Molecular & Biochemical Parasitology 169 (2010) 12–19

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Comparative structural, kinetic and inhibitor studies of *Trypanosoma brucei* trypanothione reductase with *T. cruzi*☆

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ARTICLE INFO

Article history:
Received 17 June 2009
Received in revised form 27 August 2009
Accepted 3 September 2009
Available online 10 September 2009

Keywords:
Trypanothione metabolism
Trypanosome
Thiol
Enzymology
Drug discovery

ABSTRACT

As part of a drug discovery programme to discover new treatments for human African trypanosomiasis, recombinant trypanothione reductase from *Trypanosoma brucei* has been expressed, purified and characterized. The crystal structure was solved by molecular replacement to a resolution of 2.3 Å and found to be nearly identical to the *T. cruzi* enzyme (root mean square deviation 0.6 Å over 482 Cα atoms). Kinetically, the K_m for trypanothione disulphide for the *T. brucei* enzyme was 4.4-fold lower than for *T. cruzi* measured by either direct (NADPH oxidation) or DTNB-coupled assay. The K_m for NADPH for the *T. brucei* enzyme was found to be 0.77 μM using an NADPH-regenerating system coupled to reduction of DTNB. Both enzymes were assayed for inhibition at their respective $S = K_m$ values for trypanothione disulphide using a range of chemotypes, including CNS-active drugs such as clomipramine, trifluoperazine, thioridazine and citalopram. The relative IC_{50} values for the two enzymes were found to vary by no more than 3-fold. Thus trypanothione reductases from these species are highly similar in all aspects, indicating that they may be used interchangeably for structure-based inhibitor design and high-throughput screening.

1. Introduction

Trypanosoma brucei is a parasitic protozoan of the family Trypanosomatidae (order Kinetoplastida, suborder Trypanosomatina) responsible for human African trypanosomiasis, also called sleeping sickness. The East and West African forms of the disease are caused by the *T. b. rhodesiense* and *T. b. gambiense* subspecies, respectively [1]. The disease is fatal if untreated, and the few available drugs are not ideal due to emerging drug resistance; parenteral administration; toxic side-effects and cost [2]. *T. b. brucei*, one of the causative agents of Nagana cattle disease, can serve as a model organism for drug discovery and is non-pathogenic to humans. *T. brucei* subspecies, along with all parasites of the order Kinetoplastida, possesses a novel thiol called trypanothione [N^1, N^8-bis(glutathionyl)spermidine] [3]. One of the major roles of this metabolite is to protect the parasite from oxidative stress by maintaining a reducing environment in the cell. In most other organisms, in particular mammals, it is glutathione that plays this protective role. Protection of the parasite against oxidative stress is achieved through the oxidation of the dithiol form of trypanothione ($T(SH)_2$) into the disulphide form ($T(S)_2$), followed by regeneration of $T(SH)_2$ by the NADPH-dependent enzyme trypanothione reductase (TryR) (Fig. 1) [4]. A similar mechanism involving glutathione and glutathione reductase is observed in other organisms, including humans. However, the enzymes trypanothione reductase and glutathione reductase are highly specific for their respective disulphide substrates [5] such that selective inhibition by small molecules can be readily achieved [6].

Metabolism of trypanothione and other low molecular weight thiols has been established as an attractive target for drug discovery in several trypanosomatids [7–9] and TryR from *T. b. brucei* has been specifically validated as a drug target, *inter alia*, by conditional knockout experiments [10]. However, kinetic and inhibition studies of the *T. b. brucei* enzyme have not been developed. Previously the *T. cruzi* enzyme has been used to guide drug discovery for human African trypanosomiasis (HAT), but absence of a clear correlation between inhibitor potency against *T. cruzi* TryR and cidal activity against bloodstream forms of *T. b. brucei* has raised concerns that the *T. cruzi* enzyme is not a suitable model for the *T. b. brucei* enzyme [6]. To address this issue, we report here a comprehensive comparative study of the physicochemical properties, structure, kinetics and inhibitor sensitivities of these enzymes. The information on the enzyme from *T. b. brucei* is also of particular relevance since it is identical at the amino acid level to the putative TryR from *T. b.*

Abbreviations: TryR, trypanothione reductase; $T(S)_2$, trypanothione disulphide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); HAT, human African trypanosomiasis.

☆ *Note:* Crystallographic data have been deposited on PDB with the accession code 2wba.

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doi:10.1016/j.molbiopara.2009.09.002

