Characterization of Trypanosoma brucei γ-Glutamylcysteine Synthetase, an Essential Enzyme in the Biosynthesis of Trypanothione (Diglutathionylspermidine)*

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The parasitic protozoan Trypanosoma brucei maintains redox balance by synthesizing a conjugate of glutathione and spermidine termed trypanothione. The first committed step in the biosynthesis of glutathione, and thereby trypanothione, is catalyzed by the enzyme γ-glutamylcysteine synthetase (γGCS). We have cloned and sequenced the 2037-base pair gene coding for the catalytic subunit of T. brucei γGCS. T. brucei γGCS appears to be encoded by a single copy gene. A transcript of about 2.3 kilobases was observed in procyclic trypanomastigotes. The deduced amino acid sequence of 679 residues was compared with mammalian, Caenorhabditis elegans, and yeast γ-GCS, respectively. The T. brucei γ-GCS gene was expressed in E. coli; the purified 77.4-kDa enzyme catalyzes the ligation of L-Glu to L-Cys with a $K_m$ of $10^{-3}$ M, confirming that the gene encodes the functional catalytic subunit of γ-GCS. The apparent $K_m$ values measured for the three natural substrates L-Glu, L-Cys, and ATP are 0.24, 0.69, and 0.07 mM, respectively. Unlike the mammalian enzyme, L-$\alpha$-aminobutyrate (apparent $K_m = 10$ mM) is a poor substitute for L-Cys in the T. brucei γ-GCS-catalyzed reaction. T. brucei γ-GCS is feedback-inhibited by glutathione (apparent $K_i = 1.1$ mM), and it is inactivated by cystamine and buthionine sulfoximine. The kinetic properties of recombinant T. brucei γ-GCS suggest that the substrate binding pocket and the mechanism of enzyme regulation differ from the mammalian enzyme, providing evidence that T. brucei γ-GCS could be a selective chemotherapeutic target for the treatment of trypanosomiasis.

The parasitic protozoan Trypanosoma brucei is the causative agent of African sleeping sickness in humans and nagana in cattle (1). As current chemotherapy is unsatisfactory, metabolic differences between trypanosomes and their mammalian hosts are being characterized to elucidate new potential drug targets in the parasite. Major differences have been found in the utilization of the tripeptide thiol glutathione (GSH). Mammals rely on the antioxidant GSH to protect against oxidative injury by peroxides or free radicals and detoxification of xenobiotics (2, 3). In place of GSH, trypanosomes utilize trypanothione (diglutathionylspermidine), a conjugate of GSH and spermidine, to maintain the redox balance of the cells (4). Trypanothione is synthesized in four steps via the synthesis of GSH and its subsequent conjugation to spermidine. GSH is synthesized by two enzymes, which are in common with mammalian cells, while the conjugation of GSH to spermidine is catalyzed by two trypanosome-specific enzymes (5).

The first and rate-limiting step in the biosynthesis of GSH is catalyzed by γ-glutamylcysteine synthetase (γGCS; Reaction 1).

\[
\text{γGCS} \\
\text{L-Glutamate + L-cysteine + ATP} \rightarrow \text{L-γ-glutamyl-L-cysteine + ADP + Pi.}
\]

A specific inhibitor of γGCS, buthionine sulfoximine, cures or prolongs survival of mice infected with T. brucei, implicating γGCS as a potential drug target (6). The effectiveness and selectivity of buthionine sulfoximine against T. brucei infection suggests that trypanosomes are more sensitive to GSH depletion than are mammalian cells. Trypanosomes have been reported to lack catalase (7), which in mammals breaks down hydrogen peroxide in the peroxisome. Consequently, trypanosomes possess an intracellular hydrogen peroxide concentration higher than that found in mammalian cells, which may account for the detrimental effect of GSH depletion (8). The effect of oxidative stress on T. brucei survival is also illustrated by the finding that lysis of the cattle variant of T. brucei by a component in human serum is likely to be mediated by $H_2O_2$ (9). Despite the importance of γGCS to trypanosome viability, the enzyme has never been characterized.

