Protozoa of the order Kinetoplastida differ from other organisms in their ability to conjugate glutathione (γ-Glu-Cys-Gly) and spermidine to form trypanothione (N′,N′,N′-bis(glutathionyl)spermidine), which is involved in maintaining intracellular thiol redox and in defense against oxidants. In this study, the genes from *Crithidia fasciculata*, Cf-GSS and Cf-TRS, which encode, respectively, glutathionylspermidine synthetase (EC 6.3.1.8) and trypanothione synthetase (EC 6.3.1.9) have been cloned and expressed. The deduced amino acid sequence of both Cf-GSS and Cf-TRS share 50% sequence similarity with the *Escherichia coli* glutathionylspermidine synthetase/amidase. Both genes are present as single copies in the *C. fasciculata* genome. When expressed in *E. coli* and *Saccharomyces cerevisiae*, neither protein was present in an active soluble form. However, thiol analysis of *S. cerevisiae* demonstrated that cells transformed with the Cf-GSS gene contained substantial amounts of glutathionylspermidine, whereas cells expressing both the Cf-GSS and Cf-TRS genes contained glutathionylspermidine and trypanothione, confirming that these genes encode the functional glutathionylspermidine and trypanothione synthetases from *C. fasciculata*. The translation products of Cf-GSS and Cf-TRS show significant homology to the amidase domain present in *E. coli* glutathionylspermidine synthetase, which can catalyze both synthesis and degradation of glutathionylspermidine. Glutathionylspermidine synthetase isolated from *C. fasciculata* was found to possess a similar amidase activity.

Several parasitic protozoa belonging to the order Kinetoplastida are human pathogens causing diseases such as sleeping sickness (*Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*); visceral, cutaneous, and mucocutaneous leishmaniasis (*Leishmania spp.*); and Chagas’ disease (*Trypanosoma cruzi*). Currently, there are no entirely satisfactory drugs for use against these maladies, and no vaccines exist. Many of the currently available drugs suffer from poor efficacy, unacceptable host toxicity, or drug resistance. The mode of action of most trypanocidal drugs is unknown, and currently used compounds were derived empirically, except for the DL-α-difluoromethylornithine, which is a potent inhibitor of ornithine decarboxylase (1–3). To increase the chances of success, the development of new drugs should be aimed at those steps in a metabolic pathway that are either absent or differ from analogous steps in the host.

The polyamines and glutathione (γ-L-glutamyl-L-cysteinylglycine) are found in millimolar concentrations in most biological systems. Polyamines are thought to be essential for cell proliferation and differentiation in most organisms (4–7). Glutathione has a role in several important biochemical processes that include regulation of the intracellular thiol redox balance and defense against oxidant or other chemical-induced damage (8). Unlike most prokaryotic and eukaryotic cells, in which the major thiol component is glutathione, members of the order Kinetoplastida rely on a glutathione-spermidine conjugate called trypanothione (N′,N′,N′-bis(glutathionyl)spermidine) (9, 10). Trypanothione seems to be an essential redox intermediate in the regulation of the intracellular thiol redox, analogous to glutathione in other organisms, and also to play a role in defense against oxidative stress. In fact, these parasites lack typical catalase, glutathione peroxidase (11–13), and glutathione reductase (9), which function in oxidant defense in other organisms. The parasites utilize an analogous system involving trypanothione reductase, an enzyme similar to glutathione reductase, which uses trypanothione as a cofactor (14). On the basis of these observations, trypanothione metabolism represents an attractive candidate for rational drug design, since it is uniquely found in the Kinetoplastida (15).

Biosynthesis of trypanothione involves ATP-dependent conjugation of intact glutathione to the two terminal amino functions of spermidine (16). The original report (17) of a single enzyme from *Crithidia fasciculata* converting glutathione and spermidine into trypanothione, called trypanothione synthetase, has been superseded by the identification of two distinct ATP-dependent enzymes involved in the synthesis of trypanothione (18, 19), a glutathionylspermidine synthetase (GSS)1 that forms both N′- and N″-glutathionylspermidine and a

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1 The abbreviations used are: GSS, glutathionylspermidine synthetase; TRS, trypanothione synthetase; ORF, open reading frame; HPLC, high pressure liquid chromatography; PCR, polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.
trypanothione synthetase (TRS) that catalyzes the addition of a second glutathione to either of the preceding intermediates (Fig. 1). GSS and TRS co-purify as a functional complex but can be separated by hydrophobic chromatography (18) or by extraction in aqueous two-phase systems (19).

