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Transketolase in *Trypanosoma brucei*

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ABSTRACT

A single copy gene, encoding a protein highly similar to transketolase from other systems, was identified in the *Trypanosoma brucei* genome. The gene was expressed in *E. coli* and the purified protein demonstrated transketolase activity with K_m values of 0.2 mM and 0.8 mM respectively for xylulose 5-phosphate and ribose 5-phosphate. A peroxisomal targeting signal (PTS-1) present at the C-terminus of the protein suggested a glycosomal localisation. However, subcellular localisation experiments revealed that while the protein was present in glycosomes it was found mainly within the cytosol and thus has a dual localisation. Transketolase activity was absent from the long slender bloodstream form of the parasite and the protein was not detectable in this life cycle stage, with the RNA present only at low abundance, indicating a strong differential regulation, being present predominantly in the procyclic form. The gene was knocked out from procyclic *T. brucei* and transketolase activity was lost but no growth phenotype was evident in the null mutants. Metabolite profiling to compare wild type and TKT null mutants revealed substantial increases in transketolase substrate metabolites coupled to loss of sedoheptulose 7-phosphate, a principal product of the transketolase reaction.

1. Introduction

Transketolase (TKT) is a key enzyme of the non-oxidative branch of the pentose phosphate pathway. This pathway provides ribose 5-phosphate for nucleotide biosynthesis and also NADPH used in reductive biosyntheses and defense against oxidative stress [1,2]. The pathway, in some organisms, also provides other phosphorylated sugars for anabolic purposes. Transketolase has broad substrate specificity transferring 2-carbon units between a range of ketose donors and aldose acceptors [3]. Specialised derivatives that catalyse other reactions have also evolved. These include 1-deoxylulose-5-phosphate synthase (which uses glycoaldehyde from pyruvate to generate 1-deoxyxylulose 5-phosphate) [4] and dihydroxyacetone synthase (which uses formaldehyde as an acceptor substrate and xylulose 5-phosphate as donor to produce glyceraldehyde 3-phosphate and dihydroxyacetone [5]). Transketolase has also been co-opted as a putative crystallin in vertebrate eyes [6] and also a binding partner of the regulatory protein marR in *E. coli* [7].

The structure of the enzyme has been determined from several systems including yeast [8], *E. coli* [9], maize [10] and the protozoan parasite *Leishmania mexicana* [11]. The enzyme is a homodimer and its mechanism of action has been determined using kinetic and structural analysis (reviewed in 8). In common with several other enzymes that catalyze decarboxylations of α-ketoacids or the transfer of ketol groups from donor ketoses to acceptor aldoses, transketolase requires thiamine diphosphate (TDP) as a cofactor [12,13]. The interaction between transketolase and thiamine has been thoroughly investigated in *Saccharomyces cerevisiae* [14–16].

In the pentose phosphate pathway, transketolase is usually depicted as performing two reactions, transferring a 2-carbon unit from xylulose 5-phosphate to ribose 5-phosphate to yield sedoheptulose 7-phosphate and to erythrose 4-phosphate yielding fructose 6-phosphate. Transketolase has also been shown to play a role in

Abbreviations: TKT, transketolase; PPP, pentose phosphate pathway; ENO, enolase; ALD, aldolase; 6-PGDH, 6-phosphogluconate dehydrogenase; Xu5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate.

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protection against oxidative stress in yeast [17,18] and in mammals [19–21] given its role in redirecting sugar phosphates towards the oxidative branch which generates NADPH.

Glycolysis has been studied in considerable detail in trypanosomatid parasites such as *Trypanosoma brucei* [22–24], the causative agent of sleeping sickness, *Trypanosoma cruzi* the causative agent of Chagas' disease and the Leishmania species responsible for leishmaniasis. The pentose phosphate pathway has, however, only received attention relatively recently in these organisms [25–30]. Transketolase has previously been characterized in *L. mexicana*, where it was shown to have a dual localisation between cytosolic and glycosomal compartments [11]. It was reported that in *T. brucei*, transketolase activity could be detected in the procyclic forms representative of the parasites found in the tsetse fly midgut but not bloodstream form organisms [25] and a recent proteomic analysis of the glycosomal preparations in trypanosomes [31] also found this enzyme in glycosomes of procyclic but not bloodstream form organisms. Bioinformatic analysis, based on the presence of predicted peroxisomal targeting sequences [32], also placed transketolase in glycosomes. To date, however, a detailed analysis of the enzyme in *T. brucei* has been lacking hence we set out to use genetic modification to determine roles of this enzyme in *T. brucei* and coupled this to metabolic profiling of parasites from which the gene was deleted.

2. Material and methods

2.1. Trypanosome stocks

Procyclic *T. brucei* (strain 427) cells [33] were grown at 27 °C in SDM-79 medium [34] supplemented with 5% heat-inactivated fetal bovine serum. The bloodstream form stock BS 221 (synonymous with MiTat 1.2/221; the bloodstream form of strain 427) was cultured at 37 °C in a humidified atmosphere of 5% CO_2 in HMI-9 medium [35] (BioConcept, Allschwil, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (BioConcept).

