Convenient Isolation and Kinetic Mechanism of Glutathionylspermidine Synthetase from *Crithidia fasciculata*<sup>*</sup>

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Trypanothione, the essential metabolite in the oxidant defense system of trypanosomatids, is synthesized by two distinct proteins, glutathionylspermidine synthetase and trypanothione synthetase. Glutathionylspermidine synthetase was purified to homogeneity from the trypanosomatid *Crithidia fasciculata* by aqueous two-phase systems and chromatography. The enzyme showed a specific activity of 38 μmol of glutathionylspermidine formed per min per mg of protein. Its molecular mass was 78 kDa in SDS-polyacrylamide gel electrophoresis, and it appeared predominantly monomeric in native polyacrylamide gel electrophoresis and gel filtration. The isoelectric point was at pI 4.6, and the pH optimum was near 7.6. Partial amino acid sequencing revealed homology with, but low similarity to, the glutathionylspermidine synthetase/amidase of *Escherichia coli*, and amidase activity was not detected in glutathionylspermidine synthetase of *C. fasciculata*. The kinetics of trypanosomatid glutathionylspermidine synthetase revealed a rapid equilibrium random mechanism with limiting $K_m$ values for Mg$^{2+}$-ATP, GSH, and spermidine of 0.25 ± 0.02, 2.51 ± 0.33, and 0.47 ± 0.09 mM, respectively, and a $k_{cat}$ of 415 ± 78 min$^{-1}$. Partial reactions at restricted cosubstrate supply were not detected by $^{31}$P NMR, supporting the necessity of a quaternary complex formation for catalysis. ADP inhibited competitively with respect to ATP ($K_i = 0.08$ mM) and trypanothione exerted a feedback inhibition competitive with GSH ($K_i = 0.48$ mM).

Glutathionylspermidine synthetase (GspS)<sup>1</sup> catalyzes the first of two steps of trypanothione biosynthesis, the synthesis of glutathionylspermidine (Gsp) from GSH and spermidine with the consumption of ATP (1). Trypanothione ($N^1,N^4$-bis(glutathionyl)spermidine; TSH) is a metabolite unique to trypanosomatids such as *Trypanosoma sp.*, *Leishmania sp.*, and *Crithidia fasciculata* (2). These parasites comprise pathogens causing widespread and difficult to treat tropical diseases such as African sleeping sickness (*Trypanosoma brucei gambiense* or *T. rhodesiense*), Chagas disease (*Trypanosoma cruzi*), kala azar (*Leishmania donovani*), oriental sore (*Leishmania tropica*), and mucocutaneous leishmaniasis (*Leishmania braziliensis*). Others (e.g. *Trypanosoma congolense*) affect domestic animals, whereas *C. fasciculata* is pathogenic to insects only.

Since the discovery of TSH in 1985 (3, 4), the pathways for its synthesis and utilization have attracted considerable interest as potential targets for selective therapeutic intervention (5, 6). In all trypanosomatids, TSH substitutes for GSH in the defense against hydroperoxides and derived reactive oxygen species because of its ability to reduce peroxides either enzymatically (7–9) or spontaneously (10). It thereby protects the parasitic trypanosomatids, which apparently are deficient in catalase and glutathione peroxidases (11), against oxidative stress for instance during host-defense reactions (9, 12, 13). Trypanothione disulfide thus formed is reduced by the NADPH-dependent trypanothione reductase (14, 15), a flavoprotein homologous to glutathione reductase that, together with glutathione peroxidases (16, 17), constitutes a major part of the defense system of the host (18, 19). The precursor of TSH, Gsp, may have a distinct biological role. It was first identified in *Escherichia coli* (20), where it remains unprocessed to TSH due to the apparent lack of TSH synthetase. In *E. coli*, GspS, and consequently Gsp, is prominent in the stationary phase (20, 21). Similarly, in *C. fasciculata*, Gsp increases substantially during the transition from growth phase to stationary phase, while TSH simultaneously drops (22). These fluctuations of GSH conjugates or the associated variations in cellular spermidine levels have tentatively been implicated in growth regulation (20, 21).