Glutathionylspermidine (N\(^1\)-γ-glutamyl-1-cysteinylglycyl)spermidine) has also been identified in *Escherichia coli* (20, 21), where it accumulates in stationary phase cells (22). The function of this compound in *E. coli* is not known, although it has been suggested that it is involved in regulating the levels of the precursors (spermidine and glutathione). More recently, Bollinger et al. (23–25) reported the cloning and characterization of a bifunctional *E. coli* glutathionylspermidine synthetase/amidase enzyme responsible for both the biosynthesis and degradation of glutathionylspermidine in *E. coli*.

We report here the cloning and characterization of the glutathionylspermidine synthetase (GSS) and trypanothione (Cf-TRS) synthetase genes from *C. fasciculata* and their overexpression in *E. coli* and *Saccharomyces cerevisiae*.

**EXPERIMENTAL PROCEDURES**

**Organisms and Reagents**—*C. fasciculata* clone HS6 (26) was used as source for DNA, RNA, and protein. Routine DNA manipulations were performed in *E. coli* strain JM109 and XL1-Blue. Overexpression in *E. coli* was performed in strain BL21(DE3), and overexpression in yeast was performed in *S. cerevisiae* ABYS106 strain. All chemicals were of the highest grade available and from Sigma or BDH. Restriction enzymes and DNA-modifying enzymes were from Promega.

**Peptide Sequencing**—*C. fasciculata* GSS and TRS were purified as described (18) and separated by SDS-PAGE. The bands were then transferred onto a nitrocellulose membrane and digested with trypsin, and the resulting peptides were separated by reverse phase HPLC essentially as described by Stone and Williams (27). The automated Edman degradation was performed using an ABI 477A protein sequencer with a 120A online PTH-AA analyzer by Dr. W. S. Lane (Harvard Microchemistry Facility).

**Fig. 1.** Enzymatic steps involved in the biosynthesis of trypanothione.

**Amplification of a Glutathionylspermidine Synthetase Gene Fragment by PCR**—Two degenerate oligonucleotides were designed based on the sequence of two trypsin peptides from the 90-kDa band (GSS activity) (18) (PFDPYDG (named GSS1), 5'-TTYGAYTTGNGTGAYGGG-3'; and EPLWK (named DTC2), 5'-YTTCCANADNGGTYCTC-3'). PCR was performed in a 50-μl volume containing 0.2 μm each dNTP, 500 ng of each primer, 100 ng of *C. fasciculata* total genomic DNA, and 2.5 units of *Taq* DNA polymerase with buffer (Bioline). Before adding the enzyme, the PCR mixture was heated for 10 min at 95°C and then cooled rapidly to 30°C. Following centrifugation, the enzyme was added, and the reaction mixture was covered with oil and subjected to the following for 30 cycles: denaturation for 10 s at 95 °C, rampsing at 2 °C s\(^{-1}\) to 40 °C, annealing for 1 s at 40 °C, and elongation for 30 s at 72 °C. A final 10-min extension at 72 °C was included. A 561-bp DNA fragment was identified following agarose gel electrophoresis of the PCR products. The DNA fragment was cloned into the *Sma*I site of pUC18, giving the plasmid pGSS, and sequenced with a T7 sequencing kit (Amersham Pharmacia Biotech), giving the plasmid pGSS, and sequenced as above.

**Amplification of a Trypanothione Synthetase Gene Fragment by PCR**—Two degenerate oligonucleotides were designed based on the sequence of two trypsin peptides from the 82-kDa band (TRS activity) (18) (DLNDPAEK (named TRS2), 5'-GAYTNYGAGCCNCCNGARAA-3'; and DGYYAIIG (named TRS4), 5'-CCDATDATANGTACCRTCTC-3'). PCR was performed as described above for the GSS gene. A 1089-bp DNA fragment was identified following agarose gel electrophoresis of the PCR products. The DNA fragment was cloned into the *Sma*I site of pUC18, giving the plasmid pTRS, and sequenced as above.

**Genomic Libraries: Construction and Screening**—Genomic DNA was isolated from *C. fasciculata* as described (28), digested with *Pst*I or *Sal*I, and subsequently separated by agarose gel electrophoresis. DNA in the size range 3–4 kb (*Pst*I) and 1–1.5 and 3–3.5 kb (*Sal*I) were isolated (QIAEX Extraction Kit, QIAGEN), ligated to pUC18 vector (digested with *Pst*I or *Sal*I and dephosphorylated, AppIIen), and transformed in *E. coli* XL1-Blue. The *Pst*I and *Sal*I sublibraries each contained about 20,000 recombinant plasmids.

