Glutathionylspermidine Synthetase

Glutathionylspermidine synthetase (GspS) catalyzes the first of two steps of trypanothione biosynthesis, the synthesis of glutathionylspermidine (Gsp) from glutathione (GSH) and spermidine with the consumption of ATP (1). Trypanothione (N^1, N^8 - bis(glutathionyl)spermidine, TSH) is a metabolite unique to trypanosomatids such as *Trypanosoma* species, *Leishmania* species, and *Crithidia fasciculata* (2). These parasites comprise pathogens causing widespread and difficult-to-treat tropical diseases such as African sleeping sickness (*T. brucei gambiense* or *T. brucei rhodesiense*), Chagas disease (*T. cruzi*), kala azar (*L. donovani*), oriental sore (*L. tropica*) and mucocutaneous leishmaniasis (*L. braziliensis*). Others (e.g. *T. 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 thereby formed is reduced by the NADPH-dependent trypanothione reductase (14, 15), a flavoprotein homologous to glutathione reductase which 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 of 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 (2, 20, 21). The major biological function of TSH in trypanosomatids is to serve as a reducing substrate for thioredoxin-like proteins called tryparedoxins (23). Tryparedoxins in turn may have a variety of functions in replacing thioredoxin which so far could never be identified in trypanosomatids. A prominent role of tryparedoxin consists in the regeneration of tryparedoxin peroxidase after reaction thereof with a hydroperoxide such as H_2O_2, a fatty acid hydroperoxide, or a hydroperoxide of a complete lipid (24). Thereby, GspS together with trypanothione synthetase, trypanothione reductase, tryparedoxin, and tryparedoxin peroxidase constitutes the most complex system to protect trypanosomatids against oxidative damage. By analogy, reduced tryparedoxin may also substitue for thioredoxin in other pathways, e.g. in the reduction of ribonucleotides thereby becoming essential for the entire nucleic acid metabolism in trypanosomatids.

The first enzyme catalyzing synthesis of Gsp has once been isolated in trace amounts from *C. fasciculata* (0.5 mg from 500 g wet cell mass) and characterized in terms of the apparent MW, kinetic parameters, and substrate specificity (1).

Another deduced amino acid sequence obtained from *C. fasciculata* first claimed to represent a trypanothione-synthetase-like protein(acc. number U66520) was submitted to Genbank on August 9, 1996, became available to the public in February 1997 and was reported to be the sequence of glutathionylspermidine synthetase without, however, providing any experimental data supporting this assignment. In terms of size and sequence, this putative GspS of *C. fasciculata* is not identical with the GspS of *C. fasciculata* described in the present invention. This protein differs substantially from GspS described here in molecular mass (90 (1) versus

78 - 79 kDa, respectively) and pH optimum (6.5 (1) versus 7.5). Also, the previously described enzyme reportedly hydrolyzed ATP in the absence of spermidine (1), whereas such activity was not detectable in GspS as characterized here. Taken together, these discrepancies demonstrate that the two preparations can not be considered identical or equivalent.

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 synthesis and breakdown results in an apparently futile ATP consumption, the biological role of which remains speculative (25, 26). Since *E. coli* does not produce TSH, its GspS has obviously 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.

Most importantly, we here describe a method for purification of GspS from C. fasciculata yielding an enzyme pur enough to be sequenced. The partial amino acid sequence enables the identification of the pertinent gene and the heterologous expression therof by methods known per se, thereby making GspS available for the identification of specific inhibitors useful as trypanocidal drugs. Further, we have invented a simple and convenient method to partially purify GspS from *C. fasciculata*. Such preparation does not catalyze any ATP hydrolysis in the absence of further GspS substrates and cofactors, i. e. spermidine and magnesium. This implies that GspS activity and inhibition thereof can be specifically measured simply by liberation of inorganic phosphate from ATP in such partially purified GspS. This test system is easily automatized for large scale inhibitor screening.

