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Structure of *Trypanosoma brucei* glutathione synthetase: Domain and loop alterations in the catalytic cycle of a highly conserved enzyme

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ABSTRACT

Glutathione synthetase catalyses the synthesis of the low molecular mass thiol glutathione from L-γ-glutamyl-L-cysteine and glycine. We report the crystal structure of the dimeric enzyme from *Trypanosoma brucei* in complex with the product glutathione. The enzyme belongs to the ATP-grasp family, a group of enzymes known to undergo conformational changes upon ligand binding. The *T. brucei* enzyme crystal structure presents two dimers in the asymmetric unit. The structure reveals variability in the order and position of a small domain, which forms a lid for the active site and serves to capture conformations likely to exist during the catalytic cycle. Comparisons with orthologous enzymes, in particular from *Homo sapiens* and *Saccharomyces cerevisae*, indicate a high degree of sequence and structure conservation in part of the active site. Structural differences that are observed between the orthologous enzymes are assigned to different ligand binding states since key residues are conserved. This suggests that the molecular determinants of ligand recognition and reactivity are highly conserved across species. We conclude that it would be difficult to target the parasite enzyme in preference to the host enzyme and therefore glutathione synthetase may not be a suitable target for antiparasitic drug discovery.

1. Introduction

The tripeptide glutathione (L-γ-glutamyl-L-cysteinyllycine, GSH) is an abundant non-protein thiol implicated in a variety of cellular functions. The majority of organisms use GSH, in conjunction with glutathione reductase and a glutathione peroxidase, to regulate intracellular thiol levels, to control redox metabolism and therefore protect against reactive oxygen species. GSH also contributes to the metabolism of drugs, carcinogens, and in maintaining cysteine residues in their reduced form [1,2].

Trypanosomatids are unusual in that they derive protection against oxidative damage and maintain intracellular thiol redox balance through the utilization of trypanothione, a peptide–polyamine conjugate (N^1,N^8-bis(glutathionyl)-spermidine, $T[SH]_2$) [3–5]. This protective mechanism requires the presence of a unique peroxidase pathway comprising trypanothione reductase, tryparedoxin and tryparedoxin peroxidase. The divergence from the human host in this aspect of biology is of basic interest and we have determined the structure–function relationships for the tryparedoxin peroxidase pathway components [6–10]. The protective peroxidase pathway is recognized as a potential route for the development of urgently sought antiparasitic drugs [4,5]. It is also recognized that disruption of $T[SH]_2$ biosynthesis might represent a useful strategy in this respect [11] and characterization of the component enzymes is an important first step in assessing the potential of such targets [12].

Biosynthesis of the peptide component of $T[SH]_2$ involves two enzymes. First glutamylcysteine synthetase is responsible for the ligation of cysteine to glutamate and then glutathione synthetase (GS) converts the dipeptide to GSH by addition of glycine (Fig. 1). The bifunctional enzyme trypanothione synthetase-amidase catalyses the stepwise addition of two molecules of glutathione (GSH) onto spermidine and is also capable of hydrolyzing $T[SH]_2$ and/or glutathionylspermidine back to spermidine and glutathione [13,14].

We set out to characterize the structure of *Trypanosoma brucei* glutathione synthetase (*Tb*GS) and to assess its potential as a target for design, or discovery, of a novel therapeutic agent for treatment of African trypanosomiasis. We now report the preparation of an efficient bacterial protein production system, a purification protocol, and a low resolution (3.15 Å) crystal structure. Comparisons with previously determined structures of GS from *Homo sapiens* (*Hs*GS) and *Saccharomyces cerevisae* (*Sc*GS) [15,16] have been carried out.

Abbreviations: AMP-PNP, adenylyl imidodiphosphate; GS, glutathione synthetase; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-hydroxyethyl)piperazine-N-(2-ethanesulfonic acid); MOPS, 3-(N-morpholino)-propanesulfonic acid; NCS, non-crystallographic symmetry; *Tb*, *Trypanosoma brucei*; TEV, tobacco etch virus; TLS, translation/libration/screw; TSA, trypanothione synthetase; $T[SH]_2$, trypanothione.

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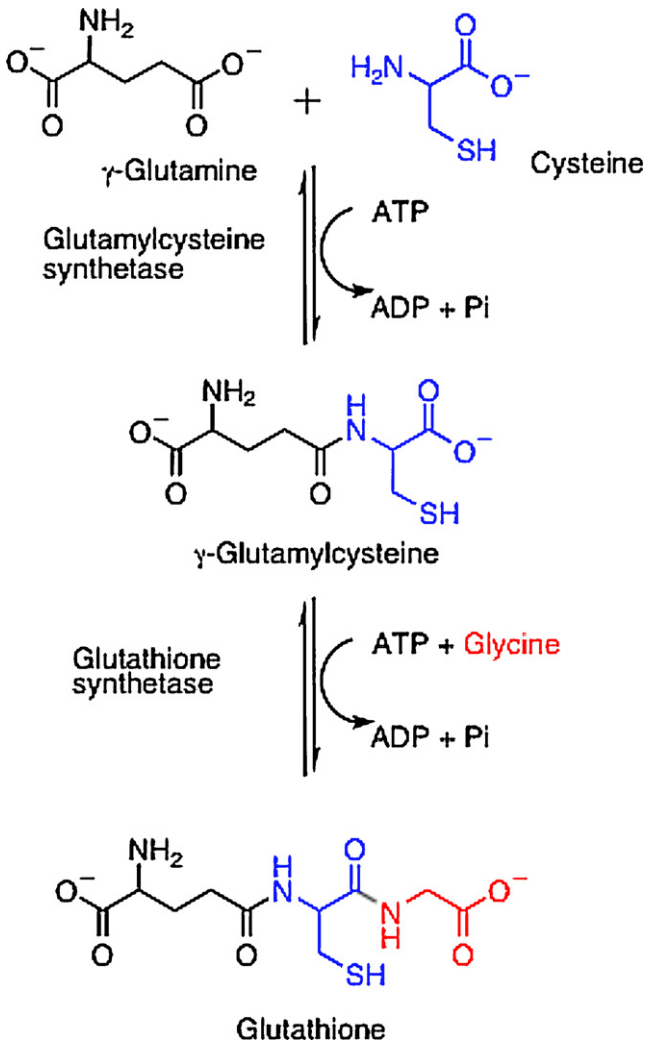

Fig. 1. Biosynthesis of glutathione.