Mammalian γGCS consists of a catalytic or heavy subunit (70 kDa) and a regulatory or light subunit (30 kDa) (10). The isolated heavy subunit retains all of the catalytic activity including feedback inhibition by GSH (11); the regulatory subunit modulates the activity of the catalytic subunit by increasing the affinity of the enzyme for L-Glu and decreasing its affinity for GSH (12). The catalytic subunit of γGCS has been cloned from rat (2), human (13), Saccharomyces cerevisiae (14), Schizosaccharomyces pombe (15), Caenorhabditis elegans (16), Escherichia coli (17), and Arabidopsis thaliana (18). Because the E. coli and plant enzymes share minimal sequence identity with the other eukaryotic enzymes, it has been suggested that they are unrelated (17, 18).

It is not known if the regulatory subunit, which has been cloned from both human and rat kidney (19, 20), is a component of the nonmammalian enzymes. The other eukaryotic γGCS frame; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
which has been purified and characterized is from the worm, *Ascaris suum*, and this enzyme was isolated as a 70-kDa monomer, suggesting that, if a regulatory domain had been present, it was not tightly associated with the catalytic subunit (21).

In order to characterize structural and functional differences between parasite and host γ-GCS we undertook the cloning of the *T. brucei* γ-GCS gene. A partial clone was obtained by PCR using degenerate oligonucleotide primers designed to conserve sequences found in the mammalian and yeast enzymes. A full-length clone of the *T. brucei* γ-GCS gene was obtained from a genomic library and encodes a 679-amino acid protein (molecular mass = 77.4 kDa) sharing 45, 41, and 36% sequence identity with the mammalian catalytic subunit, *C. elegans*, and yeast γ-GCS, respectively. Recombinant *T. brucei* γ-GCS was expressed in *E. coli* and purified. Kinetic characterization of *T. brucei* γ-GCS suggests that mediation of enzyme activity by a regulatory domain is unlikely to be necessary in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials—**Reagents for the enzyme assay were purchased from Sigma, Ni\(^{2+}\)-agarose was purchased from Qiagen, and TEV protease was purchased from Life Technologies, Inc.

**Amplification of γ-GCS by PCR—**Degenerate PCR primers were designed to two highly conserved sequences found in rat and yeast γ-GCS, corresponding to rat amino acids 240–245 and 396–407 (sense primer = 5'- AAGGATCATGGNTTYGGNATGGG-3'; antisense primer = 5'- TTGTTATTCGTCATTGNTGNYTNGTTYCTTARTG-3'; N = all bases, Y = C or T, R = A or G). PCR was performed in 67 mM Tris, pH 8.8, 6.7 mM MgCl\(_2\), 16.6 mM ammonium sulfate, 10 mM β-mercaptoethanol, 10% dimethylsulfoxide, 1 mM of each dNTP. Each PCR cycle consisted of denaturation (1 min at 94°C), annealing (1 min at 45°C), and polymerization (2 min at 72°C); 40 cycles were performed to obtain product. The PCR product was purified by electrophoresis on agarose gel and subcloned into the BamHI and EcoRI sites of pGEM-3zf (Stratagene).

DNA and RNA isolation from *T. brucei*—Genomic DNA and total RNA was isolated as described (22, 23) from procyclic 427 or 366D *T. brucei* cells cultured in SDM-79 medium or modified Steiger's medium, -mercaptoethanol, 10% dimethylsulfoxide, 1 mM of each dNTP. The cloned *BamHI/EcoRI* PCR fragment was isolated by restriction digestion and agarose gel electrophoresis, labeled with [\(^{32}P\)]dCTP by the random primer method (Boehringer Mannheim), and used to screen approximately 6.2 \(\times\) 10\(^5\) plaques from the amplified unamplified library. Phage were transferred to nylon membranes and hybridized to a full-length clone of the *T. brucei* γ-GCS gene obtained from a genomic library and encodes a 679-amino acid protein (molecular mass = 77.4 kDa) sharing 45, 41, and 36% sequence identity with the mammalian catalytic subunit, *C. elegans*, and yeast γ-GCS. The unamplified library contained 2.5 \(\times\) 10\(^6\) plaques. Each phage was screened for hybridization with the amplified *T. brucei* γ-GCS gene by Kunkel mutagenesis (31). Mutagenesis was confirmed by DNA sequence analysis. Because of the presence of an internal Ncol in the γ-GCS gene, the gene was cloned into the Ncol/HindIII sites of the expression vector in two pieces. The 5' end of *T. brucei* γ-GCS expressed from this construct (pTBGCS) is M-H\(_{\text{II}}\)AENLYFGAGML. The underlined amino acids represent the TEV protease site (30), and the residues in italics are the first 4 amino acids of *T. brucei* γ-GCS.

