 15-h time course. It appeared that only a small amount of TSH was oxidized to $T(S)_2$ during this incubation. It is unclear if either of the small peaks at 30.5 min and 31.5 min corresponds to $T(S)_2$. In summary, the data of Figure 4 clearly validate the identification of a separate Gsp synthetase and a separate TSH synthetase.

In order to overcome the problems associated with oxidation and thiol–disulfide exchange between products and reactants, a second experimental approach was undertaken where pure TSH reductase and NADPH were included in the incubations. TSH reductase reduced both TSH disulfide and Gsp disulfide directly and GSH disulfide indirectly by thiol–disulfide exchange with the reduced products. Samples were incubated as described in the Materials and methods, and the thiols were derivatized with the fluorescent reagent monobromobimane before analysis of HPLC (Fig. 5). This approach yielded an unambiguous demonstration that Gsp synthetase uses GSH as a substrate, producing only Gsp as product (Fig. 5A,B), and likewise TSH synthetase consumes GSH and N^1-Gsp, producing only TSH as product (Fig. 5C,D).

In summary, the data shown in Figures 4 and 5 clearly validate the identification of a separate Gsp synthetase and a separate TSH synthetase.

Substrate specificity results

A preliminary investigation of substrate specificity was obtained by testing analogues of spermidine and GSH at concentrations of 5 mM for each analogue and saturating conditions for other substrates needed in the reaction (Ta-