The first enzyme of TSH synthesis, GspS, has been isolated once in trace amounts from *C. fasciculata* (0.5 mg from 500 g wet cell mass) and preliminarily characterized in terms of the apparent $M_r$, kinetic parameters, and substrate specificity (1). An enzyme catalyzing the analogous reaction in *E. coli* has recently been cloned. Surprisingly, this GspS also exhibits a substantial amidase activity with Gsp as substrate. The simultaneous catalysis of Gsp synthase and breakdown results in an apparently futile ATP consumption, the biological role of which remains speculative (23, 24). Since *E. coli* does not produce TSH, its GspS obviously has to be seen in a biological context distinct from trypanosomal TSH metabolism, and also the structural and phylogenetic relationship of bacterial and trypanosomal GspS remains to be investigated.

Here we report a convenient isolation procedure for GspS from *C. fasciculata* that allows an in depth analysis of this enzyme. Various physicochemical parameters, preliminary amino acid sequence data, and the kinetic mechanism of the enzyme are presented.

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<sup>‡</sup> The abbreviations used are: GspS, glutathionylspermidine synthetase; Gsp, glutathionylspermidine; TSH, trypanothione; TS, trypanothione synthetase; DTT, dithiothreitol; bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

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Glutathionylspermidine Synthetase from *C. fasciculata*

**TABLE I**

| Purification of glutathionylspermidine synthetase | Volume | Protein | Specific activity | Purification factor | Yield  |
|--------------------------------------------------|--------|---------|------------------|---------------------|--------|
|                                                  | ml     | mg      | units/mg         | %                   |        |
| Crude extract                                    | 0.000  | 7600.0  | 0.003            | 1                   | 100    |
| First extraction into top phase                  | 170    | 221.0   | 0.092            | 31                  | 107    |
| Second extraction into top phase                 | 170    | 119.0   | 0.129            | 43                  | 81     |
| Extraction into bottom phase                     | 505    | 50.6    | 0.199            | 66                  | 53     |
| Diafiltration                                    | 1200   | 48.0    | 0.25             | 83                  | 63     |
| Resource Q                                       | 6      | 4.2     | 1.1              | 370                 | 25     |
| Poros 20 Pi                                      | 8      | 0.8     | 5.8              | 1943                | 24     |
| Poros 20 PE                                      | 5      | 0.2     | 12.2             | 4067                | 13     |
| Mono P                                           | 5      | 0.1     | 37.6             | 12533               | 19     |

**EXPERIMENTAL PROCEDURES**

**Protein Determination**

Protein concentrations were determined by the method of Bradford (25). Bovine serum albumin was used as a standard.

**Enzyme Assays**

The assays were carried out at 25.9 °C in a volume of 0.9 ml containing 50 mM bis-Tris-propane, 50 mM Tris, pH 7.5, 5 mM MgSO<sub>4</sub>, 1 mM EDTA, 5 mM DTT, 5 mM ATP, 10 mM GSH, and 10 mM spermidine (1). The assay for trypanothione synthetase (TS) was carried out as described by Smith et al. (1). Aliquots were taken after 20 min. For thiol analysis, a precolumn derivatization with the fluorescent thiol-specific reagent, monobromobimane (Calbiochem), was used as described previously (2). All samples for HPLC analysis were diluted 4-fold with water. Separation and analytical conditions were as described previously (26). HPLC analysis was performed with a Jasco-HPLC-system consisting of an autosampler (851-AS), a pump (PU-980), a ternary gradient unit (LG-980~2), and a highly sensitive fluorescence detector (FP-920), which enabled a precise analysis of the small product peak consisting of an autosampler (851-AS), a pump (PU-980), a ternary water. Separation and analytical conditions were as described previously (2). All samples for HPLC analysis were diluted 4-fold with analysis, a precolumn derivatization with the fluorescent thiol-specific pended in 250 ml of 20 mM bis-Tris-propane buffer, pH 7.5, disrupted by 2 after centrifugation, the cells were stored at 4 °C for all chromatographies. The diafiltrated protein mixture was applied onto a gel permeation chromatography column, Superose 12 (HR 10/30) (Pharmacia), equilibrated with 20 mM bis-Tris-propane buffer, pH 7.5, containing 0.15 M NaCl, and eluted with a flow rate of 0.3 ml/min. Blue dextran (2,000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), β-amylnase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and carbonic anhydrase (30 kDa) were used as standards.