2.2. Genetic manipulation of the transketolase gene in T. brucei

TKT alleles were replaced sequentially with resistance genes for the antibiotics neomycin and hygromycin. Flanking sequences upstream (311 bp) and downstream (355 bp) of the *TKT* open reading frame were amplified by PCR and cloned into plasmids either side of genes encoding neomycin resistance (*NEO)* or hygromycin resistance (*HYG).* The nucleotide sequences used to amplify the 5′ UTR were 5TKf: 5′-GGG TAC CTG CGC CAT TTC TTT TTC TCT-3′ with 5TKr: 5′-GAA GCT TCG TCT TTG GTC AAT GCT CTG-3′. The sequences of oligonucleotides to amplify the 3′UTR were: 3TKf: 5′-GGG ATC CGG TTT GCT TTT GCC ATG TTT-3′ and 3TKr: 5′-GTC TAG ATT TTG TGT GCC TAA CGA ACG-3′. The resulting deletion cassettes were released from the plasmid using restriction enzymes *Bam*HI and *Hin*dIII, purified by ethanol precipitation, and resuspended to 0.2 μg DNA μL^{-1} in water prior to electroporation into trypanosomes.

To over-express TKT in procyclic cells, the open reading frame was PCR-amplified, using the following primers: TKoverexH-f: 5′-AAG CTT ATG TCG TTT AAT GAT AAC C-3′; TKoverexB-r: 5′-CCT AGG TCA CAA ATG GGA CCG CTT C-3′ and then cloned into the plasmid pHD1485 (with PARP promoter and an N-terminal Myc-tag), transfected into 427-1313 cells constitutively expressing the tet repressor [36] which were then selected with 25 $\mu g\,mL^{-1}$ hygromycin [37]. Induction of overexpression was achieved by adding tetracycline at a concentration of 1 $\mu g\,mL^{-1}$ to cell cultures inoculated at a cell density between 0.4 and 1.0×10^6 cells mL^{-1}. Overexpression was dectected by northern blot analysis and increase in enzymatic activity.

Procyclic forms of the strain 427 were stably transformed as described [38]. Aliquots of 1 mL were transferred to each well of the 24-well plates. After an 18-h incubation to allow recovery, 0.5 mL of fresh medium containing selective antibiotic was added to each well (75 $\mu g\,mL^{-1}$ hygromycin or 45 $\mu g\,mL^{-1}$ neomycin). Independent antibiotic-resistant clonal cell lines were obtained after 10–12 days.

2.3. Cloning and heterologous expression of the T. brucei TKT gene

T. brucei transketolase (TbTKT, accession number Tb927.8.6170) was PCR amplified from genomic DNA of the Lister 427 strain using oligonucleotides oMB44 (5′-CAA GGA TCC ATG TCG TTT AAT GAT AAC C-3′) and oMB45 (5′-CCA AAG CTT TCA CAA ATG GGA CCG CTT C-3′). The PCR fragment was sub-cloned into pGEM-T Easy (Promega) and then into pET28a(+) (Novagen) using *Bam*HI and *Hind*III (underlined primer sequences) to encode an N-terminal His-tagged protein. The resultant plasmid, named pMB-G27, was transformed into *E. coli* BL21 (DE3). Overexpression was induced with 1 mM IPTG for 16 h at 16 °C and purified using Ni^{2+} resin chromatography on a BioCAD system. The purity of the preparation was assessed by SDS PAGE electrophoresis and Coomassie blue staining.

2.4. TbTKT activity assays

Before use, trypanosomes were washed by suspension and centrifugation in STEN buffer (250 mM sucrose, 25 mM Tris–HCl (pH 7.5), 1 mM EDTA and 150 mM NaCl). The cells were then lysed by incubation in TE buffer (10 mM Tris/HCl, 1 mM EDTA) containing, 0.15% Triton X-100 and complete protease inhibitor cocktail (F. Hoffmann-La Roche Ltd.) and incubated at room temperature for 20 min. The supernatant was separated from cell debris by centrifugation at 13,000 rpm at 4 °C for 30 min. The supernatant was used to determine enzyme activities immediately. Protein concentration was determined by the Bradford method (Biorad).

All assays were performed at room temperature; the reaction mixtures were equilibrated for 3–5 min at this temperature, and the reactions started by addition of the cell-free extract (1×10^7 cells $assay^{-1}$) or the purified recombinant TbTKT. The reaction mixture, was as described [11]. Kinetic parameters (K_m and V_{max}) for the recombinant protein purified from *E. coli* were derived for ribose 5-phosphate by keeping xylulose 5-phosphate constant at 2 mM and varying ribose 5-phosphate as doubling dilutions (10 mM – 0.156 mM) or ribose 5-phosphate constant at 10 mM and varying xylulose 5-phosphate as doubling dilutions (2 mM-0.03125). Alternatively a reaction where fructose 6-phosphate acts as ketose donor linked to glyceraldehyde 3-phosphate, generating erythrose 4-phosphate was used [39].

2.5. Southern and northern blot analysis

Genomic DNA from procyclic cells was digested with restriction endonuclease *Bcl*I. Southern blotting was performed by standard protocols [42]. TKT, neomycin and hygromycin resistance genes were each labeled using the DIG (digoxigenin labelling) system (Roche Diagnostics, Basel, Switzerland) and used to probe blots at high stringency according to standard procedures [40]. The signals were scanned with a Luminescent Image Analyzer LAS-1000plus machine (Fujifilm) with exposure times of 5–15 min. For northern blots total RNA was extracted from 5×10^7 to 1×10^8 cells (from procyclic and bloodstream form trypanosomes) by hot phenol extraction. 11 μg of RNA was loaded per lane, and RNA separated by electrophoresis then blotted according to standard procedures [40].