Transketolase from *Leishmania mexicana* has a dual subcellular localization

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Transketolase has been characterized in *Leishmania mexicana*. A gene encoding this enzyme was identified and cloned. The gene was expressed in *Escherichia coli* and the protein was purified and characterized. An apparent K_m of 2.75 mM for ribose 5-phosphate was determined. X-ray crystallography was used to determine the three-dimensional structure of the enzyme to a resolution of 2.2 Å (1 Å = 0.1 nm). The C-terminus of the protein contains a type-1 peroxisome-targeting signal, suggestive of a possible glycosomal subcellular localization. Subcellular localization experi-

ments performed with promastigote forms of the parasite revealed that the protein was predominantly cytosolic, although a significant component of the total activity was associated with the glycosomes. Transketolase is thus the first enzyme of the non-oxidative branch of the pentose phosphate pathway whose presence has been demonstrated in a peroxisome-like organelle.

Key words: glycosome, *Leishmania mexicana*, pentose phosphate pathway, transketolase.

INTRODUCTION

The glycolytic pathway has been studied in detail in parasitic trypanosomatids, including species of the genera *Leishmania* and *Trypanosoma*, which are important human pathogens. Many steps of this pathway are compartmentalized within the glycosome, a peroxisome-like organelle unique to this group of organisms [1]. The PPP (pentose phosphate pathway) has received less attention, although previous reports indicate that it is present and might provide targets for chemotherapy [2]. Recently, we have shown that the PPP is functional in *Leishmania mexicana* and that all of the enzymes of the pathway are present in promastigotes (the insect stage of the parasite; [3]).

Transketolase (TKT; EC. 2.2.1.1) is a key enzyme in the non-oxidative branch of the PPP that transfers a two-carbon glycolaldehyde unit from ketose-donor to aldose-acceptor sugars [4–6]. The enzyme is also involved in the photosynthetic Calvin cycle in plants and autotrophic bacteria. Within a cell, TKT normally transfers a two-carbon unit from Xu5P (xylulose 5-phosphate) to either R5P (ribose 5-phosphate) or erythrose 4-phosphate, generating glyceraldehyde 3-phosphate, sedoheptulose 7-phosphate and fructose 6-phosphate in the process. Thiamin diphosphate, the active form of vitamin B₁, is employed as a cofactor in the reaction. Together with transaldolase, TKT plays critical roles in the provision of key phosphorylated carbohydrate intermediates to the cell.

TKT activity has previously been detected in the promastigote forms of several *Leishmania* species, including *L. brasiliensis*, *L. donovani*, *L. mexicana* and *L. tropica* [7]. TKT activity was also detected in *Trypanosoma brucei* procyclic forms (insect stage) during an investigation of all of the PPP enzymes [8]. However, no TKT or R5P epimerase activity was detected in the bloodstream forms (mammalian host stage) of this parasite, indicating that a novel mechanism of two-carbon transfer might operate alongside transaldolase that is measurable in both life-cycle stages [8]. TKT

is also present in the four major developmental stages of *T. cruzi* (D. A. Maugeri and J. J. Cazzulo, unpublished work).

TKT has been shown to be present in most organisms and is usually present in the cytosol, although isoforms have been localized to the chloroplast in some plant species [9] and the endoplasmic reticulum in mammals [10]. Both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of the oxidative branch have also been reported in peroxisomes of plants and animals [11–13], and both glucose-6-phosphate dehydrogenase and 6-phosphogluconolactonase [14,15] have been identified in *T. brucei* glycosomes. However, none of the enzymes of the non-oxidative branch of the PPP has been reported so far in this organelle. The oxidative branch dehydrogenases have been proposed to play a role in production of NADPH required for various reductive biosyntheses in peroxisomes and glycosomes [2,11–15]. However, the fate of the phosphorylated carbohydrate products of these reactions in these organelles had not previously been considered.

Structural studies and site-directed mutants of the *Saccharomyces cerevisiae* TKT have been informative on the structure and activity of this enzyme (reviewed in [6]). The reaction proceeds via a Ping Pong mechanism. The ketose donor binds to the enzyme, and carbon bond cleavage is catalysed by nucleophilic attack by the C-2 deprotonated carbanion of coenzyme, ThDP (thiamin diphosphate), on the substrate's carbonyl group. The glycolaldehyde group remains covalently linked to the coenzyme while the remainder of the donor substrate is released. An aldehyde acceptor then enters the catalytic crevice. The glycolaldehyde unit is added to the acceptor substrate by nucleophilic attack, generating a new ketose that leaves the enzyme. Bivalent cations, including Ca²⁺ or Mg²⁺, are crucial in orienting the coenzyme.