**Molecular Mass Estimation by Chromatography**—Proteins were applied onto a gel permeation chromatography column, Superose 12 (HR 10/30) (Pharmacia), equilibrated with 20 mM bis-Tris-propane buffer, pH 7.5, containing 0.15 M NaCl, and eluted with a flow rate of 0.3 ml/min. Blue dextran (2,000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), β-amylnase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and carbonic anhydrase (30 kDa) were used as standards.

**Determination of Physical Parameters**

**Molecular Weight**

The molecular mass of the protein was determined by SDS-PAGE (29) using a PhastGel Gradient 8-25 (Pharmacia) with the following molecular mass standards: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and a-lactalbumin (14.4 kDa).

**Isoelectric Point**

The isoelectric point was determined by isoelectric focusing using a PhastGel IEF 3–9 (Pharmacia) with a broad pH calibration kit and by titration curve analysis with PhastGel IEF 3–9. The latter technique is a two-dimensional electrophoresis. In the first dimension, a pH gradient is generated. The gel is then rotated clockwise 90°, and the sample is applied perpendicular to the pH gradient across the middle of the gel.
mium molybdate, and Tween 20 (1) was added. Lanes containing active GspS showed a dark green color after few minutes.

The GspS content in partially purified samples was determined using SDS capillary electrophoresis (Bio-Rad) on the basis of the absorption at 280 nm and assuming an identical absorption coefficient for all proteins in the sample.

**Amino Acid Sequencing**

SDS-PAGE of purified GspS was performed at a constant current of 20 mA in a separating gel (7.5% T). For blotting, the proteins were transferred for 1.5 h onto a polyvinylidene difluoride membrane at 40 V/70 mA in a buffer containing 25 mM Tris base, 192 mM glycine, and 10% (v/v) methanol. The blot was stained with Coomassie Blue.

For peptide sequencing, the band corresponding to a molecular mass of 78 kDa was cut out. This material was washed and digested with endoproteinase Lys-C as described before (30) and separated by reversed-phase HPLC (30). Peptide peaks were detected at 214 nm and collected manually. Aliquots of 15–30 μl were applied directly to biobrene-coated, precycled glass fiber filters of a sequencer (Applied Biosystems 470A) with standard gas phase programs of the manufacturer.

**Kinetic Analysis**

All kinetic experiments were carried out at 25.0 °C in a volume of 0.9 ml containing 50 mM bis-Tris-propane, 50 mM Tris, pH 7.5, 1 mM EDTA, 5 mM DTT, and variable concentrations of ATP (0.10, 0.13, 0.18, 0.28, and 0.66 mM), GSH (0.36, 0.47, 0.66, 1.11, and 3.57 mM), and spermidine (0.36, 0.47, 0.66, 1.11, and 3.57 mM), respectively. The enzymatic tests for kinetic studies except the ADP inhibition studies were performed in the presence of phosphoenolpyruvate (10 mM) and pyruvate kinase (0.5 units). A fixed magnesium concentration of 5 mM and a GspS content of 0.072 mg (0.923 μM) was used. Aliquots were taken at 15 and 30 min. GspS activity was analyzed by product determination as described above.

31P NMR spectra were recorded on a Bruker ARX 400 NMR spectrometer (at 162 MHz and locked to the deuterium resonance of D2O) to detect potential partial reactions.

The experiments were carried out at 25.0 °C in a volume of 0.6 ml containing 50 mM bis-Tris-propane, 50 mM Tris, pH 7.5, 5 mM MgSO4, 1 mM EDTA, 5 mM DTT, in the presence of 20% D2O. Spectra were recorded at the beginning of the experiment and after the addition of the substrates (5 mM ATP, 10 mM GSH, and 10 mM spermidine).

**RESULTS**

**Enzyme Purification**—The purification strategy outlined under “Experimental Procedures” resulted in a GspS preparation with a specific activity of 37.6 units/mg and an overall yield of about 20%. The purification factor achieved was 12,500. As is seen from Table I, the phase distribution system applied proved to be highly efficient in enriching GspS.
experience. In fact, GspS thus purified appeared homogeneous (1), and also the yields compared favorably with previous chromatographic steps was about 6-fold higher than achieved by a Resource Q column.

