

**Trypanothione: A Novel Bis(glutathionyl)spermidine Cofactor
for Glutathione Reductase in Trypanosomatids**

Alan H. Fairlamb, Peter Blackburn, Peter Ulrich, Brian T. Chait, and Anthony Cerami

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Abstract. *Glutathione reductase from trypanosomes and leishmanias, unlike glutathione reductase from other organisms, requires an unusual low molecular weight cofactor for activity. The cofactor was purified from the insect trypanosomatid Crithidia fasciculata and identified as a novel glutathione-spermidine conjugate, N¹,N⁸-bis(L-γ-glutamyl-L-hemicystinyl-glycyl)spermidine, for which the trivial name trypanothione is proposed. This discovery may open a new chemotherapeutic approach to trypanosomiasis and leishmaniasis.*

The available treatments for human tropical diseases caused by parasitic trypanosomes (African sleeping sickness, Chagas' disease) and leishmanias (oriental sore, kala-azar) lag far behind the enormous advances in chemotherapy of bacterial diseases. Current drug treatment is either lacking or unsatisfactory (1), and new drugs for clinical use are too expensive to develop through classical empirical screening methods (2). Research is now being directed toward identifying key differences between the metabolism of host and parasite as targets for drug development (3).

During our studies of glutathione metabolism in trypanosomes, we have identified such a target in glutathione reductase (4). This nicotinamide adenine dinucleotide phosphate (NADP⁺)-dependent enzyme is responsible for the maintenance of glutathione in the reduced form (L-γ-glutamyl-L-cysteinyl-glycine; GSH). Unlike the mammalian enzyme, the enzyme activity in trypanosomatids was found to be highly unusual in that it had an absolute requirement for an unknown thiol-containing cofactor of low molecular weight (4). This led us to purify the cofactor and determine its structure. The discovery that the cofactor contains the polyamine spermidine covalently linked to glutathione opens exciting new avenues for drug development against these parasitic diseases.

The insect trypanosomatid *Crithidia fasciculata* was chosen as a suitable source of cofactor because it can be

grown in simple media and obtained in high yields. Purification of the cofactor was monitored enzymatically with dialyzed enzyme extracts from bloodstream *Trypanosoma brucei* by measuring oxidized glutathione (GSSG)-dependent oxidation of NADPH on addition of cofactor extract (5). Enzyme extracted from *T. brucei* was used in preference to enzyme from *C. fasciculata* because it contains negligible amounts of NADPH oxidase, which otherwise interferes with the assay (4). Crude cofactor was extracted from *C. fasciculata* (wet weight, 118 g) (6) by a combination of freeze-thawing, centrifugation, and ultrafiltration (4), yielding 3.9 g of lyophilized material containing 5440 units of cofactor (5). This material was purified by anion and cation exchange chromatography and ion-paired reversed-phase high-pressure liquid chromatography (HPLC), yielding approximately 300 μg of cofactor in an overall recovery of 24 percent (Table 1). The pure material eluted as a single sharp peak of ninhydrin-reactive material near the breakthrough of the third buffer on a Durrum-500 amino acid analyzer (7) with an elution time of 45 minutes. The purified cofactor also eluted as a single symmetrical peak at 62.5 minutes on ion-paired, reversed-phase HPLC (8). After performic acid oxidation (9) the oxidized cofactor eluted earlier in the Durrum-500 and HPLC systems, still as a single sharp peak but now at 2.0 and 6.5 minutes, respectively. This finding is consistent with the oxidation of sulfur to the more acidic sulfonate moiety.

Amino acid analysis of the performic acid-oxidized, acid-hydrolyzed material yielded stoichiometric amounts of glutamate, glycine, and cysteic acid residues (Table 2). However, when this material was analyzed by cation-exchange thin-layer chromatography (TLC) (10), an additional ninhydrin-positive spot was noted at the origin, indicating the presence of an additional highly basic amino-containing compound. Ion-paired, reversed-phase HPLC confirmed the presence of four amino-containing compounds: cysteic acid, glycine, glutamate, and a component with the same retention time as

spermidine (77 minutes) and well resolved from putrescine (46 minutes) and spermine (89 minutes). The presence of spermidine and the absence of the latter polyamines in acid-hydrolyzed samples of cofactor was confirmed by TLC of the dansyl derivatives (11). Toluene extracts of acid-hydrolyzed dansylated cofactor yielded material with a migration identical to that of authentic tris-dansyl spermidine (migration relative to dansyl-NH₂ = 0.8). Carboxymethylation of cofactor reduced in the presence of dithiothreitol or sodium borohydride followed by acid hydrolysis yielded S-carboxymethyl cysteine, indicating the presence of cystine in the original material. The spermidine content determined quantitatively by HPLC was found to be half that of the amino acids, suggesting a minimum composition of spermidine:glycine:glutamate:hemicystine residues of 1:2:2:2 and a minimum molecular weight of 721.

