Biosynthesis of the Trypanosomatid Metabolite Trypanothione: Purification and Characterization of Trypanothione Synthetase from *Crithidia fasciculata*†

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ABSTRACT: Trypanothione synthetase from *Crithidia fasciculata* has been purified ca. 14 500-fold to homogeneity in an overall yield of 40%. The pure enzyme catalyzed the synthesis of N^1- and N^8-glutathionylspermidine and N^1,N^8-bis(glutathionyl)spermidine (trypanothione) from ATP/magnesium, glutathione (GSH), and spermidine, N^1- and N^8-glutathionylspermidines being intermediates of trypanothione synthesis. The enzyme showed a sharp pH optimum of 7.5–7.75 for the synthesis of both mono- and diglutathionylspermidine conjugates. It was highly specific for its physiological substrates ATP/Mg^{2+}, GSH, spermidine, and N^1- and N^8-glutathionylspermidine with K_m values of 400 μM, 914 μM, 1.07 mM, 20 μM, and 7 μM, respectively. Trypanothione synthetase was active in the monomeric form with M_r = 87 000 and absorption maxima λ_{max} = 225 and 280 nm (A_{280}/A_{260} = 1.85). Trypanothione synthetase is a new member of the ATP-dependent class of ligases which form amide linkage with concomitant production of ADP and orthophosphate.

The tripeptide glutathione (GSH) is usually the major thiol metabolite of prokaryotic and eukaryotic cells. GSH has a role in several important biochemical processes that include regulation of the intracellular thiol/disulfide redox balance and amino acid transport. It is also a specific cofactor for enzyme systems concerned with peroxide reduction, and ribonucleotide reduction, cis/trans isomerization reactions, and it is used by higher organisms to conjugate and detoxify foreign substances (Meister, 1983). The polyamines putrescine, spermidine, and spermine are also ubiquitously distributed in living systems. Although the precise biochemical functions of polyamines remain obscure, they have been shown to be required for optimal growth in all cell types tested (Tabor, 1984). Some years ago, a curious link between the metabolism of polyamines and glutathione was established with the discovery that *Escherichia coli* synthesizes a glutathione–spermidine conjugate, N^1-glutathionylspermidine (Tabor, 1975). Of particular interest was the finding that this compound only accumulates in stationary-phase cells at which point virtually all of the free spermidine and a large part of the GSH become conjugated. The functional significance of this process in *E. coli* is not known, and attempts to characterize this glutathionylspermidine synthetase and investigate its cellular regulation have been thwarted by the reported instability of the activity.

More recently, investigation of glutathione metabolism in trypanosomes and leishmanias led to the observation that the process of GSH conjugation to spermidine also occurs in these organisms. All species of trypanosomatids examined to date possess the N^1,N^8-bis(glutathionyl)spermidine conjugate, which has been given the trivial name trypanothione [$T(SH)_2$; Figure 3]. Trypanosomatids also possess a flavoprotein disulfide reductase, trypanothione disulfide reductase, which maintains this compound in the reduced (dithiol) form (Fairlamb et al., 1985; Shames et al., 1986) and a trypanothione-dependent peroxidase activity (Henderson et al., 1987a).

The enzymes that control the biosynthesis and metabolism of trypanothione represent important targets for the development of new chemotherapeutic strategies for the treatment of the diseases caused by pathogenic trypanosomes and leishmania (Henderson & Fairlamb, 1987; Henderson et al., 1988; Fairlamb et al., 1989). We have previously shown that the biosynthesis of trypanothione involves ATP-dependent conjugation of intact GSH to the terminal amino functions of spermidine (Fairlamb et al., 1986). In an extension of these studies, we now report the purification and characterization of a single enzyme, trypanothione synthetase, which is responsible for the synthesis of glutathione–spermidine conjugates in the insect trypanosomatid *Crithidia fasciculata*.

Materials and Methods

Materials. DEAE-Sephacel was from Pharmacia. Thiolyte (monobromobimane) was purchased from Calbiochem. The following fast protein liquid chromatography (FPLC) columns were also from Pharmacia and were used on a Pharmacia FPLC system: Mono Q, phenyl-Superose, Mono P, and Superose 12. Protein molecular weight standards were from Sigma and Bio-Rad. Dihydrotrypanothione [$T(SH)_2$], N^1-glutathionylspermidine (N^1-GSHSPD), and N^8-glutathionylspermidine (N^8-GSHSPD) were chemically synthesized as described previously (Henderson et al., 1986). Trypanothione disulfide reductase was purified from *C. fasciculata* as described previously (Shames et al., 1986). *C. 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. In crude cell extracts and in partially purified enzyme preparations, synthetase activity was assayed by coupling glutathionyl spermidine or dihydrotrypanothione synthesis to NADPH oxidation. This assay is based on the ability of trypanothione disulfide reductase to reduce the disulfide form of N^1-GSHSPD and the mixed disulfide of