The bottom phase was diafiltrated and then could be loaded onto a 6.0 without loss of activity. The GspS in the phosphate-rich solution. GspS was therefore extracted from the second top phase and viscosity of the top phase in which the enzyme was dissolved. GspS was thus purified from the second top phase quantitated. The final chromatographic purification of GspS, (2) after two extractions into top phases, GspS was essentially proved to be unstable and was not purified further. This contrast markedly with a relative amidase activity of 18% (w/w) PEG6000, 13% (w/w) phosphate, pH 7.0, yielded an extraction of GspS into the top phase (Fig. 1) with a purification factor of 30 in one step.

Some residual turbidity left in the top phase of the initial extraction could be eliminated by a second extraction step, mixing the primary top phase with a bottom phase of an identical blank system. By these systems, a proteolytic activity, as observed with casein yellow, and an ATPase activity were quantitatively removed by extraction into the bottom phases. Simultaneously, GspS was completely separated from TS activity. While GspS was recovered completely in the top phase, TS activity was extracted into the bottom phase (Fig. 1), but it proved to be unstable and was not purified further. This confirms, in contrast to previous assumptions (22), the existence of two distinct enzymes involved in trypanothione biosynthesis (1). After two extractions into top phases, GspS was essentially free of interfering enzymatic activities and could be precisely quantitated. The final chromatographic purification of GspS, however, was impaired by the high phosphate concentration and viscosity of the top phase in which the enzyme was dissolved. GspS was therefore extracted from the second top phase into the bottom phase of a third system by lowering its pH to 6.0 without loss of activity. The GspS in the phosphate-rich bottom phase was diafiltered and then could be loaded onto a Resource Q column.

The specific activity of GspS obtained after additional chromatographic steps was about 6-fold higher than achieved before (1), and also the yields compared favorably with previous experience. In fact, GspS thus purified appeared homogeneous by SDS-PAGE (Fig. 2) and by titration curve analysis (Fig. 3).

Enzyme Characterization—A subunit molecular mass of GspS of 78 kDa was estimated by SDS-PAGE, and an identical value was obtained by gradient gel electrophoresis of the native enzyme. In the latter case, the identity of the 78-kDa band with GspS was confirmed by activity staining, i.e. phosphate liberation upon incubation with Mg\(^{2+}\)-ATP, GSH, and spermidine (not shown). Also, gel permeation chromatography on Superose 12 indicated a comparable molecular mass (79 kDa). A small activity peak eluted at about 170 kDa, suggesting a slight tendency of the enzyme to dimerize. In essence, however, GspS of C. fasciculata was present as a monomeric enzyme of 78 kDa. Its isoelectric point deduced from isoelectric focusing was at pH 4.6.

Functional characterization of GspS of C. fasciculata was performed with a 400-fold purified preparation, i.e. with the fraction obtained after step 6 in Table I, since the pure enzyme, even when stored at 4 °C and −20 °C, almost completely lost its activity within 1 day. According to purity analysis by capillary electrophoresis, the partially purified preparation contained 33% GspS. At this stage of purification, GspS activity was stable in 20 mM bis-Tris-propane, pH 8.0, and in the presence of 5 mM DTT for more than 2 months at pH 4.6.

FIG. 4. pH optimum of glutathionylspermidine synthetase. Product formation of Gsp was analyzed as described under “Experimental Procedures.” Values are means ± S.D. from two independent measurements done at 10 and 20 min.

18% (w/w), each tested at pH 4.0, 5.5, and 7.0 and containing 40% cell lysate. By centrifugation, the cell debris was concentrated in a gum-like interphase if the pH of the system was ≥5.5. A graphical evaluation of the experimental data (not shown) clearly demonstrated a significant increase in the partition coefficient of GspS with increasing pH and a decrease in the partition coefficient of the total protein with increasing molecular weight of PEG. The best system, containing 7.5% (w/w) PEG6000, 13% (w/w) phosphate, pH 7.0, yielded an extraction of GspS into the top phase (Fig. 1) with a purification factor of 30 in one step.