Thus, one embodiment of the invention concerns a protein characterized by its ability to catalyze the synthesis of glutathionylspermidine with a pH optimum of about 7.5.

The protein according to the invention is further characterized by an apparent molecular weight of 78,000 ± 3000 Da.

The protein is further characterized by comprising partial sequences shown in fig. 1 and being homologous to glutathionylspermidine synthetase/amidase of *Escherichia coli*.

The protein according to the invention is further characterized by being isolated from a species of the family trypanosomatidae or produced in any other species by recombinant DNA techniques making use of the partial amino acid sequences shown in fig. 1, genetic probes or primers derived thereof or encoding nucleic acid sequences thus obtained, e. g. SEQ ID NO1 (fig. 2) or any useful part thereof.

The protein according to the invention is further characterized by comprising the partial amino acid sequence deduced from the nucleic acid sequence SEQ ID NO1.

The protein according to the invention is further characterized by comprising or having a sequence which is at least 70 %, preferntially 75 % identical to that deduced from SEQ ID NO1, respectively.

The protein according to the invention is also any modification thereof genetically designed for facilitaded purification such as e. g. an carboxyterminal polyhistidine extension.

Another embodiment of the invention is a simple process to purify GspS to an extent that its activity as well as the inhibition thereof can be conveniently but specifically tested by liberation of inorganic phosphate from adenosyltriphosphate (ATP) in the presence of spermidine, glutathione and magnesium ions.

The process of the invention is characterized by making use of aqueous two phase systems containing polyethyleneglycol.

Another embodiment of the invention is the specific determinateion of GspS activity by means of the detection of inorganic phosphate from ATP by partially purified GspS.

This analytical process is characterized by not being disturbed by any other ATP hydrolyzing activity enhanced by glutathione and spermidine.

Finally, another embodiment of the invention concerns a pharmaceutical preparation having trypanocidal activity and comprising an inhibitory substance according to the invention or of a protein which can be obtained according to the process according to the invention to produce said protein.

The pharmaceutical preparation according to the invention can be characterized in that it can be obtained by use according to the invention and/or by using a test system according to the invention.

The invention is now described by means of figures and examples

Figure 1. Partial sequences of GspS from *C. fasciculata* compared to Gsp synthetase/amidas from *Escherichia coli*. The amino acid numbering corresponds to the *E. coli* sequence (23). * = identical amino acids, + = similar amino acids found in GspS from *E. coli*.

Figure 2. Partial DNA sequence encoding GspS of *C. fasciculata*. Corresponding peptide sequences in the deduced amino acid sequence, which are verified by peptide sequencing of authentic GspS from *C. fasciculata* are underlined.

Figure 3. Extraction of glutathionylspermidine synthetase in aqueous two-phase systems. GspS = glutathionylspermidine synthetase, TS = trypanothione synthetase. For extraction of GspS aqueous two-phase systems were prepared by weighing in concentrated solutions of the phase components and finally the crude extract. A poly(ethylene glycol) (PEG)/phosphate system containing 7.5% (w/w) PEG_{6000}, 13% (w/w) Na-K-phosphate, pH 7.0, and 40% crude homogenate. The mixture was gently shaken for 10 min at room temperature and separated by centrifugation at 5000 g.
With the first system we yielded an extraction of GspS into the top phase with a purification factor of 30 in one step.

1. System: GspS was extracted into the top phase of an aqueous two-phase system containing 7.5% (w/w) PEG_{6000} and 13% (w/w) Na-K-phosphate, pH 7.0. 2. System: the PEG-rich top phase of the first system containing GspS was applied to a bottom phase of an identical system containing water instead of cell lysate. 3. System: the top phase of the second phase system was added to an acidified bottom phase of a blank system. The GspS was now extracted into the phosphate-rich bottom phase.