**Enzyme Purification—** *E. coli* BL21/D3 cells (30) containing pTB-GCS were grown from single colonies at 37°C in Luria-Bertani medium, and purified. Kinetic characterization of *T. brucei* γ-GCS suggests that mediation of enzyme activity by a regulatory domain is unlikely to be necessary in vivo.

**RESULTS**

**Isolation and Characterization of Clones—**Degenerate oligonucleotide primers were designed to two amino acid sequence regions conserved between the rat kidney catalytic subunit (34) and *S. cerevisiae* γ-GCS (14), corresponding to the motif MGF-GM (residues 240–245 of rat γ-GCS) and to HENFİQST-NWQT (residues 396–407 of rat γ-GCS). A 500-bp fragment was amplified from the primers using *T. brucei* genomic DNA and cloned into the BamHI and EcoRI sites of pBluescript. Sequence analysis verified that this DNA fragment encoded an ORF with sequence similarity to rat and yeast γ-GCS. The cloned PCR fragment was labeled with \(^{32}P\) and used to screen a *T. brucei* genomic library (trypanosome genes lack introns)
Seven clones were isolated which contained overlapping DNA fragments of 1.5–5.8 kb. Clone Tb12.1 (insert size 5.5 kb) contained a full-length 2037-bp ORF which was sequenced on both strands. The ORF is predicted to encode a 679-amino-acid polypeptide of 77.4 kDa (Fig. 1).

Comparative Data Base Analysis of *T. brucei* γGCS—The deduced *T. brucei* γGCS amino acid sequence shares 36–45% sequence identity with the catalytic subunits of γGCS from rat (34), human (13), *C. elegans* (16), *S. cerevisiae* (14), and *S. pombe* (15), with 146 invariant residues identified among all six sequences. A unique 60-amino acid insertion is found in *T. brucei* γGCS at position 242. An alignment of representative eukaryotic γGCS sequences is displayed in Fig. 2A. Two other γGCS sequences have been reported for *E. coli* and *A. thaliana*; however, it has been suggested that they may not be related to the mammalian and yeast enzymes (18).

Analysis of the function of conserved amino acid residues in the γGCS family has been limited by the small number of organisms for which sequence information was available. The cloning of the *T. brucei* gene allows us to identify sequence motifs which have been conserved among organisms with diverse evolutionary backgrounds; *T. brucei* is thought to have originated as one of the most primitive eukaryotic lineages (36).

A profile was constructed from the mammalian, yeast, *C. elegans*, and *T. brucei* sequences and was used to search the Swissprot data base. *A. thaliana* γGCS was identified by this search (Z score, 3.18), whereas *E. coli* γGCS was not. *A. thaliana* γGCS shares an average sequence identity with the other eukaryotic enzymes of 18% over the full-length of the sequence. Notably, it shares 35 out of the 146 invariant residues identified among the other eukaryotic enzymes with the *T. brucei* γGCS (Fig. 2A). Previous analysis had not identified this region as one of the conserved sequence blocks (18).

RNA blot analysis confirmed that the trypanosomal γGCS gene is actively transcribed. A single 2.3-kb transcript was observed in total RNA isolated from procyclic 427 cells (Fig. 4).