After desalting by HPLC (12) the cofactor was subjected to analysis by ²⁵²Cf fission fragment-induced ionization mass spectrometry (FFII-MS) (13), a method characterized by its ability to produce quasi-molecular ions in addition to structurally informative fragments of ions from involatile compounds. The observed quasi-molecular ion species and two significant fragment ions are listed in Table 3. The data indicate a molecular weight of 721 for the cofactor, confirming the chemical analysis. FFII-MS of GSSG gave a strong fragment at mass 217 in common with the cofactor, suggesting that the arrangement of amino acid residues in the cofactor is similar to that in GSSG, but with spermidine covalently attached to it. Of the potential structures of spermidine—(i) attached to both glycine residues, (ii) attached to both glutamate residues, or (iii) attached from a glutamate residue of one tripeptide to the glycine of the other—the fragmentation data suggest that (i) is the most likely.

This compound was synthesized by active ester peptide coupling chemistry with a protected spermidine substrate,

Table 1. Purification of cofactor for trypanosomatid glutathione reductase from *C. fasciculata*.

Purification step	Cofactor units (5)	Recovery (%)
Ultrafiltrate	5441	100
Dowex AG 1-X8 (formate)	5138	94
Dowex AG 50-X4 (H ⁺)	5130	94
Amino acid analyzer (Na ⁺)	3104	57
Dowex AG 50-X4 (H ⁺)	2494	46
Reversed-phase HPLC	1325	24

Table 2. Composition of performic acid-oxidized cofactor after acid hydrolysis.

Component	Nano-moles	Ratio (to cysteic acid)
Cysteic acid*	5.19	1.0
Glycine*	5.21	1.0
Glutamate*	5.19	1.0
Spermidine†	2.50	0.5

*Measured with the Durrum amino acid analyzer.
†Measured from peak height of Fluram trace on HPLC, compared to standards.

N^1, N^4 -(methylene)spermidine (14). The synthesis involved simultaneous sequential introductions of the amino acid residues of both glutathionyl appendages with *t*-butoxycarbonyl-glycine hydroxy-succinimide ester, *t*-butoxycarbonyl-*S*-benzylcysteine-4-nitrophenyl ester, and *t*-butoxycarbonyl-glutamic- α -(benzyl ester)- γ -(hydroxysuccinimide ester); cleavage of the N^1, N^4 -methylene group (14); removed of the *t*-butoxycarbonyl groups with trifluoroacetic acid and debenzilation with anhydrous HF; and oxidation of the reduced form with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to afford N^1, N^8 -bis-(*L*- γ -glutamyl-*L*-hemicystinyl-glycyl)spermidine. This material shows retention times identical to those of the cofactor on the amino acid analyzer and by ion-paired, reversed-phase HPLC; has a common series of structurally significant ions on FFII-MS (Table 3); and substitutes for cofactor in the glutathione reductase assay with *T. brucei* enzyme, confirming the chemical structure assignment of the cofactor (Fig. 1).

Previously a related compound that contains spermidine attached to a single glutathione moiety (*L*- γ -glutamyl-*L*-cysteinylglycylspermidine) was found in *Escherichia coli*. This compound is absent in logarithmically growing cells but is synthesized during the stationary phase, where it accounts for all the intracellular spermidine and a large part of the intracellular glutathione (15). Its function is not known. Using dialyzed glutathione reductase from *T. brucei* as a biological probe, we were able to identify a similar cofactor-like activity only in extracts of trypanosomatids (*T. brucei*, *Trypanosoma cruzi*, *C. fasciculata*, *Leishmania mexicana*), but not in extracts of *E. coli* (logarithmic-phase cells), rat liver, spinach, yeast, and the protozoans *Chlamydomonas reinhardtii*, *Eimeria tenella*, *Plasmodium berghei*, and *Trichomonas vaginalis* (4). As the compound identified here has been found in all trypanosomatids, but in no other

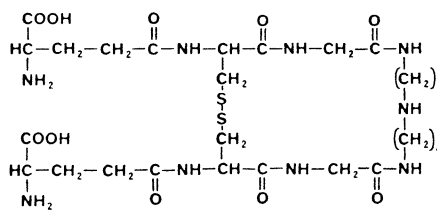
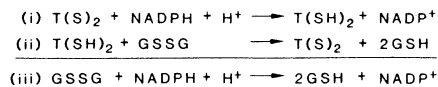


Fig. 1. Structure of trypanothione.

sources examined to date, we propose that the trivial name trypanothione be used to differentiate trypanosomatid cofactor from *E. coli* glutathionylspermidine.