Figure 4. SDS-PAGE analysis of the glutathionylspermidine synthetase fractions during purification. Lanes are as follows: 1, SDS marker proteins; 2, pooled fractions after chromatography on Mono P; 3, pooled fractions after chromatography on Poros 20 PE; 4, pooled fractions after chromatography on Poros 20 Pi; 5, pooled fractions after chromatography on Resource Q.

Figure 5. Titration curve analysis of the homogeneous glutathionylspermidine synthetase. First dimension: isoelectric focusing, run without protein sample; second dimension: native PAGE.

Figure 6. Scheme of the glutathionylspermidine synthetase as a rapid equilibrium random terreactant system.

Figure 7. pH optimum of glutathionylspermidine synthetase. Product formation of Gsp was analyzed as described under Experimental Procedures. Values are means ± standard deviations from two independent measurements done at 10 and 20 min.

Figure 8. ^{31}P NMR spectra during the reaction of the glutathionylspermidine synthetase with their substrates. (A) GspS in the presence of ATP, incubation time = 0 min, scanning time = 7 min; (B) GspS in the presence of ATP, incubation time = 5 h, scanning time = 30 min; (C) addition of the second substrate (GSH or spermidine), incubation time = 5 h, scanning time = 30 min; (D) addition of the third substrate (GSH or spermidine), incubation time = 5 h, scanning time = 30 min. The peaks are assigned to (1) inorganic phosphate, (2) ATP, γ-P, (3) ADP, β-P , (4) ADP, α-P, (5) ATP, α-P, and (6) ATP, β-P.

Figure 9. Malachite green colorimetric assay for liberation of inorganic phosphate. The malachite green colorimetric assay for liberation of inorganic phosphate (1) was used for fast detection of GspS activity.

The standard assay was carried out at 25.0°C in a volume of 0.15 ml containing 50 mM Bis-Tris-propane, 50 mM Tris, pH 7.5, 5 mM $MgSO_4$, 1 mM EDTA, 5 mM DTT, 5 mM ATP, 10 mM GSH, 10 mM spermidine, and GspS. After an incubation of 10 min the malachite green reagent was added for detection of GspS activity.

High enzymatic GspS activity is visualized by a dark green color of the assay.

Blank = standard assay with water instead of GspS; no liberation of phosphate, therefore the color of the assay is yellow instead of green.

ADP significantly inhibits GspS. The type of inhibition is competitive with respect to ATP.

Concentration of ATP (see heading) and ADP (see table).

ATP [mM]:-> 0.2 mM 0.2 mM 0.5 mM 0.5 mM 1 mM 1 mM 2 mM 2 mM 5 mM 5 mM

	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	10	10	10	10	10	10	10	10	10	10	
B	blank	5	5	5	5	5	5	5	5	5	5	
C	blank	2	2	2	2	2	2	2	2	2	2	
D	blank	1	1	1	1	1	1	1	1	1	1	
E		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	
F		0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
G		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	
H		0	0	0	0	0	0	0	0	0	0	

EXAMPLES

Example 1. Purification of GspS.

Production of starting material: *C. fasciculata* was grown in a medium previously described (27) in a 100-l fermenter at 27°C with continuous stirring (200 rpm) and aeration (0.1 vvm). Organisms were harvested in the late logarithmic growth phase by continous flow centrifugation. The pellet was resuspended with 100 mM HEPES buffer (pH 7.5) containing 1 mM DTT and 1 mM $MgSO_4$. After centrifugation the cells were stored at -20°C.

GspS Assay: The assays 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, 5 mM $MgSO_4$, 1 mM EDTA, 5 mM DTT, 5 mM ATP, 10 mM GSH, and 10 mM spermidine (1). The assay for 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 four-fold with water. Separation and analytical conditions were as described previously (28). HPLC-analysis was performed with a Jasco-HPLC-system consisting of an autosampler (851-AS), a pump (PU-980), a ternary gradient unit (LG-980-02), and a highly sensitive fluorescence detector (FP-920), which enabled a precise analysis of the small product peak in the presence of numerous other and larger ones. An external standard (0.04 mM Gsp) was used for integration calibration of the samples.