Kinetic Characterization of γGCS—The γGCS gene contained in Tb12.1 was subcloned into an expression vector which utilizes the T7 promoter to direct the expression of γGCS fused to an N-terminal six-histidine tag followed by the TEV protease cleavage site (30). The soluble protein was purified from the E. coli cell extracts as described in Experimental Procedures. A single band of approximately 75 kDa was observed by SDS-PAGE analysis (Fig. 5). The identity of the band was confirmed by N-terminal amino acid sequencing. The apparent molecular mass of *T. brucei* γGCS estimated by chro-
matography on the Superdex 200 column is 75 kDa, suggesting that it is a monomer in solution.

The purified recombinant T. brucei γ-GCS catalyzes the ligation of L-Glu with L-α-aminobutyric acid or L-Cys in the presence of ATP with a specific activity of 7.6 μmol/min/mg of protein, which corresponds to a kcat of 9.8 s⁻¹. The specific activity is similar to the activity reported for the rat catalytic subunit (16 units/mg) (12, 20) or for the single-subunit enzyme purified from A. suum (18 units/mg) (21). For T. brucei γ-GCS, the Km app for L-Glu is 6- and 75-fold lower than for the rat holoenzyme and the rat catalytic subunit, respectively, while it is 4-fold lower than for the A. suum enzyme (Table I).

In contrast, the Km app for L-α-aminobutyric acid is 10–30-fold higher than reported for the rat or the A. suum enzymes. Additionally, the selectivity of T. brucei γ-GCS for L-Cys over L-α-aminobutyrate (measured by the ratio of Km app for L-Cys to Km app for α-aminobutyrate) is 4- and 20-fold greater than that observed for the rat or A. suum catalytic subunits, respectively (Table I). The Km app for ATP is 0.071 ± 0.01 mM, similar to that reported for the rat holoenzyme (12, 20). Like rat γ-GCS (37), T. brucei γ-GCS is inactivated by both cystamine (87% inhibition was observed at 0.5 mM) and buthionine sulfoximine (66% inhibition was observed at 1 mM), and it is feedback-inhibited by GSH. GSH inhibition is competitive with respect to L-Glu.

Fig. 3. Southern blot analysis of T. brucei genomic DNA. T. brucei genomic DNA (6–10 μg) was digested with the indicated restriction endonucleases, electrophoresed, and blotted. The blot was probed with high stringency with the 32P-labeled cloned PCR fragment (10–50 ng).
with a $K_{\text{m}}^{\text{app}} = 1.1 \pm 0.2$ mM slightly higher than the reported cellular concentrations of GSH in T. brucei (38).

**DISCUSSION**

We have cloned the T. brucei $\gamma$GCS gene which codes for a 679-amino acid, 77.4-kDa protein. Although many trypanosome genes are found in multiple copies and in tandem arrays (39), Southern and comparative Dot Blot analyses suggest that T. brucei $\gamma$GCS is a single copy gene. Northern blot analysis demonstrates that the T. brucei $\gamma$GCS gene is transcribed in procyclic trypanosomes and expression of the $\gamma$GCS gene in E. coli confirms that the T. brucei gene encodes the functional catalytic subunit of $\gamma$GCS.

T. brucei $\gamma$GCS shares a 36–45% amino acid sequence identity with the other eukaryotic enzymes from mammals (rat and human), yeast (S. cerevisiae and S. pombe) and C. elegans. Profile analysis which included the T. brucei $\gamma$GCS sequence identified A. thaliana but not E. coli $\gamma$GCS in the search, suggesting that A. thaliana $\gamma$GCS may be structurally related to the other eukaryotic enzymes. Of the amino acids which are invariant in all of the eukaryotic enzymes, 7 invariant positively charged amino acids and a single Cys were identified. The structures of other enzymes which bind GSH (e.g. GSH S-transferase (40) and GSH reductase (41)) reveal that Arg residues are found as common components of the substrate and GSH carboxylate binding sites, suggesting that the conserved Arg residues maybe involved in the binding of L-Glu and L-Cys by $\gamma$GCS.