The cloned GSS1/DTC2 PCR fragment was isolated following digestion of pGSS with EcoRI-*Pst*I and separation by agarose gel electrophoresis, labeled with \(^{32}P\)dCTP by the random primer method (Ready
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**Fig. 2. Map of the yeast expression vectors.** A, pRB1438-Cf-GSS; the gene Cf-GSS is under control of the GAL1 promoter. B, pRB1438-Cf-TRS; the gene Cf-TRS is under control of the GAL1 promoter. C, pRB1438-Cf-GSS/Cf-TRS; the gene Cf-GSS is under control of the GAL1 promoter and Cf-TRS Gal10 promoter. The expression is induced with 4% galactose. This plasmid carries the URA3 marker for selection in yeast cells and also contains elements for selection and propagation in E. coli.

Prime, Amersham Pharmacia Biotech, and used to screen approximately 10,000 clones from the C. fasciculata PstI sublibrary. Colonies were transferred to nylon membranes (Amersham Pharmacia Biotech) and hybridized to the labeled probe at high stringency as described for the sublibraries. Colonies were transferred to nylon membranes and hybridized to the labeled probe at high stringency as described for the PstI sublibrary. Positive clones were isolated and subsequently amplified.

The cloned TRS2/TRS4 PCR fragment was isolated following digestion of pTRS with EcoRI–HindIII and separation by agarose gel electrophoresis, labeled with [32P]dCTP and washed as described above for the screening of sublibraries. Sequencing of double-stranded DNA was accomplished by the dideoxynucleotide chain termination method (29) as described in the T7 sequencing kit from Amersham Pharmacia Biotech, using the dye terminator (ABI PRISM dye terminator kit, Perkin-Elmer). DNA and confirmed by automated cycle sequencing using the dye terminator method (ABI PRISM dye terminator kit, Perkin-Elmer). DNA and amino acid sequences were analyzed with the Lasergene system (DNASTAR Inc.) program and the Pileup program from the Genetics computer Group Wisconsin Sequence Analysis Software Package.

Expression of Glutathionylperoxidine and Trypanothione Synthetases in S. cerevisiae—A S. cerevisiae strain (ABY8106) expressing the Cf-GSS gene was obtained by transformation with the gene cloned in the expression vector pET-3a (Novagen). The gene was obtained by PCR amplification of the C. fasciculata open reading frame in plasmid pT7-Cf-GSS using oligonucleotide ExpGSS5′ (5′-CCA CAT ATG TCG TCG GTG-3′) for the sense strand containing an NdeI cloning site (underlined) incorporating an initiation codon (ATG). For the antisense strand, oligonucleotide ExpGSS3′ (5′-CGG GAA TTC TTA TGT TTC-3′) was designed, which includes a stop codon with a BamHI restriction site (underlined) just downstream. The PCR product was blunt end-ligated into the SmalI site of pUC18 (SureClone kit from Amersham Pharmacia Biotech), giving the plasmid pUC18-Cf-GSS. The insert was removed from this subclone by digestion with Ndel–BamHI and ligated with the pET-3a vector, creating plasmid pET-Cf-GSS. E. coli strain BL21(DE3) expressing the Cf-TRS gene was obtained by transformation with the gene cloned in the expression vector pET-3a. The gene was obtained in a similar fashion to Cf-GSS with the following primers: ExpTRS5′ (5′-AAA GAA TTC CAT ATG GCC TCC GCT GAG-3′) for the sense strand and ExpTRS3′ (5′-GGA TCC ATC ATC CAT ATC GCC-3′) for the antisense strand. The PCR product was blunt end-ligated into the SmalI site of pUC18 (SureClone kit from Amersham Pharmacia Biotech), giving the plasmid pUC18-Cf-TRS. The insert was removed from this subclone by digestion with NdeI–BamHI and ligated with the pET-3a vector, creating plasmid pET-Cf-TRS. BL21(DE3)/pET-Cf-GSS or BL21(DE3)/pET-Cf-TRS were grown at 37 °C with vigorous aeration in Luria-Bertani broth (LB) containing 100 μg ml⁻¹ ampicillin. When cultures reached an A₆₀₀ of 0.6, isopropyl-β-D-thiogalactopyranoside was added to a concentration of 0.4 mM to induce expression of the recombinant Cf-GSS or Cf-TRS protein. Cultures were grown an additional 3–4 h and then harvested by centrifugation. Cells were lysed in 50 mM bis-Tris propane buffer (pH 6.8) by flash-freezing in liquid nitrogen, followed by rapid thawing and then sonication (six 45-s pulses interrupted with cooling on ice). Cell debris was separated by centrifugation at 15,000 × g.