Extraction in Aqueous Two-Phase Systems: 250 g cells were suspended in 250 ml of 20 mM Bis-Tris-propane puffer, pH 7.5, disrupted by freezing in liquid nitrogen and thawing. The crude homogenate was subjected to an aqueous two-phase extraction at room temperature. All other operations were performed at 4°C.

For extraction of GspS aqueous two-phase systems (total mass 900 g) were prepared by weighing in concentrated solutions of the phase components and finally the crude extract (Fig. 3). A poly(ethylene glycol) (PEG)/phosphate system containing 7.5% (w/w) PEG_{6000}, 13% (w/w) Na-K-phosphate, pH 7.0, and 40% crude homogenate (or water in the blank systems) was used. The mixture was gently shaken for 10 min at room temperature and separated by centrifugation at 5000 g. The top phase was sucked off and applied to a bottom phase of a blank system. After mixing, centrifugation, and separation of the phases the PEG-rich top phase of the second phase extraction was mixed with a blank bottom phase, adjusted to pH 6.0 with HCl. This third system was mixed again, centrifuged, and separated. Now the GspS was found in the phosphate-rich bottom phase.

Diafiltration: The phosphate-rich third bottom phase and other pooled enzyme fractions were diafiltrated with an omega membrane with a cut-off of 30 kDa (Filtron Minisette) using a Pro Flux M12 (Amicon) at 0.2 MPa and a 500-fold volume of 2 mM Bis-Tris-propane buffer, pH 8.0.

Resource Q Chromatography: A BioLogic-System (Bio-Rad) was used at 4°C for all chromatographies. The diafiltrated protein mixture was applied onto a Resource Q column (6 ml) (Pharmacia) equilibrated with 2 mM Bis-Tris-propane buffer, pH 8.0. After washing with 10 column volumes of equilibration buffer, the bound proteins were eluted at a flow rate of 1 ml/min with a gradient of 0.0 to 0.4 M KCl (100% B) as follows: t = 0 min, B = 0%; t = 20

min, B = 15%; t = 40 min, B = 15%; t = 60 min, B = 30%; t = 120 min, B = 30%; t = 150 min, B = 100%. The GspS eluted at 0.27 M KCl and the pooled active fractions were diafiltrated with 2 mM Bis-Tris-propane buffer, pH 6.0.

Poros 20 Pi Chromatography: The diafiltrated proteins were applied onto Poros 20 Pi (0.46 x 10 cm, 1.7 ml) (Perseptive Bioystems) equilibrated with 2 mM Bis-Tris-propane buffer, pH 6.0. After washing with 10 column volumes of equilibration buffer, bound proteins were eluted at a flow rate of 4 ml/min with a gradient of 0 to 1 M NaCl (100% B) as follows: t = 0 min, B = 0%; t = 8 min, B = 35%; t = 16 min, B = 35%; t = 17 min, B = 37%; t = 21 min, B = 37%; t = 25 min, B = 100%. GspS eluted at 0.7 M NaCl.

Poros 20 PE Chromatography: Pooled active fractions were adjusted to 1 M ammonium sulfate and applied onto a hydrophobic interaction chromatography column Poros 20 PE (0.46 x 10 cm, 1.7 ml) (Perseptive Biosystems) equilibrated with 20 mM Bis-Tris-propane buffer, pH 8.0, containing 1 M ammonium sulfate, washed with 10 column volumes of equilibration buffer, and eluted with a linear gradient of 1 to 0 M ammonium sulfate and a flow rate of 4 ml/min over 7.5 min. GspS eluted at 0.75 M ammonium sulfate. Pooled active fractions were diafiltrated with 10 mM Bis-Tris-propane buffer, pH 6.8.