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In partially purified GspS, we did not observe any TS activity, at a detection limit of ≥1% of the corresponding GspS rate. We could also exclude an amidase activity of C. fasciculata GspS, which had been described for the corresponding E. coli enzyme (23, 24), since Gsp was not hydrolyzed by C. fasciculata GspS under experimental conditions that would have detected a hydrolytic activity at a 1% level of the synthetase activity. TSH hydrolysis was also not detected (not shown). These findings contrast markedly with a relative amidase activity of 18% (pH 7.5) or 35% (pH 8.5) of the synthetase activity reported for E. coli GspS. Also, the pH optimum of the C. fasciculata enzyme (Fig. 4) is higher by nearly 1 pH unit (pH 7.6–7.8) than that of E. coli GspS (pH 6.8).

Amino Acid Sequencing—As already observed by Smith et al. (1), N-terminal amino acid sequencing proved unsuccessful, obviously due to an N-terminal blocking group. After proteolytic cleavage with endoproteinase Lys-C, however, a total of 11 peptides could be recovered from HPLC in a quality to allow sequencing. Of these peptides, seven could unambiguously be aligned to the deduced GspS sequence of E. coli recently pub-
lished by Bollinger et al. (23) (Table II). GspS of *E. coli* and of *C. fasciculata* thus appeared to be phylogenetically related. However, based on the limited sequence information, the sequence similarity between these enzymes, with only 40% identity, appears rather low.

**Kinetic Pattern**—The analysis of the kinetic mechanism by steady-state kinetics were performed by means of direct product (Gsp) detection at fixed time points. The time points were set to yield less than 15% consumption of the limiting substrate but more than 0.002 mM Gsp for a reliable quantification. In the beginning, nonlinear Lineweaver-Burk plots were obtained that could be attributed to product (ADP) inhibition (see below) and, at an ATP concentration above 1 mM, also to substrate inhibition (not shown). When ADP accumulation was avoided by coincubation with phosphoenolpyruvate/pyruvate kinase and the concentrations of ATP were kept constant at levels below 1 mM, linear double-reciprocal plots were observed. Fig. 5, A–C, illustrates enzyme-normalized Lineweaver-Burk plots, each showing the rate dependence of the variable substrate at five fixed variable concentrations of the cosubstrate and a fixed level of the third substrate. Hereby Mg\(^{2+}\) -ATP was considered as a single substrate, since an excess of Mg\(^{2+}\) (5 mM) over ATP (maximum of 0.66 mM) guaranteed complete ATP complexation. From this set of primary data, the secondary and tertiary plots could be derived to fit the general Dalziel equation for three-substrate reactions (32).

\[
e^v = \phi_A + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_C}{[C]} + \frac{\phi_{AB}}{[A][B]} + \frac{\phi_{AC}}{[A][C]} + \frac{\phi_{BC}}{[B][C]} + \frac{\phi_{ABC}}{[A][B][C]} + \text{etc.}
\]

(Eq. 1)

All pertinent kinetic coefficients and constants describing the catalytic mechanism could be deduced. Even by inspection of the primary plots, an enzyme substitution or “ping-pong”

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**TABLE III**

**Dissociation constants and their S.D. values for the glutathionylspermidine synthetase**

The dissociation constants are defined as \(K_{ATP} = [E][ATP]/[E \cdot ATP]\), \(K_{GSH \cdot ATP} = [E \cdot GSH][ATP]/[E \cdot GSH \cdot ATP]\), etc. Limiting \(K_a\) values corresponded to the dissociation constants of the quaternary complexes (\(K_{GSH \cdot Sperm \cdot ATP}\), etc.). In an ideal rapid equilibrium random mechanism, the four dissociation constants characterizing the affinity of a given substrate to the enzyme or its complexes should be identical. Pertinent experimental values are indeed not significantly different.

| Constant                     | Value (mM) |
|------------------------------|------------|
| \(K_{ATP}\)                  | 0.23 ± 0.07|
| \(K_{GSH \cdot ATP}\)       | 0.35 ± 0.15|
| \(K_{Sperm \cdot ATP}\)     | 0.17 ± 0.05|
| \(K_{GSH} \cdot Sperm \cdot ATP\) | 0.26 ± 0.04|
| \(K_{GSH}\)                  | 1.47 ± 1.14|
| \(K_{ATP} \cdot GSH\)       | 1.84 ± 0.72|
| \(K_{GSH} \cdot Sperm\)     | 1.78 ± 0.85|
| \(K_{ATP} \cdot Sperm \cdot GSH\) | 2.62 ± 0.79|
| \(K_{Sperm}\)                | 0.45 ± 0.13|
| \(K_{ATP} \cdot Sperm\)     | 0.34 ± 0.14|
| \(K_{GSH} \cdot Sperm\)     | 0.64 ± 0.24|
| \(K_{ATP} \cdot GSH \cdot Sperm\) | 0.47 ± 0.08|