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N^1-GSHSPD and GSH, but not glutathione disulfide (GSSG) (Henderson et al., 1987b). The assay mixture contained 0.1 M Bis-Tris–propane (pH 7.8), 0.5 mM EDTA, 0.25 mM NADPH, 0.5 unit mL^{-1} *C. fasciculata* trypanothione reductase, 10 mM GSSG, 10 mM GSH, 10 mM spermidine, 5 mM $MgSO_4$, 2 mM ATP, 10 mM phosphoenolpyruvate (PEP), and 10 units mL^{-1} pyruvate kinase. In this system, trypanothione synthetase catalyzed ATP-dependent conjugation of GSH to spermidine, and the products, N^1-GSHSPD or $T(SH)_2$, became oxidized to the corresponding disulfide or mixed disulfide forms by nonenzymatic thiol/disulfide exchange with GSSG; these products were then reduced specifically by trypanothione reductase at the expense of NADPH. This enzymatic amplification assay is nonlinear with time but provides a sensitive indicator of trypanothione synthetase activity during the early stages of the enzyme purification. At a later stage in the purification, after the removal of high endogenous ATPase activity, it was possible to couple trypanothione synthetase specific hydrolysis of ATP to ADP + P_i to NADH oxidation by use of phosphoenolpyruvate (PEP), pyruvate kinase, and lactate dehydrogenase. In this case the assay mixtures contained 0.1 M Tris (pH 7.5), 0.5 mM EDTA, 5 mM DTT, 5 mM $MgSO_4$, 10 mM PEP, 10 mM GSH, 10 mM spermidine, 2 mM ATP, 10 units mL^{-1} pyruvate kinase (PK), and 10 units mL^{-1} lactate dehydrogenase (LDH). Absorbance changes were measured on a Varian CARY spectrophotometer with a thermostated cuvette chamber. One unit of activity is defined as the amount of enzyme required to convert 1 μmol of NADH to NAD^+ per minute at 25 °C. A pH–activity profile covering the range pH 6.0–8.0 was obtained with an equimolar mixture of Bis-Tris–propane and Tris buffers (50 mM each) and was independent of the concentration of coupling enzymes.

Protein concentrations were determined by the method of Bradford (1976) as supplied by Bio-Rad. Bovine serum albumin was used as standard.

Product Determination and Estimation of Specific Activity in Whole Cells. In order to determine the products and the specific activity of the enzyme in whole cells, GSH, spermidine, and ATP were incubated for fixed time periods with enzyme extracts. Reactions were terminated by addition of monobromobimane (thiolite), which converted the free thiol groups to the fluorescent *S*-bimane derivatives. Enzyme-catalyzed conversion of GSH to N^1-GSHSPD, N^8-GSHSPD, or $T(SH)_2$ could then be quantified by HPLC analysis and comparison with synthetic standards (Fairlamb et al., 1987).

Purification of Trypanothione Synthetase. As the enzyme activities are not stable to freezing, the following operations were carried out on freshly harvested cells as rapidly as possible at 4 °C. All buffer solutions contained 1 mM DTT and 1 mM EDTA. Trypanothione synthetase was purified ca. 14 500-fold to apparent homogeneity as follows:

(*1*) *Ammonium Sulfate Fractionation. C. fasciculata* (wild type) were grown with vigorous aeration and were harvested when the cell density reached (3–5) × 10^7 organisms mL^{-1}. Cells were concentrated approximately 40-fold on a Millipore Pellicon cell concentrator and pelleted by centrifugation at 5000*g*. The cells were washed with phosphate–saline–glucose solution and resuspended in 20 mM potassium phosphate, pH 7.0 (3× packed cell volume) containing 10 mM benzamidine, 10 mM 1,10-phenanthroline, 20 μg mL^{-1} leupeptin, and 30 μg mL^{-1} phenylmethanesulfonyl fluoride. The cell suspension was sonicated during ten 1-min pulses with intermittent 1-min cooling periods. After sonication, cellular debris was removed by centrifugation, and the supernatant was adjusted to 3% (w/v) with streptomycin sulfate and then to 30% saturation with $(NH_4)_2SO_4$. After stirring for 45 min, the solution was centrifuged, and the resulting supernatant was then adjusted to 55% saturation with $(NH_4)SO_4$. After stirring for 1 h, the solution was again centrifuged, and the resulting pellet was dissolved in the minimum quantity of 20 mM Bis-Tris–propane (pH 7.4). The enzyme solution was then dialyzed extensively against this buffer solution.