Mono P Chromatography: The diafiltrated fraction was applied onto a Mono P HR 5/20 column (4 ml) (Pharmacia) for anion exchange chromatography. The column was equilibrated with 10 mM Bis-Tris-propane buffer, pH 6.8. After washing with 10 column volumes of equilibration buffer, bound proteins were eluted with a gradient of 0 to 1 M NaCl (100% B) as follows: t = 0 min, B = 0%; t = 20 min, B = 25%; t = 40 min, B = 25%; t = 60 min, B = 50%; t = 80 min, B = 50%; t = 100 min, B = 100%. The flow rate was 1 ml/min. GspS eluted at 0.45 M NaCl.

Results: The purification strategy outlined above resulted in a GspS preparation with a specific activity of 37.6 U/mg at an overall yield of about 20%. The purification factor achieved was 12,500. As is seen from table 1, the phase distribution system applied proved to be highly efficient in enriching GspS.

The optimized procedure was based on a factorial design of phase compositions (31), i.e. PEG_{6000}/phosphate (7.5/13% (w/w)), PEG_{4000}/phosphate (8/14% (w/w)), PEG_{1550}/phosphate (9/18% (w/w)), each tested at pH 4.0, 5.5, and 7.0 and containing 40% cell lysate. By

centrifugation the cell debris were 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) PEG_{6000}, 13% (w/w) phosphate, pH 7.0, yielded an extraction of GspS into the top phase (Fig. 3) 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 trypanothione synthetase (TS) activity. While GspS was recovered completely in the top phase, TS activity was extracted into the bottom phase (Fig.3), but proved to be unstable and was not purified further. After two extractions into top phases GspS was essentially free of interfering enzymatic activities and could be precisely quantitated by ATP hydrolysis in the presence of glutathione and spermidine. 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 diafiltrated and then could be loaded onto a Resource Q column. GspS thus purified appeared homogeneous by SDS-PAGE (Fig. 4) and by titration curve analysis (Fig. 5).

Example 2. Determination of physical parameters of GspS.

Molecular mass estimation by gel permeation 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-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and carbonic anhydrase (30 kDa) were used as standards.

Gel permeation chromatography on Superose 12 indicated a molecular mass of 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 79 kDa.

Electrophoresis: The subunit molecular weight was determined by SDS-PAGE (28) using a PhastGel Gradient 8-25 (Pharmacia) with the following molecular weight standards: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

A subunit molecular mass of GspS of 78 kDa was estimated by SDS-PAGE.

The native molecular weight was determined by native PAGE using a PhastGel Gradient 8-25 (Pharmacia) with the same following molecular weight standards and additionally: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and lactate dehydrogenase (140 kDa).

A molecular weight of 78 kDa was obtained by gradient gel electrophoresis of the native enzyme. 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).

The isoelectric point was determined by isoelectric focusing using a PhastGel IEF 3-9 (Pharmacia) with a broad pI 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 (29).

For detection of GspS activity after isoelectric focusing the gels were cut into two pieces, one was silver stained for protein detection and the second was incubated for 15 min at room temperature in a solution of 100 mM HEPES, pH 7.0, 5 mM $MgSO_4$, 1 mM EDTA, 5 mM DTT, 10 mM GSH, 10 mM spermidine, and 2 mM ATP. After 15 min 2.5 ml of a staining solution containing malachite green, ammonium molybdate and Tween 20 (1) was added. Lanes containing active GspS showed a dark green colour after few minutes.

The isoelectric point deduced from isoelectric focusing was at pH 4.6.

Example 3. Amino acid sequencing.

SDS-PAGE of purified GspS was performed at a constant current of 20 mA in a separating gel (T = 7.5%). For blotting, the proteins were transferred for 1.5 h onto a PVDF-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 fibre filters of an sequencer (Applied Biosystems 470A) sequencer with standard gas-phase programs of the manufacturer.