Expression of Glutathionylperoxidine and Trypanothione Synthetases in S. cerevisiae—A S. cerevisiae strain (ABY8106) expressing the Cf-GSS gene was obtained by transformation with the gene cloned in the expression vector pRB1438 under the control of the GAL1 promoter (kindly provided by Dr. P. Andrews, Department of Biochemistry, University of Dundee). The coding sequence was removed from the plasmid pUC18-Cf-GSS by digestion with BamHI and ligated with the pRB1438 vector, creating plasmid pRB1438-Cf-GSS (Fig. 2). The orientation of the gene was determined using the SacI restriction site.

A S. cerevisiae strain (ABY8106) expressing the Cf-TRS gene and one co-expressing both the Cf-GSS and Cf-TRS genes were obtained by transformation with the gene cloned in the expression vector pRB1438 under the control of the GAL1 and/or GAL10 promoters. The coding
sequence was removed from the plasmid pUC18-Cf-Cf by digestion with BamHI or EcoRI and ligated into both the pBR1438 and pBR1438-Cf-Cf vectors, creating the plasmids pRB1438-Cf-GSS and pRB1438-Cf-TRS, respectively (Fig. 2). The orientation of the gene was determined using the KpnI and XhoI restriction sites.

ABYS106/pBR1438-Cf-GSS cells, ABYS106/pBR1438-Cf-TRS cells, and ABYS106/pBR1438-Cf-GSS/Cf-TRS cells were grown at 26 °C with vigorous aeration in synthetic complete medium with 2% raffinose and galactose was added to a concentration 10^7 cells ml^-1. galactose was added to a 2157-bp ORF, which was sequenced on both strands.

**RESULTS**

**Isolation and Characterization of the Glutathionylspermidine and Trypanothione Synthetase Genes from C. fasciculata**

Cf-GSS—Two degenerate oligonucleotides were designed from two of the above peptide sequences (see “Experimental Procedures”). A 561-bp fragment was amplified from the primers GSS1/DTC2 using C. fasciculata genomic DNA and subsequently cloned into pUC18, giving the plasmid pGSS. Sequence analysis verified that this DNA fragment encoded an open reading frame (ORF) with sequence similarity to E. coli glutathionylspermidine synthetase/amidase (23).

**Trypanothione Biosynthesis in C. fasciculata**

**Cf-GSS**—Two degenerate oligonucleotides were designed from two of the above peptide sequences (see “Experimental Procedures”). A 561-bp fragment was amplified from the primers GSS1/DTC2 using C. fasciculata genomic DNA and subsequently cloned into pUC18, giving the plasmid pGSS. Sequence analysis verified that this DNA fragment encoded an open reading frame (ORF) with sequence similarity to E. coli glutathionylspermidine synthetase/amidase (23).

**Cf-TRS**—A 1089-bp fragment was amplified from C. fasciculata genomic DNA using the primers TRS2/TRS4 (designated from two of the above peptide sequences (see “Experimental Procedures”).
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C. fasciculata genomic DNA was digested with several restriction enzymes and probed with the labeled TRS2/TRS4 PCR fragment (see below). The probe hybridized to fragments of ~1.3 and ~3.5 kb when DNA was digested with SalI, suggesting that these fragments contained all of the coding sequence. The TRS2/TRS4 PCR fragment was labeled with [32P]dCTP and used to screen the C. fasciculata genomic SalI sublibraries (see "Experimental Procedures"). Two different clones corresponding to the ~1.3- and ~3.5-kb fragments were isolated. Reconstruction of these two clones gave the plasmid pTcf-TRS, which contains an ORF encoding a protein of 652 amino acids with a predicted molecular mass of 74,516 Da (Fig. 3), again smaller than the apparent molecular mass (82 kDa) previously described (18). A PCR product overlapping the SalI restriction site was cloned to confirm the sequence at this site. Seven of the nine peptide sequences determined from the 82-kDa band (underlined in Fig. 3) were found in the predicted protein product.