(*2*) *DEAE-Sephacel Chromatography.* The dialyzed protein solution was centrifuged (30000*g* for 30 min) and then applied to a DEAE-Sephacel column (4 × 40 cm; flow rate, 2 mL min^{-1}) that had been equilibrated previously with 20 mM Bis-Tris–propane. After being washed with 1 L of buffer, the bound proteins were eluted with a linear salt gradient (0–0.28 M KCl in Bis-Tris–propane, 1.1 L in each chamber) and then with Bis-Tris–propane containing 0.28 M KCl; 13-mL fractions were collected. Trypanothione synthetase eluted at 0.27 M KCl. Active fractions were pooled and concentrated on an Amicon concentrator (PM-10 membrane) to approximately 0.2 unit/mL, the concentrate was adjusted to 80% saturation with $(NH_4)_2SO_4$, and precipitated proteins were pelleted by centrifugation.

(*3*) *Mono Q Ion Exchange Chromatography.* The ammonium sulfate precipitated DEAE fraction was redissolved in 20 mL of 20 mM Bis-Tris–propane and dialyzed against this buffer. The resulting solution was filtered through a 0.2 μm Millipore filter disc and applied to a Pharmacia Mono Q 10/10 column that had been equilibrated with the same buffer. Proteins were eluted by application of a salt gradient as follows: buffer A, 20 mM Bis-Tris–propane; buffer B, 20 mM Bis-Tris–propane containing 0.4 M KCl. Flow rate = 1.5 mL min^{-1}; t = 0 min, % B = 0; t = 5 min, % B = 0; t = 90 min, % B = 100. Under these conditions, trypanothione synthetase eluted at 75%B, which is equivalent to 0.3 M KCl.

(*4*) *FPLC Chromatofocusing.* The protein fraction from Mono Q ion exchange chromatography was dialyzed against 5 mM histidine, pH 7.0, and after equilibration, it was adjusted to 25 mM histidine, pH 6.0. The resulting solution was applied to a Pharmacia Mono P column, and the bound proteins were eluted by application of Pharmacia Polybuffer 74 (1:8 dilution), pH 4.0, over 1 h (flow rate, 0.75 mL min^{-1}), which generated a linear pH gradient from pH 6.0 to pH 4.0. Fractions were collected in tubes that contained 50 mM Bis-Tris–propane, pH 7.8. Trypanothione synthetase eluted at pH 4.7 on this system and was >90% pure as judged by SDS–PAGE electrophoresis.

(*5*) *Superose 12 Gel Filtration.* Final purification of trypanothione synthetase was effected by gel filtration. After concentration, the chromatofocused protein fraction was fractionated on a Pharmacia Superose 12 column with 50 mM Bis-Tris–propane containing 150 mM NaCl; flow rate = 0.35 mL min^{-1}. Active fractions were pooled and used for further analysis or were stored in 45% glycerol solution at −20 °C. In this form, the specific activity of trypanothione synthetase decreased less than 10% over 3 weeks.

(*6*) *Phenyl-Superose FPLC.* In an alternative isolation procedure, the ammonium sulfate pellet from the DEAE-Sephacel chromatography was dissolved in 20 mM Bis-Tris–propane containing 0.9 M $(NH_4)_2SO_4$. The solution was filtered through 0.4- and 0.22-μm Millipore disc filters and applied to a Pharmacia phenyl-Superose 10/10 column that had been equilibrated with the same buffer. Partially inactivated trypanothione synthetase was eluted as follows: buffer A, 20 mM Bis-Tris–propane containing 0.9 M $(NH_4)_2SO_4$; buffer B, 20 mM Bis-Tris–propane. Flow rate = 0.5 mL min^{-1}; t = 0 min, % B = 0; t = 5 min, % B = 0; t = 10 min,

Table I: Purification of Trypanothione Synthetase from *C. fasciculata*

step	vol (mL)	protein (mg)	units[a]	sp act. (units mg^{-1})	purification (*x*-fold)
crude	500	16600	22.5[b]	1.36×10^{-3}	
3% streptomycin sulfate, 30–50% $(NH_4)_2SO_4$	150	4650	18.6[b]	4×10^{-3}	3
DEAE-Sephacel	250	175	20.76[c]	0.118	87
Mono Q	35	21.8	13.7[c]	0.628	461
Mono P	8	1.81	10[c]	5.52	4062
gel filtration, Superose 12	2	0.456	9[c]	19.73	14512

[a] Measured with spermidine. [b] By HPLC analysis. [c] By PK–LDH assay.

% B = 40; t = 90 min, % B = 100. Under these conditions, the synthetase eluted at 80% B, which is equivalent to 0.18 M $(NH_4)_2SO_4$. Active fractions were pooled and concentrated (Amicon PM-10 membrane) and then dialyzed extensively against 5 mM histidine, pH 7.0, and applied to the chromatofocusing column as described.