2. Materials and methods

2.1. Protein preparation and crystallization

The gene encoding *Tb*GS was amplified from genomic DNA using the primers 5′-catatgGTGTTAAAATTGTTGCTG and 5′-ctcgagTTACGCTACAACCGCTAA (lower case sequences correspond to the restriction sites used for cloning—Nde1/Xho1). Following TOPO cloning (Invitrogen) of the polymerase chain reaction product, the gene was ligated into a pET15b (Novagen) expression vector modified to encode a Tobacco Etch Virus (TEV) protease cleavage site. The resulting plasmid was transformed into *Escherichia coli* Rosetta (DE3) pLysS. Cultures were grown at room temperature in autoinduction medium [17] containing 50 μg/ml ampicillin and 12 μg/ml chloramphenicol for two days, and cells harvested by centrifugation. The pellet was resuspended in 25 mM HEPES pH 7.5, 500 mM NaBr, 2 mM β-mercaptoethanol, 1 mM $MgCl_2$, and 5 mM imidazole, and cells lysed using a French Press. Cell debris was removed by centrifugation at 40,000 × *g*.

*Tb*GS was purified by nickel affinity chromatography on a 5 mL HisTrap column (GE Healthcare) using an imidazole gradient. Following overnight dialysis at 4 °C into 25 mM HEPES pH 7.5 and 250 mM NaBr, gel filtration chromatography using a Superdex200 26/60 column (GE Healthcare) completed the purification. Gel filtration indicated that in solution *Tb*GS exists as a dimer of approximate mass 120 kDa. The high level of purity (>95%) was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The sample was dialysed into 50 mM MOPS pH 7.0 and 100 mM NaBr then concentrated to 6 mg mL^{-1} using a Vivaspin 20 (Sartorius) to provide a stock solution for crystallization. The yield was 8 mg of purified *Tb*GS per litre of bacterial culture.

Table 1
Crystallographic statistics.

Space group	$P2_12_12_1$
Unit cell lengths (Å)	92.5 125.0 242.9
Maximum resolution (Å)	3.15
Unique reflections	49689
Completeness (%)	99.9 (100)[a]
$\langle I/\sigma(I)\rangle$	9.0 (2.7)
Mosaicity (°)	0.4
Redundancy	4.0 (4.1)
R_{merge}[b]	11.6 (50.6)
Refinement statistics	
Resolution range (Å)	39.5–3.15
No. of reflections	47141
R_{work}[c]	21.5
R_{free}[d]	28.8
Protein residues	1996
Ligands	4 GSH, 7 sulfates, 22 waters
r.m.s. deviations from ideal geometry	
Bond lengths	0.012
Bond angles	1.5
B values (Å^2)	
From Wilson plot	77.9
Mean B over all atoms	68.4
Ramachandran favoured/allowed/outliers[e] (%)	90.4/8.2/1.4

[a] Values in parentheses refer to the highest resolution bin (3.32–3.15 Å).
[b] aa $R_{merge} = \sum h \sum i |I(h,i) - \langle I(h)\rangle| \sum h \sum i\, I(h,i)$.
[c] $R_{work} = \sum h\, k\, l ||F_o| - |F_c|| / \sum |F_o|$, where F_o is the observed structure factor amplitude and the F_c is the structure-factor amplitude calculated from the model.
[d] R_{free} is the same as R_{work} except determined using 5%, of the data that are not included in any refinement calculations.
[e] Ramachandran analysis performed using MolProbity [22].

*Tb*GS was crystallized by the hanging drop vapour diffusion method. Protein was prepared to a final concentration of 6 mg mL^{-1} with 4 mM adenylyl imidodiphosphate (AMP-PNP), 4 mM GSH, 2 mM dithiothreitol and 2 mM $MgCl_2$. A drop containing 1 μL of this protein mixture was mixed with 1 μL of reservoir containing 14% polyethylene glycol 4000, 100 mM citrate buffer pH 5.4, and 200 mM $(NH_4)_2SO_4$. Crystals, orthorhombic blocks, grew within a few days at 20 °C. They were first characterized in-house using a Rigaku 007 rotating anode X-ray generator coupled to a RAXIS IV^{++} image plate detector, cryoprotected by soaking for 5 s with mother liquor substituted with 15% ethylene glycol and diffracted to 3.5 Å. The crystals were stored in liquid N_2 for use at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) for data collection.

2.2. X-ray data collection, processing, structure solution and refinement

Diffraction data were measured on beamline ID14-4 of the ESRF using an ADSC Quantum-4 charge coupled device detector. Data were processed using XDS and scaled using XSCALE [18]. Data statistics are summarised in Table 1. The structure was solved by molecular replacement using the program PHASER [19] with the structure of *Sc*GS [1M0W; 16] as the search model. Four molecules (two dimers) were located per asymmetric unit, giving a *Z*-score of 54.2. Refinement was performed using REFMAC5 [20] utilizing both non-crystallographic symmetry (NCS) and Transla-