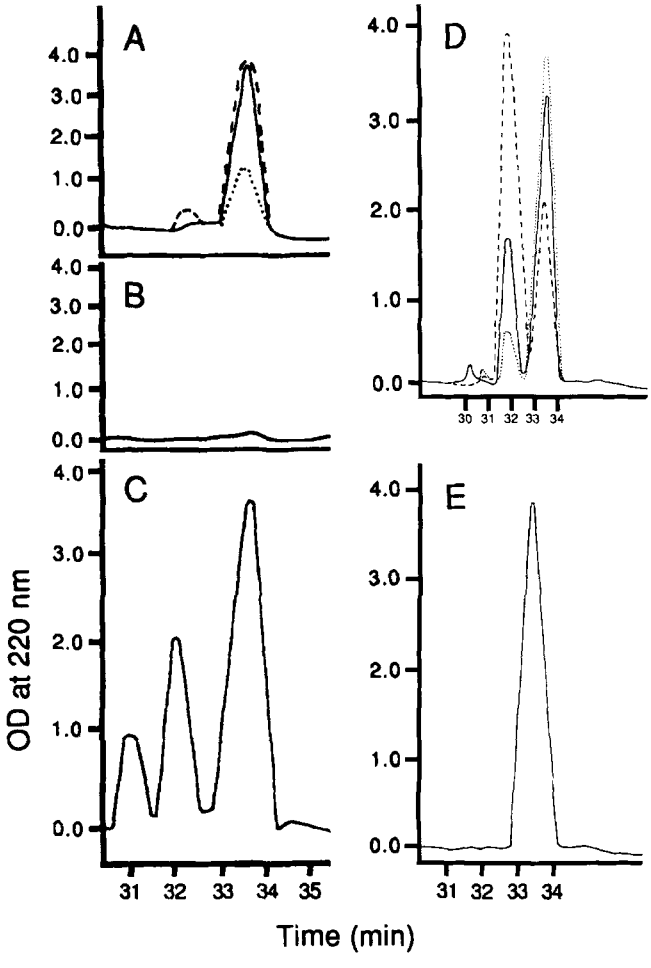


Fig. 4. Determination of enzymatic product formation by UV detection using HPLC analysis. Incubations and separations were performed as described in the Materials and methods. **A**: Time course of product formation by Gsp synthetase after: 3 h (· · · · ·), 6.5 h (———), and 20.5 h (– – – – –) of incubation. **B**: A parallel incubation as in A except without addition of Gsp synthetase was analyzed at 3 h. No formation of Gsp or TSH or $T(S)_2$ was detected. **C**: Separation of TSH (oxidized and reduced forms) and Gsp standard compounds. Separate injections of each species permitted assignment of the peak eluting at 31 min as $T(S)_2$, the peak at 32 min as TSH, and the peak at 33.5 min as Gsp. Samples of 33 nmol $T(S)_2$, 88 nmol of TSH, and 300 nm ol of N^1-Gsp were utilized in this separation. **D**: Time course of product formation by TSH synthetase after 3 h (· · · · ·), 5.5 h (———), and 15 h (– – – – –) of incubation. **E**: A parallel incubation as in D except without addition of TSH synthetase was analyzed at 3 h.

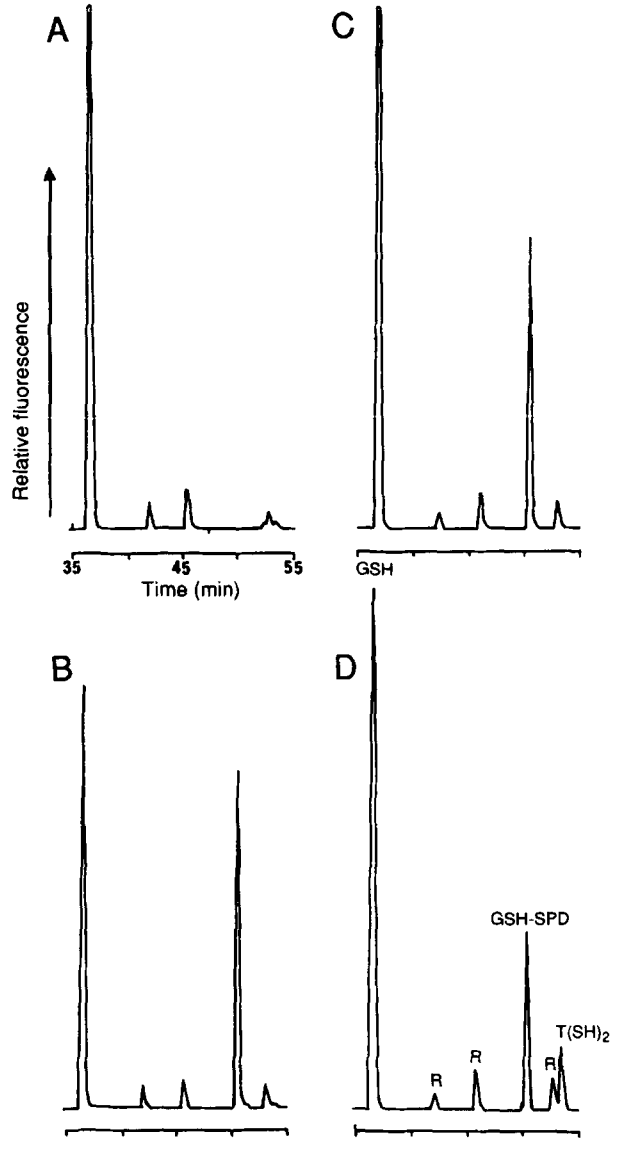


Fig. 5. Determination of product formation by fluorescence measurements using HPLC analysis. Incubations, derivatizations, and separations were performed as described in the Materials and methods. **A** and **B**: A 10-μL injection and 0.0 and 1.0 incubation, respectively, of Gsp synthetase with GSH and spermidine. **C** and **D**: A 20-μL injection and 0.0 and 3.0 h incubation, respectively, of TSH synthetase with GSH and Gsp. The retention times of authentic monobromobimane derivatives and reagent peaks (R) are given in D.

ble 2). These findings suggest that all three amine groups on spermidine are needed for efficient catalysis by the Gsp synthetase. It is not yet determined if N^1- or N^8-Gsp is formed preferentially. Both the glutamate and glycine residues of GSH appear to be important for the recognition of substrate by both enzymes, with a slight difference noted between the glycine preference of Gsp synthetase over TSH synthetase (Table 2). Ophthalmic acid (L-gamma-glutamyl-L-alpha-aminobutyrylglycine) was a good substrate for both enzymes, indicating that the sulfur of the cysteine was not essential for recognition.