Molecular Weight Determination. The molecular weight of trypanothione synthetase was determined by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) with the following molecular weight standards: *E. coli* β-galactosidase, 116000; rabbit muscle phosphorylase *b*, 97400; bovine serum albumin, 66000; egg albumin, 45000; bovine erythrocyte carbonic anhydrase, 29000. The molecular weight of the native protein was determined by gel permeation chromatography on a Pharmacia Superose 12 column. Gel filtration standards were as follows: bovine thyroglobulin, 670000; bovine γ-globulin, 158000; chicken ovalbumin, 44000; horse myoglobin, 17000; vitamin B_{12}, 1350.

Kinetic Analysis. Analyses were carried out in 50 mM Tris buffer, pH 7.5. All solutions were preincubated at 25 °C. Michaelis constants (K_m) and maximum initial velocities (V_{max}) for GSH, spermidine, N^1-GSHSPD, and N^8-GSHSPD were determined by coupling ATP hydrolysis to NADH oxidation as described above. Initial velocity measurements were made at 12 substrate concentrations, which ranged from 0.1 to 5 mM for GSH and spermidine and from 0.005 to 0.1 mM for N^1-GSHSPD and N^8-GSHSPD. The Michaelis constant for ATP was determined by direct measurement of phosphate according to a modified Fiske–Subbarow procedure as follows: Trypanothione synthetase was incubated in 50 mM Tris, pH 7.5, containing 10 mM $MgSO_4$, 5 mM spermidine, 5 mM GSH, and 1 mM DTT and ATP. After 10 min the reactions were terminated by the addition of 10% TCA, and the amount of ATP hydrolysis (relative to control incubations minus GSH) was quantified as described previously (Chen et al., 1956).

Kinetic constants (Tables II and III) were calculated by Hanes–Woolf plots and linear regression analysis. Standard deviations (SD) were ≤10% of the value quoted in each case.

Results

Identification and Purification of Trypanothione Synthetase. Crude lysates of *C. fasciculata* were found to contain enzyme activity that catalyzed ATP/Mg-dependent synthesis of N^1-GSHSPD and $T(SH)_2$ when incubated with spermidine and GSH. The products formed by the crude extracts were analyzed by HPLC (Fairlamb et al., 1987) at fixed time points over 15 min. From these measurements linear rates of N^1-GSHSPD and $T(SH)_2$ production by the crude cell lysate were determined to be 0.66 and 0.35 nmol min^{-1} (mg of protein)$^{-1}$, respectively.

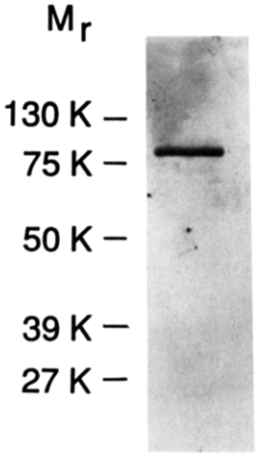

FIGURE 1: Analysis of trypanothione synthetase by SDS–polyacrylamide gel electrophoresis.

Following ammonium sulfate fractionation, chromatography on DEAE-Sephacel, and FPLC using ion exchange, chromatofocusing, and gel permeation columns, trypanothione synthetase was purified ca. 14500-fold in an overall yield of 40% (Table I). The purified enzyme appeared as a single band on SDS–polyacrylamide gel electrophoresis (Figure 1). This enzyme catalyzed the synthesis of both N^1-GSHSPD and $T(SH)_2$ from spermidine, as well as $T(SH)_2$ from either N^1-GSHSPD or N^8-GSHSPD.

An alternative purification scheme that included hydrophobic chromatography on a phenyl-Superose column gave rise to a partially inactivated trypanothione synthetase. This chromatographic step (described above) effected 10–15-fold purification. However, the enzyme lost ca. 65% of its native activity when assayed with spermidine, GSH, and ATP/Mg^{2+}, and its ability to convert N^1-GSHSPD or N^8-GSHSPD (even when assayed at 0.1 mM) to $T(SH)_2$ was abolished. Surprisingly, binding of spermidine and GSH was largely unaffected (apparent K_m values being 1.20 and 1.00 mM, respectively). It appears that the phenyl-Superose matrix caused some irreversible conformational change in the protein which primarily affected the binding of either monoglutathionylspermidine isomer in the active site.

Physical and Kinetic Characterization. Trypanothione synthetase, purified as described (Table I), and also partially inactivated protein from phenyl-Superose chromatography were found to have a molecular weight of 87000 by SDS–polyacrylamide gel electrophoresis (average of five separate determinations). The molecular weight of native enzyme, as determined by gel permeation chromatography on a Superose 12 column, was observed to be 81000. These data suggest that native trypanothione synthetase is active in the monomeric form. The pure protein had absorption maxima (λ_{max}) at 225 and 280 nm (A_{280}/A_{260} = 1.85), indicating the absence of covalently bound cofactors.