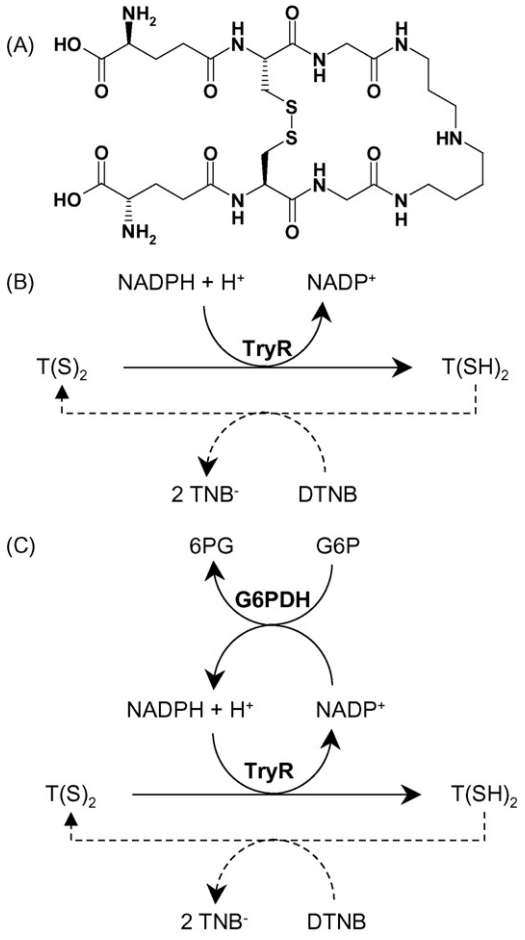

Fig. 1. Trypanothione reductase assay principle. Panel A, structure of trypanothione disulphide. Panel B, in the DTNB-coupled assay trypanothione is recycled to trypanothione disulphide by the reduction of DTNB. Formation of thionitrobenzoate anion (TNB^-) is monitored at 412 nm. Panel C, in a modification of the DTNB-coupled assay NADPH is recycled by the enzyme glucose-6-phosphate dehydrogenase.

gambiense, the causative agent of over 90% of reported HAT cases [11].

2. Materials and methods

2.1. Organisms and reagents

Routine plasmid manipulations were performed in *Escherichia coli* strain JM109 and over-expression in strain BL21 Star (DE3)pLysS (Invitrogen). All chemicals were of the highest grade available from Sigma, BDH and Molecular Probes. Restriction enzymes and DNA-modifying enzymes were from Promega or Roche.

2.2. Cloning and expression TbTryR in E. coli

The complete open reading frame of *TbTRYR* was amplified by PCR from genomic DNA from *T. b. brucei* strain S427 (MITat 1.4) using primers based on a putative TryR gene sequence deposited in GeneDB (**Tb10.406.0520**). The primers used for amplification were: 5′-CAT **ATG** TCC AAG GCC TTC GAT TTG G-3′ and 5′-GGA TCC **TTA** CAG GTT AGA GTC CGG AAG C-3′, incorporating the NdeI and BamHI restriction sites (underlined), respectively, with the start and stop codons in bold.

PCR amplification was done in triplicate. After sequencing, the PCR product of ~1.49 kb was then cloned (via a TOPO cloning vector) into the NdeI/BamHI site of pET3a to generate plasmid pET3a-*Tb*TryR. A 4 L culture of BL21 Star (DE3)pLysS/pET3a-*Tb*TryR was grown to test expression and purification. The cells were grown at 37 °C in LB media, containing 50 μg ml^1 carbenicillin for selection of pET3a and 12.5 μg ml^1 chloramphenicol for the selection of pLysS, at 37 °C with moderate agitation (200 rpm). A larger scale expression in a 30 L culture was grown in a fermenter (Infors HT) using the same media and antibiotics at 37 °C. When the cultures reached an A_{600} of ~0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM. Cultures were grown for an additional 16 h and then harvested by centrifugation at 3480 × *g* at 4 °C for 30 min and washed in phosphate buffered saline (137 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4).

2.3. Purification of TbTryR

E. coli cells were lysed using a one-shot cell disruptor (Constant Systems Ltd.). Purification of recombinant *Tb*TryR was achieved by a combination of ammonium sulphate purification, affinity chromatography on 2′5′-ADP Sepharose, and anion exchange chromatography essentially as described previously [12]. Purity was assessed by SDS-PAGE.

*Tb*TryR was used directly from this procedure for crystallography, analysis of flavin content and measurement of extinction coefficient. The remainder of the TryR was precipitated with 70% saturating ammonium sulphate and aliquotted for storage at 4 °C for subsequent use in kinetic experiments. Protein concentration was measured using the method of Bradford with bovine serum albumin as a standard [13].

2.4. Assessment of oligomeric state

*Tb*TryR (600 μg) was applied to a gel filtration column (Superdex 200 10/300 GE Healthcare) previously equilibrated with 25 mM HEPES pH 7.5 containing 100 mM NaCl. Elution of the column was monitored at 280 nm using an Akta purifier. Molecular weight was inferred from comparison with standards (BioRad gel filtration standard) on a plot of elution volume versus Log molecular weight. Samples of the recombinant enzyme were also analysed by analytical ultracentrifugation (Analytical ultracentrifugation service, College of Life Sciences, University of Dundee).

2.5. Absorbance spectra and determination of absorption coefficient

All spectra were carried out in a UV-1601pc temperature-regulated spectrophotometer (Shimadzu) using 1-cm path-length quartz cuvettes (200 μl sample volume). Enzymes were extensively dialysed against 40 mM HEPES pH 7.4, 1 mM EDTA. Absorbance spectra were acquired over a range of 200–800 nm. The enzyme-associated flavin was liberated by thermal denaturation at 100 °C for 20 min in the presence of 10 mM $MgCl_2$. Denatured protein was removed by microcentrifugation and the concentration of free flavin determined from its absorption coefficient at 450 nm (11.3 mM^{-1} cm^{-1}). The absorption coefficient of oxidised *Tb*TryR was calculated from the absorbance at 463 nm/[FAD] in triplicate samples. The absorption coefficient of NADPH-reduced *Tb*TryR at 530 nM was calculated from the absorbance at 530 nm/[FAD].

2.6. Enzyme assays

TryR was assayed spectrophotometrically either by monitoring the trypanothione-dependent oxidation of NADPH at 340 nm [14], or by the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) at 412 nm (see Fig. 1B) [15]. Assays at 340 nm were carried out at 25 °C in 500 μl volume acrylic cuvettes and changes in