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As described under “Experimental Procedures.” Each rate was determined from two different time points. The five fixed variable cosubstrate concentrations were chosen as follows: 0.36 mM (●), 0.47 mM (■), 0.66 mM (▲), 1.11 mM (□), and 3.57 mM (▲). A, the ATP concentration was varied from 0.10 to 0.66 mM at five fixed GSH concentrations. The spermidine concentration was kept constant at 0.47 mM. B, the GSH concentration was varied from 0.36 to 3.57 mM at five fixed spermidine concentrations. The ATP concentration was kept constant at 0.13 mM. C, the spermidine concentration was varied from 0.36 to 3.57 mM at five fixed GSH concentrations. The ATP concentration was kept constant at 0.13 mM.
mechanism could be excluded, since the slopes were clearly convergent. Fitting the experimental data to the general Dalziel equation yielded values significantly different from zero for all individual terms, thus ruling out a compulsory order mechanism. We therefore have to classify the kinetic mechanism of GspS as an equilibrium random order mechanism. Whether the complexation of the individual substrates occurs absolutely independently of each other or whether the binding substrates mutually affect affinities of cosubstrates is less easily decided. The apparent $K_m$ values for the different substrates, however, are not significantly affected by the concentrations of the respective cosubstrates. Consequently, the deduced dissociation constants of the corresponding binary, ternary, and quaternary complexes are very close for a given substrate and not significantly different (Table III). This would indeed imply a mutually independent random addition of substrates. However, with regard to the inevitable scatter of data, the possibility cannot be excluded that some route leading to the quaternary complex is slightly favored. However, a rapid equilibrium random order mechanism, as depicted in Fig. 8, conforms best to the experimental data. Based on this assumption, the limiting $K_m$ values are defined as the dissociation constants of the quaternary complexes, numerically $0.25 \pm 0.02$, $2.51 \pm 0.33$, and $0.47 \pm 0.09$ ms for $\text{Mg}^{2+}$, ATP, GSH, and spermidine, respectively. The rate-limiting velocity constant can then be calculated to be $k = 1/\phi_0 = 415 \pm 78$ min$^{-1}$.

A quaternary complex mechanism implies that all three substrates must be assembled at the enzyme before a reaction can proceed. To check this hypothesis, we subjected the enzyme to long term exposure with $\text{Mg}^{2+}$ and monitored a potential partial reaction by $\text{P}^{31}$ NMR. Fig. 9 demonstrates that with all combinations of substrates no ATP turnover could be observed within 5 h unless the third substrate was added. These findings strongly support the assumption of a quaternary complex mechanism and explain the absence of any ATPase activity of GspS. Neither can the presumed catalytic intermediate glutathionylphosphate be formed in any detectable amount by an incomplete catalytic complex.

As already mentioned, ADP significantly inhibits GspS, which renders it difficult to measure GspS activity in the absence of an ATP-regenerating system. The type of inhibition is competitive with respect to ATP (Fig. 6). A $K_i$ of 80 $\mu$M was calculated, which is in the range of physiological ADP concentrations. GspS also proved to be feedback-inhibited by TSH with a $K_i$ of 480 $\mu$M (Fig. 7), which is competed by GSH.

**DISCUSSION**

The purification scheme of GspS from *C. fasciculata* presented here allowed detailed functional analysis of the enzyme without interference from other enzymatic activities. The initial aqueous phase extraction procedures proved to be advantageous in separating the two enzymes involved in the synthesis of trypanothione into two different phases and in the complete separation of GspS from an ATPase activity present in the crude extract. We thereby implicitly confirmed that trypanothione biosynthesis of trypanosomatids, like the analogous GSH biosynthesis (33, 34), is achieved in two consecutive steps by two distinct proteins (1) and not by a single enzymatic entity as had been presumed formerly (22). In agreement with Smith *et al.* (1), we could not detect any trypanothione synthetase activity in our GspS preparation. A similar rapid equilibrium random mechanism as here established for GspS (Fig. 8) has already been described for GSH-synthesizing enzymes (33, 34). This kinetic pattern therefore appears not to be uncommon in nonribosomal amide-forming ligases.