The pure enzyme catalyzes the sequential addition of two molecules of GSH to the terminal amino functions of spermidine. During the course of this conversion the intermediate N^1-GSHSPD isomer accumulates when spermidine, GSH, and ATP are present at saturating concentration. To investigate the pH dependence of the reaction, formation of both final and intermediate products was monitored by HPLC analysis (Figure 2). After a short lag (reflecting the buildup to saturation of the intermediate monoglutathionylspermidine products), formation of both mono- and diglutathionylspermidine adducts was linear within the time course of the analysis. The enzyme displayed pH optima for

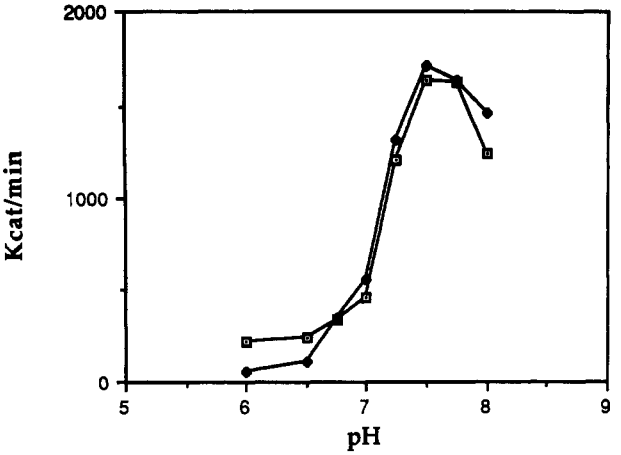

FIGURE 2: Simultaneous formation of N^1-GSHSPD (□) and $T(SH)_2$ (●) from spermidine, GSH, and Mg/ATP as a function of pH. Product formation was quantified by HPLC analysis. Each rate was determined from six time points between 1 and 10 min.

Table II: Catalytic Parameters of Trypanothione Synthetase

substrate	K_m (μM)	relative K_{cat}	K_{cat}/K_m (M^{-1} s^{-1})
spermidine	1070	1	2.67×10^4
GSH	914	1	3.13×10^4
ATP/Mg^{2+}	400	1	7.15×10^4
N^1-GSH-SPD	20	0.35	5.00×10^5
N^8-GSH-SPD	7	0.85	3.47×10^6

both reactions at 7.5–7.75 with the relative rates of product formation being identical above pH 6.75. This is a reflection of the relative catalytic parameters (Table II) and is consistent with a preferred enzymatic route to $T(SH)_2$ via N^8-GSHSPD (Figure 3). Interestingly, at a pH of less than 6.75 the rate of formation of N^1-GSHSPD exceeds the synthesis to $T(SH)_2$; these rates, as discussed below, also reflect product distribution in vivo.

At pH 7.5, the trypanothione synthetase displayed normal Michaelis–Menten saturation kinetics for the physiological substrates, GSH, ATP, spermidine, N^1-GSHSPD, and N^8-GSHSPD. In addition, the reaction was found to be dependent on Mg^{2+}; however, this substrate did not display normal saturation kinetics, suggesting that the active reactant may be the Mg/ATP complex. As described previously (Fairlamb et al., 1986), other nucleotide triphosphates would not substitute for ATP. In addition, when glutathione disulfide (GSSG) was tested as a substrate, the rate of reaction was less than 1% that supported by GSH.

Apparent K_m values for ATP, GSH, spermidine, N^1-GSHSPD, and N^8-GSHSPD were obtained (Table II), and a preliminary investigation of the substrate specificity of the enzyme was investigated with analogues of spermidine (Table III). In addition, some partial reactions of the enzyme were investigated. When the enzyme was incubated in the PK–LDH-coupled enzyme assay system alone or in the presence of spermidine, there was negligible ATPase activity. However, when incubated with GSH, the enzyme catalyzed ATP hydrolysis. At saturating GSH, trypanothione synthetase catalyzed ATP hydrolysis at ca. 6% of the rate supported by both GSH and spermidine. Under conditions in which all substrates were present in saturating concentrations, the specific activity of trypanothione synthetase at pH 7.5 was determined to be 19.73 μmol (of ATP) min^{-1} mg^{-1}. Taking the molecular weight of the pure protein as 87 000, this yields a turnover number of 1715 min^{-1} with spermidine as substrate. This is a K_{cat} for total production of glutathionylspermidine conjugates. When N^1-GSHSPD and N^8-GSHSPD were used, the turnover