Amino Acid Sequence Analysis

The predicted translation products of Cf-GSS and Cf-TRS from C. fasciculata show significant homology to each other (47% similarity and 27% identity) and to the glutathionylspermidine synthetase/amidase from E. coli (50% similarity and 29% identity) (23). The deduced amino acid sequences of Cf-GSS and Cf-TRS also show significant similarity (~25%) in their C-terminal 500 amino acids with three hypothetical 45-kDa proteins, two from E. coli (YgiC (31) and YjfC (32)) and one from Haemophilus influenzae (HIO929 (33)) (Fig. 3).

Another interesting feature arising from the alignment of these different proteins is that the Cf-GSS amino acid sequence has five significant insertions (Cf-GSS amino acids 54–71, 241–269, 325–328, 466–479, and 590–629) (Fig. 3) when compared with the E. coli GSS and Cf-TRS, while the Cf-TRS protein has a size more similar to E. coli GSS. The significance of a large insertion in the C-terminal domain of the Cf-GSS protein, where the potential synthetase activity of the E. coli GSS is predicted to reside (23, 24), is unknown.

Significant homology was found in the N-terminal domain of Cf-GSS, Cf-TRS, and the E. coli GSS proteins. This domain is responsible for the amidase activity in E. coli GSS (23, 24), suggesting that both Crithidia proteins may also possess this activity. Indeed, amidase activity was present in GSS purified from C. fasciculata using glutathionylspermidine as a substrate (Fig. 4). As for the E. coli GSS (23), amidase activity was optimal at pH 7.5, while the synthetase activity was optimal at pH 6.5. Under identical conditions, no formation of glutathionylspermidine from trypanothione was detected (not shown).

Genomic Organization and Expression

C. fasciculata genomic DNA was digested with several restriction enzymes and probed with the labeled Cf-GSS and Cf-TRS coding sequences.

The Cf-GSS probe hybridized to only a single band when DNA was cut with enzymes that do not cut within the coding sequence (BamHI, HindIII, KpnI, XbaI, and PstI), whereas the probe hybridized to two fragments when the DNA was cut with enzymes that cut at a single site within the coding sequence (SacI, NotI, and EcoRV) and five fragments (of which two are the same size) when the DNA was cut with SalI, which cut four times in the coding sequence (Fig. 5, A1 and B1).

Cf-TRS also gave a similar restriction profile when hybridized to genomic DNA digested with enzymes that do not have sites within the coding sequence (BamHI, HindIII, EcoRV, KpnI, and XbaI) or digested with enzymes that cut at a single site (SacI, NotI, and SalI) or two sites (PstI) within the coding sequence (Fig. 5, A2 and B2).

These results suggest that Cf-GSS and Cf-TRS are both single copy per haploid genome of C. fasciculata.

Northern blot analysis revealed that Cf-GSS RNA is not an abundant transcript (data not shown). Moreover, attempts to identify the 5′-minionexon splice acceptor site on the pre-mRNAs of Cf-GSS and Cf-TRS by reverse transcription PCR for both glutathionylspermidine and trypanothione synthetases were unsuccessful, probably due to the poor expression of these genes.

Overexpression of Cf-GSS and Cf-TRS in E. coli

Cf-GSS was cloned into an expression vector (pET-3a) for overproduction of the protein in E. coli. Growth of BL21(DE3)/pET-Cf-GSS strain in the presence of isopropyl-β-D-thiogalactopyranoside resulted in overproduction of a protein that migrated with an apparent molecular mass of 90 kDa (Fig. 6). Thus, although the predicted molecular mass of Cf-GSS is 80 kDa, the migration of this protein in SDS-PAGE shows an apparent molecular mass of ~90 kDa, which is identical to the size described by Smith et al. (18), suggesting an aberrant migration in SDS-PAGE. Matrix-assisted laser desorption-ionization time-of-flight analysis on the native GSS from C. fas-
C. fasciculata revealed a nominal molecular mass of 80,641 Da, which correlates well with the predicted nominal molecular mass (80,336 Da), confirming the aberrant migration of both the native and recombinant GSS.

E. coli strain BL21(DE3)/pET-CfTRS strain overproduced a protein that migrated with an apparent molecular mass of about 82 kDa (Fig. 6), which is also larger than the mass predicted from the primary amino acid sequence (74 kDa) but identical to that described by Smith et al. (18) for TRS. The reason for the aberrant migrations of these proteins is not known.