Spermidine (1 mM) did not detectably inhibit the activity of TSH synthetase using 10 mM GSH and 100 μM N^1-Gsp as substrates. TSH (1 mM) did competitively inhibit catalysis of Gsp synthetase with 10 mM GSH and 10 mM spermidine. At 200 μM concentrations, glutathi-

onylphosphinate (Fairlamb & Smith, unpubl.), butathionylsulfoximine (Fairlamb & Smith, unpubl.), and methotrexate, which inhibits glutathionyl synthetase at 100 μM concentrations (Kato et al., 1987), did not detectably inhibit either Gsp synthetase or TSH synthetase. An analog to Gsp, deoxyspergualin, which was found to be a competitive inhibitor of TSH reductase at 3 mM concentrations (Krauth-Siegel et al., 1992), was tested against both enzymes to study substrate recognition, and no inhibition was seen.

Discussion

The trypanosomatid parasites (*Trypanosoma* and *Leishmania*) cause many diseases in both humans and domestic animals. In humans, *T. cruzi* is the agent of the often-fatal South American Chagas disease for which there is no effective chemotherapy. African sleeping sickness is found in many regions of Africa, caused by either *T. brucei gambiense* or *T. brucei rhodesiense*, whereas in cattle, *T. congolense* causes the corresponding disease known as nagana. *Leishmania* are widely spread in nature, causing a variety of diseases, including human visceral leishmaniasis (kala azar, *L. donovani*), human cutaneous leishmaniasis (oriental sore, *L. tropica*), and mucocutaneous leishmaniasis (espundia, *L. braziliensis*) (Markell & Voge, 1981). The enzymes that effect the synthesis of TSH in these pathogenic organisms represent important and potentially specific targets for the development of antiparasitic chemotherapy.

We report here the purification and characterization of two separate enzymes that, via two independent ATP hydrolysis steps, catalyze the sequential synthesis of Gsp and TSH. Previous to this finding, cell lysates of *C. fasciculata* were shown to catalyze conversion of spermidine, N^1-Gsp, or N^8-Gsp to TSH (Fairlamb et al., 1986). We confirm most of the purification steps used in the previous purification protocol of TSH synthetase (Henderson et al., 1990); we improve upon the isolation procedure by using other chromatographic techniques. Most notably, it was the analysis of every protein-containing fraction from the phenyl Superose column step that showed the existence of the two enzymes, Gsp synthetase and TSH synthetase. We rule out the previous hypothesis that one enzyme, "TSH synthetase," catalyzes both steps of the biosynthetic reaction (Henderson et al., 1990).

The finding of Gsp synthetase in the stationary phase of *E. coli* (Tabor & Tabor, 1975) permits the postulate that the parasite Gsp synthetase may be similar and in turn that TSH synthetase, so far known to be unique to trypanosomatid parasites, could have evolved from Gsp synthetase. Amino acid sequence analysis and a comparison of *E. coli* and trypanosomatid DNA sequences of the genes for the encoded enzymes will allow us to investigate these possibilities. Both Gsp synthetase and TSH synthetase as purified from *C. fasciculata* are N-terminally blocked and require amino acid analysis on fragments from tryptic digest mapping (in progress).

The two enzymes have somewhat distinct but overlapping pH optima: Gsp synthetase had 80% activity at pH 7.5 and 100% activity at pH 6.5. TSH synthetase had 80% activity at pH 6.5 and 100% activity at pH 7.5. These differential activities could explain the relative differences in concentrations of Gsp and TSH in stationary and growth phases as proposed earlier (Henderson et al., 1990). The concentration of N^1-Gsp increases threefold during the conversion of growth phase (cellular pH = 7.2) to stationary phase (cellular pH = 6.3) in *C. fasciculata*. There is a threefold decrease in TSH levels in the transition from growth phase to stationary phase. It has been suggested that the interconversion of Gsp to TSH in the trypanosomatids allows for the mobilization of spermidine in the cell, possibly serving a role in the regulation of cell growth and division (Shim & Fairlamb, 1988). Whether these differences in product amounts depend on the relative rates of each enzyme at different phases of growth remains to be investigated.