N-terminal amino acid sequencing proved unsuccessful obviously due to a 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. Out of these peptides, 7 could unambigously be aligned to the deduced GspS sequence of *E. coli* recently published by Bollinger et al. (25) (Fig. 1). GspS of *E. coli* and of *C. fasciculata* thus appeared to be phylogenetically related. But based on the limited sequence information, the sequence similarity between these enzymes, with only 40% identities, appears rather low.

Example 4. 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, 0.66 mM ATP), GSH (0.36, 0.47, 0.66, 1.11, 3.57 mM GSH), and spermidine (0.36, 0.47, 0.66, 1.11, 3.57 mM spermidine), 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 min and 30 min. GspS activity was analyzed by product determination as described in example 1.

The experimental data thus obtained 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 quarternary complexes are very close for a given substrate and not significantly different. This would indeed imply a mutually independent random addition of substrates. But with regard to the inevitable scatter of data it can not be exluded

that some route leading to the quarternary complex is slightly favoured. However, a rapid equilibrium random-order mechanism, as depicted in Fig. 6, complies best with the experimental data. Based on this assumption, the limiting K_m values are defined as the dissociation constants of the quarternary complexes, numerically 0.25 ± 0.02, 2.51 ± 0.33, and 0.47 ± 0.09 mM for Mg^{2+}-ATP, GSH, and spermidine, respectively. The rate limiting velocity constant then can be calculated to be 415 ± 78 min^{-1}.

A quarternary complex mechanism implies that all three substrates must be assembled at the enzyme before a reaction can proceed. In order to check this hypothesis, we subjected the enzyme to long-term exposure with Mg^{2+}-ATP plus one of the additional substrates and monitored a potential partial reaction by ^{31}P NMR.

^{31}P NMR spectra were recorded on a Bruker ARX 400 NMR spectrometer, at 162 MHz and locked to the deuterium resonance of D_2O, 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 $MgSO_4$, 1 mM EDTA, 5 mM DTT, in the presence of 20% D_2O. 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).

Fig. 8 demonstrates that with all combinations of substrates no ATP turnover could be observed within 5 hours unless the third substrate was added. These findings strongly support the assumption of a quarternary 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. A K_i of 80 µM was calculated which is in the range of physiological ADP concentrations. GspS also proved to be feed-back inhibited by TSH with a K_i of 480 µM, which is competed by GSH.

Example 5. pH-optimum of GspS.

The activity of GspS was measured essentially as described in example 4 but at pH values ranging from 6 - 8. GspS shows a flat pH optimum near pH 7.5 (Fig. 7).

Example 6. Use of malachite green colorimetric assay for liberation of inorganic phosphate in GspS preparations partially purified according to example 1.

The liberation of inorganic phosphate from ATP can be easily visualized by malachite green (Fig. 9). The test is amply used to monitor ATP hydrolyzing activities and is correspondingly unspecific. Surprisingly, the GspS preparation after aqueous two phase extraction, as described in example 1, proved to be free of any significant spontaneous ATP-hydrolyzing activity. This finding enabled us to use this fast and convenient test to measure specifically GspS activity which is accompanied by release of inorganic phosphate from ATP in the presence of glutathione, spermidine, and magnesium ions. The test can be easily adapted to mass screening as outlined below.

The malachite green colorimetric assay for liberation of inorganic phosphate (1) was used for fast detection of GspS activity during purification after column chromatography and for GspS localization on gels.

The disclosure comprises also that of EP 96 120 014.4 (copy attached).

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Glutathionylspermidine Synthetase

Claims

1. A protein catalyzing the synthesis of glutathionylspermidine characterized by a pH optimum of said synthesis of about pH 7.5 and by a molecular mass of 78,000 ± 3,000 Da.

2. A protein according to claim 1 characterized by SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 and/or SEQ ID NO 7 as partial peptide sequences shown in Fig. 1.