Some characteristics of our GspS, however, differ from previous reports on this enzyme. Smith *et al.* (1) attributed a molecular mass of 90 kDa to GspS of *C. fasciculata* and 82 kDa to TS, whereas our GspS migrated in SDS and native gradient gels with an apparent molecular mass of 78 kDa. Clearly, the clarification of this discrepancy must await completion of full-length cDNA cloning to estimate the maximum size of GspS. Furthermore, Smith *et al.* (1) reported a sharp pH optimum at 6.5, whereas we found an optimum at about 7.5 and an inflection point of the pH dependence curve near 6.8, suggestive of a histidine residue participating in catalysis upon dissociation. Finally, a substantial rate of a partial reaction (i.e. spermidine-
independent liberation of phosphate from ATP, presumed to result from the hydrolysis of the intermediate glutathionylphosphate) was reported for both GspS and TS of *C. fasciculata* (1). Any formation of an intermediate at a measurable rate and quantity would, however, hardly be compatible with the kinetic pattern of GspS worked out here. The deduced kinetic mechanism of GspS (i.e. a rapid equilibrium random ternary complex mechanism) does not conflict with the assumption of an activation of GSH in the form of glutathionylphosphate during the transition state, but such a partial reaction would most likely require the assembly of all substrates at the active site. We also could experimentally rule out, in the absence of spermidine, any significant accumulation of glutathionylphosphate, which should have been detected by $^{31}$P NMR near 0.6 ppm after 5 h of incubation (Fig. 9). Yet neither an acylphosphate nor any ATP hydrolysis was observed unless all substrates were present.

Comparing GspS of *C. fasciculata* and *E. coli* reveals substantial differences. The limited sequence information of *C. fasciculata* so far available (see peptides reported and ongoing DNA sequencing) proves homology with the *E. coli* enzyme, but the extent of conserved residues appears to be restricted to about 40%. The divergence between the bacterial and the trypanosomal enzyme is obviously associated with pronounced functional diversification. Bollinger *et al.* (23, 24) reported a dual function for *E. coli* GspS, the Gsp synthetase activity and a Gsp amidase activity possibly residing in distinct domains of the protein. In concert, these two activities result in futile
consumption of ATP without any meaningful anabolic outcome. The biological role of the E. coli Gsp synthetase/amidase is therefore discussed in the context of modulation of spermidine and/or GSH levels in response to growth conditions. In the GspS of C. fasciculata, despite its homology and comparable size, the amidase activity appears to have been lost during evolution. The influence of physiological effectors (i.e. product inhibition by ADP and feedback inhibition by TSH) classify the trypanosomatid GspS as a typical representative of the key enzymes of anabolic processes.

In conclusion, we have functionally and in part chemically characterized GspS, the key enzyme for the synthesis of TSH, which is believed to be the crucial mediator of the oxidant defense system in trypanosomatids (6). Trypanosomatid GspS does not exhibit any homology with known proteins in vertebrates and exhibits only limited similarity with the Gsp synthetase/amidase of E. coli. In view of the uniqueness of the protein and the widely discussed importance of TSH for trypanosomatid vitality and resistance to oxidant drugs, GspS might be considered as a potential target for a specific trypanocidal therapy. The convenient access to a stable partially purified enzyme preparation, the functional characterization, and the amino acid sequence data presented here should improve the basis for future developments to this end.