The results from the Superose 6 column run at low salt vs. high salt point to the possibility of a two enzyme complex in vivo. The finding that each enzyme's activity decreases significantly after purification on the phenyl Superose or Superose 6 column suggests complex formation enhances the activity of both TSH synthetase and Gsp synthetase. Because both enzymes are in such low amounts in the parasite, it will be necessary either to start purifications from several kilograms of *C. fasciculata* cells to get sufficient enzymes for further analysis or to clone and overexpress the proteins in suitable vectors in *E. coli*.

A more thorough kinetic analysis will help in determining the mechanistic processes for each of these amide-forming enzymes. TSH synthetase has about an eightfold lower k_{cat} than Gsp synthetase and could be rate-limiting in vivo. The preference of Gsp synthetase over TSH synthetase for the GSH analog, H-Abu-Gly-OH (Table 3), will serve to better define the specific activities of both of these enzymes early on in the purification and could be useful in distinguishing between the mechanisms of the two enzymes. From the experiments on stimulation of ATP hydrolysis by GSH alone for both enzymes, glutathionyl phosphate intermediates may be indicated. Perhaps, like several other ATP-dependent ligases, an amide bond between GSH and spermidine or GSH and Gsp is catalyzed through the transient formation of an acyl phosphate on the glycyl residue of GSH with subsequent nucleophilic attack by either the N^1 or N^8 position of the spermidine or Gsp. Studies on which regioisomer is preferred, N^1- or N^8-Gsp, need to be performed.

In conclusion, it is now possible to begin mechanistic characterization of both TSH synthetase and Gsp synthetase enzymes in hopes of preparing and testing specific

derivatives of ATP-synthetase inhibitors like the phosphinates and sulfoximines (Duncan & Walsh, 1988).

Materials and methods

Materials

Chromatography resins were from the following sources: Q fast-flow Sepharose, Mono P, Superose 6, and phenyl Superose were from Pharmacia and used on a Pharmacia FPLC system. The Sephacryl S-200 gel filtration column and DEAE-Sephacel were from Pharmacia. Protein molecular weight standards were from Bio-Rad and GIBCO BRL. $T(SH)_2$ and N^1-Gsp were chemically synthesized as described previously (Henderson et al., 1986) and obtained from Bachem. *Crithidia fasciculata* was grown and harvested as described previously (Le Trang et al., 1983). All other reagents and chemicals were of the highest grade commercially available.

Enzyme assays

At all stages during the purification, except crude cell lysate and ammonium sulfate cuts, it was possible to couple TSH synthetase and Gsp synthetase to specific hydrolysis of ATP to ADP + Pi to NADH oxidation by use of phosphoenolpyruvate (PEP), PK, and LDH. As described by Henderson et al. (1990), the assay mixtures contained 50 mM Bis-Tris-propane, 50 mM Tris, 5 mM $MgSO_4$, 1 mM EDTA, 5 mM DTT, 10 mM PEP, 10 mM GSH, 10 mM spermidine, 200 μM N^1-Gsp, 2 mM ATP, 10 units/mL PK, and 10 units/mL LDH at pH 6.5 or at pH 7.5. Both Gsp synthetase activity and TSH synthetase activity copurified up until the phenyl Superose column step, at which point specific substrates were needed for activity. Absorbance changes were measured on a Perkin-Elmer spectrophotometer at 340 nm. The assay is sensitive to micromoles of NADH consumed, and one unit of activity is defined as the amount of enzyme required to convert 1 μmol of NADH and NAD^+ per minute at 25 °C.