Trypanothione may function in the trypanosomatid glutathione reductase-catalyzed reaction as follows:



where T(S)_2 and T(SH)_2 represent oxidized and reduced trypanothione, respectively. In reaction (i) oxidized trypanothione is reduced enzymatically in the presence of NADPH to form reduced trypanothione. This would be consistent with our previous finding (4) that enzymatically reduced cofactor could be inactivated by treatment with *N*-ethylmaleimide, whereas the oxidized form could not. In reaction (ii) reduced trypanothione undergoes thiol-disulfide exchange with GSSG, reforming oxidized trypanothione. The sum of these reactions (iii) illustrates that trypanothione acts catalytically as a cofactor in the overall NADPH-dependent reduction of GSSG. Reduced trypanothione will also undergo thiol-disulfide exchange with cystine and the mixed disulfide of coenzyme A and GSH, accounting for the apparent lack of specificity of the enzyme reaction noted in our previous studies (4). The enzyme-catalyzing reaction (i) is the functional equivalent of glutathione reductase in other organisms. Purification and sequence determination of this enzyme will provide information on the relatedness of the

trypanosomatid enzyme with the highly conserved sequence of glutathione reductase from other organisms. It is not known whether reaction (ii) is enzyme-catalyzed or not. However, the second reaction can proceed nonenzymatically, since chemically reduced trypanothione can be readily reoxidized by the addition of GSSG or DTNB.

We have been unable to isolate a mixed disulfide of trypanothione and DTNB, suggesting that rapid intramolecular disulfide exchange of the putative intermediate T(SH)S-TNB must occur. This situation resembles that observed for the reaction between dithiothreitol and DTNB, where an intermediate adduct forms only transiently, in contrast to the reaction products of DTNB with cysteine, homocysteine, and GSH, all of which form stable RS-TNB adducts (16). Space-filling molecular models of trypanothione suggest an explanation for this. Both oxidized and reduced forms can assume the conformation of a parallel β -pleated sheet by the formation of three hydrogen bonds (between C=O and NH functions of the peptide) and two salt bridges (between the α -carboxyl and α -amino groups of each glutamate residue). If this conformation were preferred in solution, then the proximity of the sulfhydryl groups would be maintained, so that rapid intramolecular rearrangement of the putative T(SH)S-SR intermediate would occur, expelling RSH and cyclizing trypanothione to the stable oxidized form, T(S)_2 .

Glutathione contents in the range 3 to 5 nmol per milligram of protein have been reported for *T. brucei*, *T. cruzi*, and *C. fasciculata* (17). In contrast, the content of trypanothione is about ten times lower in *C. fasciculata* (about 0.2 nmol per milligram of protein, calculated from Table 1). Thus, as in most other cells (18), glutathione represents the major low molecular weight thiol in trypanosomes. It participates directly or indirectly in many areas of cellular metabolism, and perturbation of its status can be deleterious to many organisms (18). However, certain mutants of *E. coli* defective in glutathione metabolism are apparently able to grow normally, suggesting that glutathione is not essential for growth to *E. coli* under laboratory conditions (19). This is not the case in *T. brucei*, since inhibition of glutathione synthesis in vivo by buthionine sulfoximine confers prolonged survival times or cures in infected mice (20). Furthermore, depletion of GSH levels in Melarsen oxide-treated *T. brucei* renders them more susceptible to heme lysis (21).

The unique nature of the trypanothione-

Table 3. Structurally significant ions of cofactor and synthetic trypanothione produced by FFII-MS. M, cofactor molecule.

Ion species	Ratio of mass to charge		
	Measured cofactor	Measured trypanothione	Calculated*
$(\text{H}_2\text{NCHCOONaCH}_2\text{CH}_2\text{CONHCHCH}_2\text{Na})^+$	217.1	217.1	217.1
$(\text{M} + \text{Na} - \text{HCOOH})^+$	698.5	698.5	698.3
$(\text{M} + \text{H})^+$	722.6	722.4	722.3
$(\text{M} + \text{Na})^+$	744.3	744.4	744.3
$(\text{M} + 2\text{Na} - \text{H})^+$	766.7	766.5	766.3
$(\text{M} + 3\text{Na} - 2\text{H})^+$	788.5	788.7	788.2

*Monoisotopic value.

mediated reduction of glutathione represents a target that is common to all trypanosomal and leishmanial diseases. In the absence of this essential cofactor, or if its reductase were inhibited, these parasites would be unable to maintain their intracellular pool of glutathione in the reduced form. On the basis of previous studies (20, 21), depletion of total GSH or alterations in the GSH:GSSG ratio within the cell would be expected to be deleterious to these organisms. Several potential sites for drug development can be identified, including inhibition of synthesis of trypanothione and inhibition of trypanothione reductase. The details of the pathway of synthesis of trypanothione remain to be elucidated, but initial studies with [³H]spermidine have shown that this polyamine can be incorporated into trypanothione. Since α -difluoromethylornithine inhibits spermidine synthesis in trypanosomes (22), the selective toxicity of this drug, now undergoing clinical trials (23), could be due in part to inhibition of trypanothione synthesis.