3. A protein according to claim 1 or 2 characterized in that it can be isolated from a species of the family of trypanosomatids (Trypanosomatidae), especially *Trypanosoma* sp., *Leishmania* sp., *Herpetomonas* sp., *Leptomonas* sp., *Blastocrithidia* sp., *Crithidia* sp., *Phytomonas* sp., especially C. *fasciculata*.

4. A protein according to any of claims 1 to 3 characterized in that it can be produced by genetic engineering methods known per se, making use of SEQ ID NO 1, SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6 and/or SEQ ID NO 7 as partial

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amino acid sequences shown in Fig. 1 or SEQ ID NO 8 shown in Fig. 2 or a part or variant thereof.

5. A protein according to claim 1, 3, or 4 **characterized** by SEQ ID NO 8 as partial deduced amino acid sequence shown in Fig. 2 or by any sequence being homologous to SEQ ID NO 8, having the same number of amino acids as SEQ ID NO 8 and being identical in more than 70 %, especially more than 75 % of the amino acid residues.

6. A protein according to claim 1, 3, or 4 **characterized** by being in part encoded by SEQ ID NO 9 as partial DNA sequence shown in Fig. 2 or by any other DNA sequence having the same number of nucleotides and being identical to SEQ ID NO 9 in more than 70 % and especially 75 % of the nucleotides, wherein said other DNA sequence can optionally be hybridized with SEQ ID NO 9 at a temperature of at least 25 °C and at a NaCl concentration of 1M.

7. Process for the recovery of a protein according to any of the preceding claims, **characterized** in that
(i) cells belonging to Trypanosomatidae are homogenized,
(ii) the resulting homogenate is extracted by means of an aqueous multiphase system,
(iii) the phase containing the protein wanted is separated and
(iv), if wanted, the protein is isolated from said phase.

8. Process according to claim 7, **characterized** in that the extraction is carried out by means of a 2-phase system and/or that
(a) one of the aqueous phases contains an organic polymer or a mixture of organic polymers, especially polyethyleneglycol (PEG) having a molecular weight of more than 1500 Da and especially more than 6000 Da; and
(b) the other phase is an aqueous solution of a salt or a mixture of salts.

9. Process according to claim 8, **characterized** in that phase (a) contains PEG having a molecular weight of about 6000 Da, and/or that phase (b) is a phosphate solution preferably of a pH of about 7.

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10. Process according to any of claims 7 to 9, characterized in that before an optional isolation of the protein wanted, the protein contained in said phase separated in step (iii) is extracted by means of another aqueous phase, followed, if wanted, by at least one additional extraction by means of an aqueous phase up to a substantially complete separation of enzyme activities cleaving ATP, other than the protein wanted.

11. Process according to claim 10, characterized in that the other aqueous phase is free of PEG or has a lower PEG concentration.

12. Process according to claim 10 or 11, characterized in that the extraction is carried out by lowering of the pH preferably down to pH 6 or less and/or that an aqueous buffer solution according to claim 8 or 9 is used as the other aqueous phase.

13. Process according to claims 7 to 12 characterized in that cells belonging to *Trypanosoma*, *Leishmania*, *Herpetomonas*, *Leptomonas*, *Blastocrithidia*, *Crithidia*, especially *Crithidia fasciculata*, and/or *Phytomonas* are used, preferably a-pathogenic cells.

14. Use of an aqueous phase containing a protein or an isolated protein according to any of claims 7 to 13 as a protein preparation for the detection of compounds having trypanocidal activity (GspS inhibitors).

15. Use according to claim 14, wherein the detection is carried out by means of an ATP cleavage as an indicator of the activity of a protein according to any of claims 1 to 6 catalyzing the synthesis of glutathionylspermidine.

16. GspS inhibitors which can be detected by means of a use according to claim 14 or 15 and, if wanted, isolated by a classical isolation method.

17. Pharmaceutical preparation comprising an GspS inhibitor according to claim 16 and a usual carrier and/or adjuvant.

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