Fortunately, both proteins were found exclusively in the insoluble fraction (Fig. 6), and attempts to solubilize and renature the active enzymes from 6 M urea were unsuccessful. Expression using an E. coli strain overexpressing thioredoxin (34), expression as fusion proteins with glutathione S-transferase (pGEX, Amersham Pharmacia Biotech), and expression of fragments of the protein all failed to yield soluble or active protein. The co-expression of both CfGSS and CfTRS in E. coli did not solve the problem of insolubility either.

S. cerevisiae Overexpressing CfGSS and CfTRS Proteins

CfGSS and CfTRS were cloned into a yeast expression vector (pRB1438) for overproduction of the synthetases in S. cerevisiae strain ABYS106. Analysis of SDS-PAGE gels of insoluble and soluble extracts of galactose-induced cultures of ABYS106/pRB1438-CfGSS cells, ABYS106/pRB1438-CfTRS cells, or ABYS106/pRB1438-CfGSS/CfTRS showed no overexpression of protein (data not shown), and no enzymatic activity could be detected in the soluble fraction, suggesting that both proteins were poorly expressed in yeast.

Thiol Analysis in S. cerevisiae Overexpressing CfGSS and CfTRS Proteins

Reduced thiols from galactose-induced ABYS106/pRB1438-CfGSS cells, ABYS106/pRB1438-CfTRS cells, and ABYS106/pRB1438-CfGSS/CfTRS cells were separated and quantified by HPLC after derivatization with monobromobimane. As expected, the majority of cellular thiol in yeast is glutathione...
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(37.5 nmol (10⁻⁸ cells)) (35), and no glutathionylspermidine or trypanothione-bimane adducts were found in the control S. cerevisiae ABYS106 (Fig. 7, A and E). The two small peaks (U1 and U2) visible on the chromatograph elute at a time consistent with their being bimane adducts of γ-L-glutamyl-L-cysteine and L-cysteinylglycine, respectively. Two peaks representing the majority of cellular thiols in ABYS106/pRB1438-Cf-GSS cells were identified (Fig. 7B). Peak 1 eluted with a retention time corresponding to the glutathione-bimane adduct (27.8 nmol (10⁻⁸ cells)), while peak 2 corresponded to the bimane adduct of glutathionylspermidine (17.5 nmol (10⁻⁸ cells)) (Fig. 7, B and E). Although the analytical method did not allow us to separate the N¹- and N⁸-isomers, the presence of these peaks demonstrates that the Cf-GSS gene encodes the GSS activity. No trypanothione was detected, confirming that the enzyme does not possess TRS activity as reported previously (18). Three peaks were found in ABYS106/pRB1438-Cf-GSS/Cf-TRS cells. Peaks 1 and 2 correspond to the two described above (21.8 and 6.6 nmol (10⁻⁸ cells), respectively) and peak 3 corresponds to the bimane adduct of trypanothione (4.8 nmol (10⁻⁸ cells)) (Fig. 7, C and E), confirming that the Cf-TRS gene encodes the trypanothione synthetase protein. No trypanothione was found in the cells expressing only the Cf-TRS gene (Fig. 7, D and E), since S. cerevisiae cannot make glutathionylspermidine as a substrate for this enzyme and Cf-TRS cannot make glutathionylspermidine (18). These findings conclusively demonstrate the enzymatic function of each of the encoded proteins.