At the Mono P and phenyl Superose steps of the purification procedure, the NADH oxidation assay results were repeated and confirmed by using a malachite green colorimetric assay for the presence of inorganic phosphate (Geladopoulos et al., 1991). At either pH 6.5 or 7.5, samples were incubated for 1 h at 25 °C in the presence of 100 mM HEPES, 5 mM $MgSO_4$, 1 mM EDTA, 5 mM DTT, 10 mM GSH, 10 mM spermidine, 5 mM N^1-Gsp, 2 mM ATP, and 0.1 unit/mL of both or either enzymes. Inorganic phosphate release in the assay mixture, after addition of ammonium molybdate and malachite green dye solution, was detected at 630 nm and was sensitive to nanomoles of Pi release.

Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard.

Product determination by UV detection

HPLC using a Waters 625 LC system on a C_{18} protein and peptide column (240 × 4 mm with a particle size of 5 μm) with a Waters 468 spectrophotometer set to detect 220 nm was performed on standards (GSH, DTT, TSH, $T(S)_2$ and N^1-Gsp) and on samples. Samples were incubated in 100 mM HEPES, 5 mM $MgSO_4$, 1 mM EDTA, 5 mM DTT, and 2 mM ATP at pH 7.0 for the indicated times at room temperature with 10 mM GSH and 10 mM spermidine or 0.3 mM N^1-Gsp in the presence or absence of Gsp synthetase (1 μg of 0.53 units/mg specific activity) or TSH synthetase (3 μg of 0.13 units/mg specific activity), respectively. During a 60-min run, a linear gradient over 30 min at a flow rate of 0.5 mL/min was run from 100% solvent A (0.1 M NaH_2PO_4 and 0.1 mM EDTA at pH 2.55 with Waters PIC B Low UV reagent) to 100% B (0.1 M NaH_2PO_4 with 30% acetonitrile at pH 3.0 with Waters PIC B Low UV reagent).

Product determination by fluorescence detection

Gsp synthetase (8.2 mU) or TSH synthetase (1.2 mU) were incubated in a standard system (1 mL) containing 50 mM Bis-Tris-propane, 50 mM Tris, 5 mM $MgSO_4$, 1 mM EDTA, pH 6.5, 2 mM ATP, 0.5 mM NADPH, and 0.3 U of *C. fasciculata* TSH reductase containing either 1 mM GSH and 10 mM spermidine or 0.5 mM GSH and 0.2 mM N^1-Gsp at 30 °C. At the times indicated, 10-μL aliquots were diluted 10-fold with 40 mM HEPES/NaOH, 2 mM EDTA, pH 8.0, and thiols were derivatized by the addition of an equal volume of monobromobimane in ethanol and heated at 70 °C for 3 min. Samples were deproteinized by the addition of an equal volume at 4 M methanesulfonic acid, pH 1.5, and analyzed by HPLC as described previously (Shim & Fairlamb, 1988).

Purification of TSH synthetase and Gsp synthetase

Most of the purification column steps were described by Henderson et al. (1990) with minor modifications. All buffer solutions contained 1 mM DTT and 1 mM EDTA. All operations were carried out at 4 °C. The procedure was started with ca. 500 g of *C. fasciculata*, as the proteins represented 0.005% of total cell-soluble protein.

Ammonium sulfate fractionation

A 30–55% ammonium sulfate cut was dissolved in ca. 100 mL of 20 mM Bis-Tris-propane buffer, pH 7.4, and dialyzed against the same buffer. The dialysate was centrifuged and the supernatant was saved for the next step.

DEAE-Sephacel chromatography

The protein mixture was next applied to a DEAE-Sephacel column (2.5 × 25 cm, 490 mL) that had been equilibrated in 20 mM Bis-Tris-propane, pH 7.4. After

being washed with 2 L of equilibration buffer, the bound proteins were eluted with a linear salt gradient of 0.0–0.4 M KCl in 2 L of buffer at pH 7.4 over 16 h. A high ATPase activity eluted at about 0.2–0.25 M KCl and was readily detected by the NADH consumption assay. The TSH synthetase and Gsp synthetase activities coeluted at about 0.28 M KCl and were readily detectable by the NADH consumption assay. The pooled fractions were diluted with an equal volume of 20 mM Bis-Tris-propane at pH 7.4.