ALAN H. FAIRLAMB

PETER BLACKBURN

PETER ULRICH

BRIAN T. CHAIT

ANTHONY CERAMI

Rockefeller University, New York 10021

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5. Cofactor was assayed enzymatically in 20 mM Hepes buffer (pH 7.25) containing 150 mM KCl, 1 mM EDTA, dialyzed *T. brucei* enzyme extract (438 μ g), 1 mM GSSG, and 0.15 mM NADPH (total volume, 0.5 ml; 25°C) following the oxidation of NADPH spectrophotometrically at 340 nm (4). After the background rate of NADPH oxidation was measured for 3 to 5 minutes, cofactor was added and the reaction monitored for a further 6 minutes. After correcting for background, the absorption per minute at 340 nm is proportional to the amount of cofactor added, up to a maximum of 2 nmol of NADPH oxidized per minute per assay. One unit of cofactor is defined as 1 nmol of NADPH oxidized per minute per milliliter under these conditions.
6. *Crithidia fasciculata* (wild-type) was grown commercially by New England Enzyme Center essentially as described by N. Le Trang *et al.* [*J. Biol. Chem.* **258**, 125 (1983)]. It was supplied as a washed, frozen paste.
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8. Separations by HPLC were carried out on a Beckman/Altex model 430 HPLC system with a Beckman/Altex C₁₈ reversed-phase column (4.5 by 250 mm; particle size, 5 μ m). Consecutive linear gradients of 0 to 10 percent solvent B (60 minutes) and 10 to 50 percent solvent B (60 minutes) were applied: solvent A contained 0.25 percent (weight to volume) camphorsulfonate (lithium salt; pH 2.64) and solvent B contained 0.25 percent (weight to volume) camphorsulfonate plus 50 percent (by volume) isopropanol. In

- analytical runs the column eluent was monitored on-line by absorption at 210 nm and amino compounds were detected by fluorescence after postcolumn derivatization with fluorescamine [S. Udenfriend *et al.*, *Science* **178**, 871 (1972); M. Weigle *et al.*, *J. Am. Chem. Soc.* **94**, 5927 (1972)].
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10. Samples were chromatographed on Fixion 50X8 Chromatoshets (Chromatronix) using 0.117M sodium citrate and 1.0M NaCl (pH 5.8) as solvent. Compounds were detected with cadmium-ninhydrin reagent [T. Devenyi, *Acta Biochim. Biophys. Acad. Sci. Hung.* **11**, 1 (1976)].
11. Samples were derivatized with 5-dimethylamionaphthalene-1-sulfonyl chloride and separated on silica gel G by ascending chromatography with cyclohexaneethyl acetate (1:1) (two runs) and cyclohexaneethyl acetate (3:2) (one run) as described by N. Seiler and B. Knodgen [*J. Chromatogr.* **164**, 155 (1979)].
12. Samples were desalted by means of the instrumentation described in (8) on a Beckman/Altex C₁₈ reversed-phase column (4.5 by 250 mm; pore size, 5 μ m) with a linear gradient of 0 to 50 percent solvent B (60 minutes); solvent A contained 0.1 percent (by volume) trifluoroacetic acid and solvent B 0.1 percent (by volume) trifluoroacetic acid and 75 percent (by volume) acetonitrile.
13. Cofactor was desalted as described above and concentrated by evaporation under reduced pressure. Approximately 20 μ g was electrosprayed onto aluminized polyester film and mass spectra were obtained with the Rockefeller University ²⁵²Cf fission fragment ionization time-of-flight mass spectrometer [B. T. Chait, B. F. Gisin, F. H. Field, *J. Am. Chem. Soc.* **104**, 5157 (1982)].
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16. The TNB adducts of GSH, homocysteine, and cysteine were synthesized by reacting these

- compounds with a tenfold molar excess of DTNB in triethylamine HCl buffer (pH 8.5) for 15 minutes. The mixtures were dried under vacuum and acidified with 0.1 percent (by volume) trifluoroacetic acid, and the pure compounds were isolated by reversed-phase HPLC. The molar extinction coefficients at 330 nm (pH 3.0) were determined to be approximately 8500 liter mol⁻¹ cm⁻¹ on the basis of amino acid analyses of the performic acid-oxidized adducts. The TNB adducts were stable at pH 3 for at least 2 months at 4°C.
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