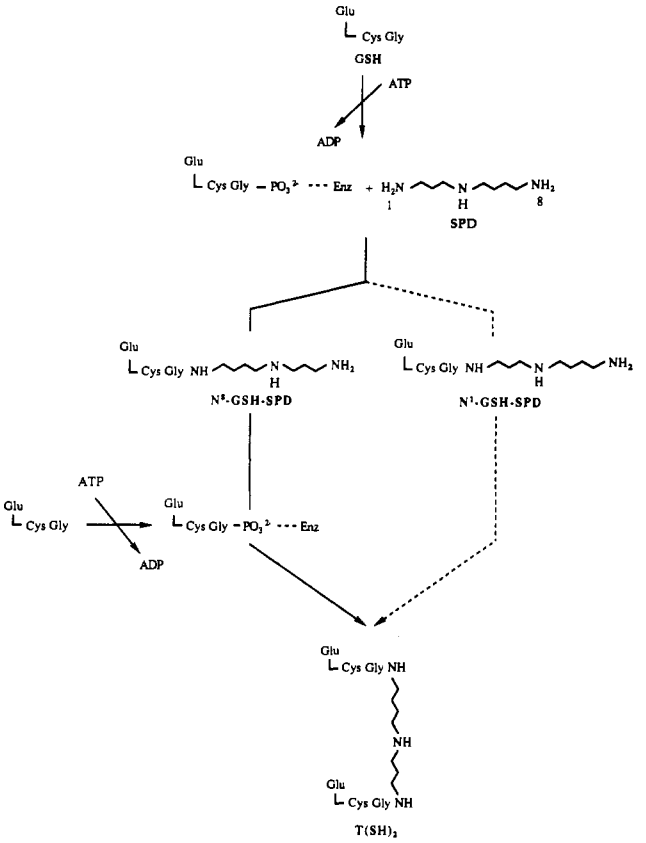

FIGURE 3: Proposed enzymatic formation of N^1-GSHSPD and $T(SH)_2$ from GSH, ATP/Mg^{2+}, and spermidine.

Table III: Catalytic Parameters of Spermidine Analogues

spermidine analogues[a]	relative K_{cat}
spermidine	1
spermine	0.20
H_2N HN	0.30
H_2N NH_2	0.04
H_2N NH_2	0.06
H_2N N NH_2 (CH_3)	0.176
H_2N NH_2	0.075

[a] Measured at 5 mM.

numbers were 600 and 1457 min^{-1}, respectively.

Discussion

Trypanothione, N^1,N^8-bis(glutathionyl)spermidine, is a novel dithiol metabolite that is present in the pathogenic trypanosomatids responsible for African sleeping sickness, South American Chagas' disease, and visceral and cutaneous leishmaniasis (Kala-Azar). These and other trypanosomatids appear to have replaced classical glutathione-dependent processes with trypanothione-dependent counterparts. For this reason, the enzyme that effects trypanothione biosynthesis in these pathogenic organisms represents an important and potentially specific target for the development of much-needed new chemotherapy. As a first step in this direction we have now characterized the biosynthesis of this molecule and purified the enzyme, trypanothione synthetase, to homogeneity. The insect trypanosomatid *C. fasciculata* was chosen as an initial biochemical source and model of the human pathogens because of its nonpathogenic nature and ease of culture. In addition, this organism has proved a reliable model in previous

studies directed at other aspects of trypanothione metabolism (Shames et al., 1986).

In an earlier investigation of glutathione conjugation to spermidine in *C. fasciculata*, cell lysates of this organism were shown to catalyze conversion of spermidine, N^1-GSHSPD, or N^8-GSHSPD to trypanothione. As N^1-GSHSPD could be detected in this and other trypanosomatid species, it was postulated to be a biosynthetic intermediate of trypanothione (Fairlamb et al., 1986). The biosynthesis of trypanothione and the distribution of glutathionylspermidine conjugates in *C. fasciculata* and other trypanosomatids are now readily interpreted in terms of the catalytic properties of trypanothione synthetase (Figure 3). At neutral pH, this enzyme will convert spermidine to the N^1- and N^8-glutathionylspermidine conjugates. Given intracellular spermidine concentrations in the millimolar range (Fairlamb et al., 1986), which is similar to the apparent K_m for this substrate, the much higher affinity of trypanothione synthetase toward the monoglutathionylspermidine conjugates (K_m = 20 and 7 μM for N^1-GSHSPD and N^8-GSHSPD, respectively) dictates that these products will effectively compete with spermidine for the enzyme and be converted to trypanothione. Although intermediate monoglutathionylspermidine (primarily N^1-GSHSPD) can (and does) accumulate intracellularly, under conditions of normal growth the enzyme is committed to trypanothione synthesis.