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REFERENCES
1. Smith, K., Nadeau, K., Bradley, M., Walsh, C., and Fairlamb, A. H. (1992) Protein Sci. 1, 874–883
2. Shim, H., and Fairlamb, A. H. (1988) J. Gen. Microbiol. 134, 807–817
3. Fairlamb, A. H., Blackburn, P., Ulrich, P., Chait, B. T., and Cerami, A. (1985) Science 227, 1485–1487
4. Fairlamb, A. H., and Cerami, A. (1985) Mol. Biochem. Parasitol. 14, 187–198
5. Schirmer, R. H., Muller, J. G., and Krauth-Siegel, R. L. (1995) Ang. Chemie (Int. Ed.) 34, 141–154
6. Fairlamb, A. H. (1996) Biochemist, 11–16
7. Penketh, P. G., and Klein, R. A. (1986) Mol. Biochem. Parasitol. 20, 111–121
8. Penketh, P. G., Kennedy, W. P. K., Patton, C. L., and Saritorelli, A. C. (1987) FEBS Lett. 2, 427–431
9. Henderson, G. B., Fairlamb, A. H., and Cerami, A. (1987) Mol. Biochem. Parasitol. 24, 39–45
10. Carnieri, R. G. S., Moreno, S. N. J., and Docampo, R. (1993) Mol. Biochem. Parasitol. 61, 79–86
11. Boveris, A., Sies, H., Martino, E. E., Docampo, R., Turrens, J. F., and Steppan, A. O. M. (1980) Biochem. J. 188, 643–648
12. Babior, B. M., Kipnes, B. S., and Curnutte, J. T. (1973) J. Clin. Invest. 52, 741–744
Convenient Isolation and Kinetic Mechanism of Glutathionylspermidine Synthetase from *Crithidia fasciculata*

Kerstin Koenig, Ulrich Menge, Michael Kiess, Victor Wray and Leopold Flohé

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Convenient isolation and kinetic mechanism of glutathionylspermidine synthetase from Crithidia fasciculata.*

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Since the discovery of trypanothione (N1,N8-bis(glutathionyl)spermidine), it has been debated whether its biosynthesis in Crithidia fasciculata requires one or two enzymes. After two related genes implicated in trypanothione synthesis had become available, the correct assignment of sequence to function remained controversial because heterologous expression did not yield active proteins (Tetaud, E., Manai, F., Barrett, M. P., Nadeau, K., Walsh, K. T., and Fairlamb, A. H. (1998) J. Biol. Chem. 273, 19383–19390). In 2002, however, Oza et al. (Oza, S. L., Ariyanayagam, M. R., and Fairlamb, A. H. (2002) Biochem. J. 364, 679–686) reported the functional expression of a gsps DNA sequence of C. fasciculata (GenBank™ accession number U66520.2) that did not comply with the partial amino acid sequences of glutathionylspermidine synthetase (GspS), as published in our above article.

A deep-frozen sample of the GspS preparation that had been isolated from C. fasciculata and functionally characterized by us was therefore reinvestigated. It still displayed GspS activity and no trypanothione synthetase (TryS) activity, as originally stated. Matrix-assisted laser desorption ionization time-of-flight analysis of a tryptic digest, however, did not reveal the presence of any of the peptide sequences previously reported but instead revealed many mass peaks complying with the GspS sequence. Coverage was 45% of the deduced GspS sequence and 70% of theoretically detectable peptide masses. Major mass signals that could not be attributed to the GspS sequence were absent. Therefore, the sequence assignment to GspS by Oza et al. is the correct one.

The partial sequences previously reported by us are contained in the expression product of trys (GenBank™ accession number AY603101), the homologous trypanothione synthetase that catalyzes both steps of trypanothione biosynthesis (Comini, M. A., Menge, U., Wissing, J., and Flohé, L. (2005) J. Biol. Chem. 280, 6850–6860). Thus C. fasciculata is equipped with two enzymes, one catalyzing the formation of glutathionylspermidine only and the other one being competent to catalyze both steps of the trypanothione synthesis.

How the wrong assignment of partial TryS sequences to GspS occurred could no longer be assessed with certainty. According to our laboratory notes, peptide sequencing was not performed with the same batch that had been functionally characterized. The sequenced sample had been purified to electrophoretic homogeneity by means of the chromatographic scheme described for the isolation of GspS but had not been subjected to the initial liquid/liquid extraction that separates and denatures most of the TryS. This may explain how ultimately an inactive TryS protein was obtained and sequenced instead of the presumed GspS. Leopold Flohé, who supervised this work, takes the full and sole responsibility for the erroneous interpretation of the sequence information.

*Amended by Marcelo Comini, Ulrich Menge, and Leopold Flohé.
Physiological roles of the intermediate conductance, Ca\(^{2+}\)-activated potassium channel Kcnn4.

Ted Begenisich, Tesuji Nakamoto, Catherine E. Ovitt, Keith Nehrke, Carlo Brugnara, Seth L. Alper, and James E. Melvin

Page 47684, Fig. 4A: There was an error in the labeling above the lanes in panel A. The correct figure is shown below.