**DISCUSSION**

In contrast to most prokaryotic and eukaryotic cells, in which the major thiol component is glutathione, the Kinetoplastida possess a unique low molecular mass thiol, trypanothione (9, 10), which is synthesized from glutathione and spermidine via N¹- or N⁸-glutathionylspermidine, involving two distinct ATP-dependent enzymes, GSS and TRS (18, 19). To date, only trypanosomatids have been found to contain trypanothione (15). However, E. coli can synthesize the metabolic intermediate N¹-glutathionylspermidine (21), but not trypanothione (22). The function of glutathionylspermidine in E. coli is unknown, but, since it only accumulates in stationary phase cells, it has been proposed that it might be involved in the regulation of intracellular levels of the precursors, spermidine and glutathione (21). Also, because of its net positive charge at physiological pH, glutathionylspermidine may be more effective at protecting DNA against radical- or oxidant-induced damage than negatively charged glutathione, as has been proposed for trypanothione (36). E. coli GSS has been shown to possess an amidase activity, permitting it to both synthesize and break down glutathionylspermidine (23). Amidase activity is also present in the C. fasciculata GSS (Fig. 4), and alignment of the N-terminal regions of E. coli GSS and of Cf-GSS with Cf-TRS suggests conservation of an amidase function in Cf-TRS as well (Fig. 3). Moreover, Cys-59 of E. coli GSS, which is implicated in the catalytic mechanism of the amidase activity (25), is conserved in both sequences (Fig. 3). Whether Cf-TRS is able to hydrolyze trypanothione and/or glutathionylspermidine is not known at present. However, such a bifunctional activity could regulate the level of glutathionylspermidine and trypanothione in the parasite, since both thiols vary in a reciprocal fashion during the growth phases of C. fasciculata (26). The mechanism by which these two opposing activities are regulated to avoid a futile cycle resulting in hydrolysis of ATP is not fully understood, although pH may be a factor (23).

Despite a considerable amount of effort, we have been unable to obtain functionally active recombinant Cf-GSS or Cf-TRS in E. coli. However, we have been able to conclusively demonstrate that the genes do encode the two different synthetases by expressing them in yeast. Cells transformed with Cf-GSS produced glutathionylspermidine, and those containing both this gene and Cf-TRS produced trypanothione. The fact that no glutathionylspermidine or trypanothione was found in cells transformed with Cf-TRS alone is consistent with our previous report that Cf-TRS is unable to make detectable glutathionylspermidine (18).

It is important to stress at this juncture that some of our findings are in conflict with a recent report by Köning et al. (19). In our hands, GSS purified from C. fasciculata migrates anomalously on SDS-PAGE with an apparent molecular mass of 90 kDa, whereas the gene sequence predicts a protein with a molecular mass of 80.3 kDa. Mass spectrometry on the purified 90-kDa protein yields a nominal molecular mass of 80.6 kDa, which, allowing for the unknown N-terminal blocking group (18, 19), is in excellent agreement with the molecular mass predicted from the sequence of the gene. Expression of Cf-GSS in E. coli yields an inactive protein that migrates anomalously at 90 kDa. Thus, our results are internally consistent with our previous finding that GSS is associated with a 90-kDa peptide (18).
In light of these comments, it is difficult to explain why Koenig et al. (19) isolated a 78-kDa protein from the same clonal isolate of C. fasciculata possessing GSS activity but lacking glutathionylspermidine amidase or TRS activity. TRS activity is reported to be separated from GSS activity in the first step of their purification scheme, which involves aqueous two-phase extraction at room temperature in the absence of any protease inhibitors (19). Thus, it is curious that the sequences of all seven peptides from their 78-kDa protein with apparent GSS activity correspond exactly to our TRS sequence. In light of these comments, it is difficult to explain why Koenig et al. (19) isolated a 78-kDa protein from the same clonal isolate of C. fasciculata possessing GSS activity but lacking glutathionylspermidine amidase or TRS activity. TRS activity is reported to be separated from GSS activity in the first step of their purification scheme, which involves aqueous two-phase extraction at room temperature in the absence of any protease inhibitors (19). Thus, it is curious that the sequences of all seven peptides from their 78-kDa protein with apparent GSS activity correspond exactly to our TRS sequence.

The relationship of the TRS gene to the GSS gene would implicate that it has probably evolved from it via mutation following duplication. In addition to significant homology to each other and to the E. coli GSS, three hypothetical proteins from E. coli (31, 32) and H. influenzae (33), present in the sequence data bases have significant homology to this protein family. Homology is confined to the carboxyl terminus, suggesting that these enzymes may possess only a synthetase activity (23, 24). The function of the three hypothetical proteins is hard to predict, since alignment of all of these proteins did not unambiguously identify sequence motifs involved in recognition of Mg$^{2+}$, ATP, glutathione, or spermidine.

Both reactions depend on ATP and Mg$^{2+}$, and the GSS reaction may proceed through the synthesis of a glutathionylsphosphate intermediate as described previously for other amide bond-forming reactions such as d-alanine:d-alanine ligase (37), glutamine synthetase (38, 39), and glutathione synthetase (40). Phosphinate and phosphonate analogues (which are known to inhibit this class of enzymes) have been shown to inhibit GSS activity (41–44) and may form the basis for novel chemotherapeutic agents. We are currently isolating the corre-