On consideration of the relative catalytic efficiency of turnover of N^1-GSHSPD and N^8-GSHSPD, it is clear that the N^8-isomer will not accumulate in the incubation mixtures or within the cell because the preferred enzymatic route to trypanothione is via this intermediate. These results are supported by our previous analysis (Fairlamb et al., 1968) in which the relative rates of conversion of N^8-GSHSPD and N^1-GSHSPD to $T(SH)_2$ in crude extracts of *C. fasciculata* was determined to be 1.64:1 (analyses carried out at saturating concentration) and in which the intracellular ratio of the N^1- to N^8-isomer was determined to be 10:1. This interpretation is also supported by a related study of glutathionylspermidine conjugates in *C. fasciculata* (Shim & Fairlamb, 1988) in which the intracellular concentrations of the conjugates was determined and in which the intracellular ratio of N^1-GSHSPD to $T(SH)_2$ was observed to change from 0.6 in logarithmic-phase cells to 6.0 in stationary-phase cells. It seems likely that this change may simply reflect a drop in the intracellular pH in which case the rate of N^1-GSHSPD synthesis would exceed that of synthesis of $T(SH)_2$. Recent ^{31}P NMR studies which show that the intracellular pH of *C. fasciculata* does decrease from 7.2 to 6.3 during late-log phase lend support to this suggestion (Grota et al., 1989). The commitment of the enzyme toward trypanothione synthesis is evident on analysis of its specificity toward amine substrates (Table III). 1,4-Diaminobutane (putrescine), 1,3-diaminopropane, and 1,8-diaminooctane are poor substrates while 1-propyl-1,3-diaminopropane and N,N'-bis(3-aminopropyl)-1,4-diaminobutane (spermine) compete effectively with spermidine. In this context it is relevant to note that putrescine is ubiquitously found in trypanosomatids but these organisms do not synthesize spermine.

A more detailed kinetic and mechanistic analysis of trypanothione synthetase is clearly warranted but is beyond the scope of this study. The present experiments however suggest that trypanothione synthetase may be related to a number of known ATP-dependent ligases. These include glutamine synthetase (Stadtman, 1974), τ-glutamylcysteine synthetase (Meister, 1974), and D-alanine:D-alanine ligase (ADP) (Duncan & Walsh, 1988), all of which, like trypanothione synthetase, catalyze amide bond formation at the expense of ATP hydrolysis to ADP and orthophosphate (P_i). This being the case, glutathione conjugation to spermidine most likely proceeds by a mechanism that involves initial binding of GSH and subsequent glycylcarbonyl activation via an acyl phosphate intermediate which undergoes nucleophilic attack by the N^1- or N^8-amino function of spermidine (or monoglutathionylspermidine). The finding that trypanothione synthetase catalyzes ATP hydrolysis to ADP and P_i on incubation with GSH, but not with spermidine alone, is consistent with this mechanism. Under these conditions, the intermediate GSH-PO_3^{2-} is most likely hydrolyzed by water within the catalytic center as attempts to isolate this product (under conditions in which analogous acyl phosphates are stable) proved unsuccessful.

In conclusion, with the purification of a highly active trypanothione synthetase it is now possible to begin molecular characterization of this novel enzymatic process which has no known mammalian counterpart. Further investigation of the catalytic mechanism and the specificity of trypanothione synthetase toward GSH, spermidine, and analogues of these compounds could, in principle, lead to the synthesis of specific inhibitors (e.g., sulfoximine or alkylphosphinate derivatives) of the enzyme which may have pharmacological relevance. Further characterization of the enzyme would also be facilitated by gene cloning, amino acid sequencing, and its expression in a suitable vector. Our research efforts are now focused in these directions.

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References

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.
Chen, P. S., Jr., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.
Duncan, K., & Walsh, C. T. (1988) *Biochemistry 27*, 3709–3714.
Fairlamb, A. H., Blackburn, P., Ulrich, P., Chait, B. T., & Cerami, A. (1985) *Science 227*, 1485–1487.
Fairlamb, A. H., Henderson, G. B., & Cerami, A. (1986) *Mol. Biochem. Parasitol. 21*, 247–257.
Fairlamb, A. H., Henderson, G. B., Bacchi, C. J., & Cerami, A. (1987) *Mol. Biochem. Parasitol. 24*, 185–191.
Fairlamb, A. H., Henderson, G. B., & Cerami, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 2607–2611.
Grote, R. M., Edwards, M. R., Norton, R. S., & O'Sullivan, W. J. (1989) *International Symposium on Molecular Parasitology*, Taiwan, Abstract S-23.
Henderson, G. B., & Fairlamb, A. H. (1987) *Parasitol. Today 3*, 312–315.
Henderson, G. B., Ulrich, P., Fairlamb, A. H., & Cerami, A. (1986) *J. Chem. Soc., Chem. Commun.*, 593–594.
Henderson, G. B., Fairlamb, A. H., & Cerami, A. (1987a) *Mol. Biochem. Parasitol. 24*, 39–45.
Henderson, G. B., Fairlamb, A. H., Ulrich, P., & Cerami, A. (1987b) *Biochemistry 26*, 3023–3027.
Henderson, G. B., Ulrich, P., Fairlamb, A. H., Rosenburg, I., Pereira, M., Sela, M., & Cerami, A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5374–5378.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Le Trang, N., Meshnick, S. R., Kitchener, K., Eaton, J. W., & Cerami, A. (1983) *J. Biol. Chem. 258*, 125–130.
Meister, A. (1974) *Enzymes (3rd Ed.) 10*, 671–697.