Q fast-flow Sepharose chromatography

This mixture was then applied to a Q sepharose column (0.9 × 15 cm; 38 mL) that had been equilibrated with 20 mM Bis-Tris-propane buffer. Over a 200-mL linear gradient of 0.0–0.4 M KCl, fractions were tested, pooled, and concentrated to about 5 mL with an Amicon concentrator (PM-30 membrane).

Sephacryl S-200 gel filtration

After being equilibrated with 20 mM Bis-Tris-propane containing 0.15 M KCl, the column (1.2 × 70 cm) was loaded with sample. Active fractions were pooled and concentrated by an Amicon concentrator to about 10 mL.

Mono P (HR 5/20) FPLC

Sample was applied to a 20-mM Bis-Tris-propane equilibrated column with a flow rate of 1 mL/min and fractionated by using buffer B (20 mM Bis-Tris-propane with 0.4 M KCl) as follows: $t = 0$ min, %B = 0; $t =$ 10 min, %B = 40; $t = 60$ min, %B = 100. Both enzymes fractionated together up to and through this step of purification. Enzyme activities were measured by NADH consumption and by Pi release assay. About 500 μg of both proteins were obtained, and some material was stored in 45% glycerol at −20 °C for use on the Superose 6 column. The rest of the material (27%) was used for the next purification step. Overall yield at this stage was 30%.

Phenyl Superose (HR 10/10) chromatography

In a total volume of 6 mL, Mono P-purified material was equilibrated in 50 mM phosphate buffer at pH 7.0 with 0.8 M $(NH_4)_2SO_4$ and applied to a Pharmacia phenyl Superose HR 10/10 column in a flow rate of 1 mL/min. A linear gradient was used over 120 mL from 0.8 M to 0.0 M $(NH_4)_2SO_4$. Active fractions were then tested for the ability to catalyze ATP hydrolysis in the presence of GSH and spermidine (Gsp synthetase) or in the presence of GSH and N^1-Gsp (TSH synthetase) by both enzyme assays. We noted that both proteins lose some activity after being run over this column.

Superose 6 (HR 10/30) gel filtration

With 1 unit of Mono P-purified material, an FPLC column was run in 50 mL of 20 mM Bis-Tris-propane buffer at pH 7.4 with 0.0 M NaCl or 0.15 M NaCl at a flow rate of 0.3 mL/min. Using gel filtration standard proteins of aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran (2,000 kDa) under the same conditions, a species of ca. 400 kDa (corresponding to a multimeric complex of both enzymes) and a species of ca. 170 kDa (corresponding to a hetero- or homodimers of both or one enzyme) was detected and run on SDS-PAGE. Running the same column equilibrated under the similar conditions except now in 0.8 M NaCl with 1 unit of the Mono P-purified sample equilibrated in 0.8 M NaCl, the two enzymes were resolved as monomers of 82 kDa (TSH synthetase) and 90 kDa (Gsp synthetase).

MW determination

The MW of Gsp synthetase and TSH synthetase was determined by SDS-PAGE with standards of myosin heavy chain (200 kDa), phosphorylase B (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa).

Kinetic analysis

Analyses were carried out in the same buffer system used in the NADH consumption assay at pH 6.5 for Gsp synthetase and at pH 7.5 for TSH synthetase. Michaelis constants (K_m) and maximum initial velocities (V_{max}) for GSH, spermidine, and N^1-Gsp were determined by coupling ATP hydrolysis to NADH oxidation as described above. Initial velocity measurements were made at six substrate concentrations, which ranged from 0.1 to 10 mM for GSH and spermidine and from 0.005 to 1 mM for N^1-Gsp. Kinetic constants (Table 2) were calculated from Lineweaver–Burk plots.

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