A role for a conserved Cys is suggested by the observation that the eukaryotic enzymes, including as we have demonstrated, T. brucei $\gamma$GCS, are inactivated by cystamine. Cystamine inactivation of $\gamma$GCS is reversed by dithiothreitol providing evidence that the mechanism of inactivation is through disulfide bond formation to a Cys residue (42–44). L-Glu protects the enzyme from cystamine inactivation suggesting that the susceptible Cys residue is in or near the active site (45). As the only conserved Cys residue, Cys$^{319}$ is likely to mediate the response to cystamine. The reaction catalyzed by $\gamma$GCS is thought to proceed by transfer of the $\gamma$-phosphate of ATP to L-Glu to form a $\gamma$-glutamylphosphate intermediate; this intermediate is attacked by L-Cys to form products (46). Thus the $\gamma$-phosphate of ATP must be positioned in close proximity to the L-Glu binding site. While there is no universal ATP binding site, a glycine-rich loop (e.g. the P-loop) is a common component of phosphate binding sites (47, 48). $\gamma$GCS does not contain a classic P-loop motif but it does contain a conserved glycine-rich sequence which directly precedes the invariant Cys and is characterized by the motif M(A/G)FGMGXXCLQ.

The substrate specificity profile of recombinant T. brucei $\gamma$GCS differs significantly from the profiles reported for the rat or A. suum enzymes and suggests it will be possible to design selective inhibitors of the parasite enzyme. T. brucei $\gamma$GCS has a stronger preference for L-Cys over $\alpha$-aminobutyrate when compared to the rat enzyme, while the A. suum enzyme has a slight preference for $\alpha$-aminobutyrate. These results suggest that the T. brucei binding pocket for L-Cys must differ from the binding pockets of the other enzymes.

In addition to these differences, the T. brucei $\gamma$GCS catalytic subunit has a much greater apparent binding affinity for L-Glu than the other eukaryotic enzymes.

**Table 1**

| Enzyme          | Apparent $K_m$ or $K_i$ (mM) | $K_m$ (mM) | $K_i$ (mM) |
|-----------------|------------------------------|------------|------------|
|                 | L-Glu | L-$\omega$-Aminobutyrate | L-Cys | GSH |
| T. brucei       | 0.24 ± 0.022 | 10.4 ± 1.01 | 0.69 ± 0.13 | 1.1 ± 0.20 |
| Rat catalytic subunit$^a$ | 18 | 0.8 | 0.2 | 1.8 |
| Rat holoenzyme$^a$ | 1.4 | 1.2 | 0.2 | 8.2 |
| Ascaris suum$^b$ | 0.9 | 0.3 | 0.4 | 0.1 |

$^a$ Values from Huang et al. (12).

$^b$ Values from Hussein and Walter (21).
than the rat catalytic subunit (Table I). In the rat holoenzyme, the effect of the regulatory subunit is to increase the affinity of the catalytic subunit for l-Glu by reducing the $K_m^{app}$ from 18 mM to 1 mM, and to moderate the feedback inhibition by GSH by increasing the $K_i^{app}$ from 2 to 10 mM (12, 20). Our results demonstrate that T. brucei $\gamma$GCS does not require a regulatory subunit to create a high affinity l-Glu binding site. While for T. brucei $\gamma$GCS the apparent $K_j$ for GSH is similar to that reported for the rat catalytic subunit and lower than that for the rat holoenzyme (Table I), the intracellular concentration of GSH has also been reported to be lower in T. brucei cells (0.4 mM) (38) relative to mammalian cells (5 mM) (2). Thus, with respect to their cellular environments, monomeric T. brucei $\gamma$GCS should be as efficient as the holoenzyme complex of the mammalian enzyme. While our results do not rule out a second subunit component of T. brucei $\gamma$GCS, they do lead to the conclusion that the role of any such regulatory subunit must be markedly different from that reported for the rat enzyme. The most striking difference between the T. brucei and rat $\gamma$GCS sequences is the unique 60-amino acid insertion in the T. brucei enzyme at position 242. Perhaps this insertion is involved in replacing the role of the regulatory subunit in promoting high affinity binding to l-Glu. Further analysis of both the recombinant and native T. brucei $\gamma$GCS will allow us to address these questions.

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