Meister, A., & Anderson, M. (1983) *Annu. Rev. Biochem. 52*, 711-760.
Shames, S. L., Fairlamb, A. H., Cerami, A., & Walsh, C. T. (1986) *Biochemistry 25*, 3519-3526.
Shim, H., & Fairlamb, A. H. (1988) *J. Gen. Microbiol. 134*, 807-817.
Stadtman, E. R., & Ginsburg, A. (1974) *Enzymes (3rd Ed.) 10*, 755-807.
Tabor, H., & Tabor, C. W. (1975) *J. Biol. Chem. 250*, 2648-2654.
Tabor, C. W., & Tabor, H. (1984) *Annu. Rev. Biochem. 53*, 749-790.

Molecular Organization and Structural Stability of β_s-Crystallin from Calf Lens†

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ABSTRACT: β_s-Crystallin has been purified to homogeneity. Its structural features and conformational behavior have been studied in solution. Protein secondary structure was estimated by curve fitting of far-UV circular dichroism spectra, which gave 16% α-helix, 45% β-sheet, 12% bends, and 27% remainders. This result indicates that the structural organization of β_s-crystallin is reasonably similar to that of other β and γ family members. A comparison assessed between β_s- and γ_2-crystallin by the use of predictive methods (flexibility and volume plots) reveals that the two proteins differ in respect to their local flexibility and packing, although they show similar overall organization. The interdomain and the C-terminal regions were found to be more flexible in β_s-crystallin. This finding can be explained by the presence of smaller amino acid residues within these structural districts. The location of one out of four tryptophans, i.e., Trp-162, in a flexible and exposed region of the protein was found to be the origin of the fluorescence heterogeneity. In fact, the fluorescence emission maximum of the native protein, centered at 328 nm, is due to two emitting centers, whose emission maxima are located at 323 and 330 nm, respectively, as evidenced by acrylamide quenching of fluorescence. The effect of perturbing agents, such as pH and guanidine hydrochloride, on the conformational behavior of β_s has also been evaluated by numerous spectroscopic techniques. The range of pH stability was between 6.5 and 8. Above this interval, a conformational change takes place. In the acid region, the protein is unstable and precipitates irreversibly. The conformational resistance to guanidine hydrochloride has also been shown to be weak. GdnHCl denaturation curves were neither superimposable nor ascribable to a very cooperative transition. This result suggests a non-two-state denaturation equilibrium reflecting the presence of structural domains. The main conclusion of our work is that the protein shows a very narrow range of stability. This result indicates that an inherent structural stability may not be a general property of lens proteins. Therefore, the evolutive hypothesis about a specific recruitment of stable proteins in the lens architecture may need reconsideration.

The eye lens is remarkable for its high protein content (Lindley et al., 1985). It is organized in a complex supramolecular system, responsible for the lens optical properties. The lens can thus be considered a real "proteic" lens, whose supramolecular structure is a tridimensional network of proteins. Their role and function are still barely known. The main fraction of these proteins is represented by the "crystallins", a group formed by three major species of proteins designated as α-, β-, and γ-crystallins (Lindley et al., 1985). These proteins are believed to act as the primary determinant of the lens tissue's optical properties (e.g., transparency, refractive index, optical anisotropy, etc.), but no biological activity has been associated with them. Of course, numerous other minor protein species are also present in the lens, such as some enzymes, membrane proteins, filamentous proteins, etc. (Alcala & Maisel, 1985). No clear data are available regarding the relative distribution and role of different proteins within the supramolecular network of the normal lens. Only recently, systematic and detailed structural studies of each proteic component of normal lens have begun [e.g., see Tardieu et al. (1986), Mandal et al. (1987), and Wistow et al. (1983)].

The β fraction is composed of β_L (organized in dimers and trimers) and of β_H (organized in higher aggregates) components (Siezen & Argos, 1983). The γ fraction is composed of several low molecular weight monomeric isoproteins (Pulcini et al., 1989), the biosynthesis of which is modulated during the differentiation (Slingsby & Croft, 1973; Slingsby & Miller, 1983; Siezen et al., 1985). β_s is a very peculiar minor component of the β fraction, since it is the only monomeric low molecular weight soluble protein of this fraction (Lindley et al., 1985). β- and γ-crystallins form a superfamily of related proteins, which are likely derived from a common gene (Wistow et al., 1981). They have been found to be homologous by sequence analysis. This finding is very interesting because only the γ_2 structure is known at the X-ray level (Wistow et al., 1983). The possible organization of the common structural unit shared by β and γ family members should be a four-motif β-barrel structure (Quax-Jeuken et al., 1985). We have focused our attention on β_s, the molecular properties of which are still unknown. This protein resembles γ components as far as solubility, molecular weight, and monomeric structure are concerned, but it is classically classified into the β fraction.

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