Enzymes of the PPP have been proposed as potential targets for chemotherapy in parasitic protozoa [2]. In the present study, we set out to characterize TKT, a key enzyme in the non-oxidative branch of the PPP, in the trypanosomatid *L. mexicana*. In particular, we

Abbreviations used: ALAT, alanine aminotransferase; GPI, glucose phosphate isomerase; HK, hexokinase; ICDH, isocitrate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; PDB, Protein Data Bank; PPP, pentose phosphate pathway; PTS, peroxisome-targeting signal; R5P, ribose 5-phosphate; ThDP, thiamin diphosphate; TKT, transketolase; Xu5P, xylulose 5-phosphate.

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were interested in its subcellular localization, given that enzymes of the oxidative branch of the PPP can be found, along with the early stages of glycolysis, in the glycosome as well as the cytosol.

MATERIALS AND METHODS

Materials

All reagents used for biochemical analysis were of the highest grade available from Sigma (Poole, Dorset, U.K.). D-Xu5P was prepared as described previously [16]. Triose phosphate isomerase and glycerol-3-phosphate dehydrogenase were from Boehringer Mannheim. Oligonucleotides were from MWG Biotech (Milton Keynes, U.K.).

Growth of *L. mexicana* promastigotes

Promastigotes of *L. mexicana*, strain MNYC/BZ/62/M379, were cultured *in vitro* in HOMEM growth medium [17] supplemented with 20 % (v/v) heat-inactivated fetal-calf serum. Cultures were routinely maintained at 26 °C. Numbers were determined using a Neubauer haemocytometer.

Protein preparation from *L. mexicana*

Leishmania promastigotes were harvested by centrifugation at 1000 *g* for 10 min and washed in iso-osmotic buffer [25 mM Tris/HCl (pH 8.0)/1 mM EDTA/0.25 M sucrose] three times. Cells were resuspended in 100 µl of lysis buffer {20 mM Tris/HCl (pH 8.0)/0.25 % Triton X-100/0.25 M sucrose containing 10 % proteinase cocktail inhibitors [4-(2-aminoethyl)benzenesulphonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane, bestatin, leupeptin and aprotinin; Sigma} and left on ice for 30 min. The cells were centrifuged at 12 000 *g* for 15 min, and the supernatant was used as soluble protein. Protein concentration was determined using the Bio-Rad protein assay that is based on the method of Bradford [18].

DNA and RNA extraction from *L. mexicana* promastigotes

Genomic DNA was isolated from mid-exponential phase *L. mexicana* promastigotes using a method adapted from the *T. brucei* DNA mini-prep method [19]. RNA from these parasites was obtained using TRIzol[®] reagent (Life Technologies, Gibco BRL) and cDNA was synthesized from the RNA using Ready-To-Go T-Primed First-Strand Kit (Amersham Biosciences), according to manufacturer's specifications.

Cloning of the *L. mexicana* TKT gene

The *L. mexicana* TKT gene was cloned using PCR with primers designed on the basis of various regions of the gene. The PCR reaction components were: 1 × PCR buffer, 1.5 mM MgCl₂, 0.5 mM dNTPs, 5 % DMSO, 2.5 units of DNA *Taq* polymerase (all from Promega), 10–100 pmol of each primer (MWG-Biotech; further details are provided below) and 1–100 ng of *L. mexicana* DNA/cDNA. PCR was performed using a GeneAmp 2400 PCR System (PerkinElmer) using the following procedure: 95 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min, for 25 cycles. Oligonucleotides based on sequences within the ThDP-binding consensus motif (pThDP, GGNGAYGGNTGYYWNATGGARGG) and a well-conserved TKT motif [5] (pTKT1, TGRTGNGTNGG-NCCRTCCTCNCNAG) were used to amplify a central 1 kb region of DNA of the TKT gene sequence from *L. mexicana* DNA. The 5'-region of the gene was amplified from *L. mexicana*

cDNA using reverse transcriptase. Nested primers were used to amplify DNA from the spliced leader sequence present on the 5' of mRNA transcripts of trypanosomatids (pTKTSL1, TAA-CGCTATATAAGTATCAGTTTC; and pTKTSL2, AGTATCAG-TTTCTGTACTTTATTG) and antisense primers were designed on the basis of *L. mexicana* TKT-specific sequence (pTPP1, CGATCAGTGGAACCCATGGCC; and pTPP2, CCGTGAA-GGAGAGGCTTGTCG). The 3'-region of the *L. mexicana* TKT was amplified using PCR with nested primers designed on the basis of an *L. major* sequence with similarity to TKT obtained from the Sanger Centre *L. major* sequencing database (accession number AL390114). The nested primers were pLmex1 (GACGACGACGTCCGCGCTGTGTT), pLmex2 (GAAGCTC-CCGACGAACTCC), pLmajor1 (CGCGTTGCTGTGTGAGCG-TA) and pLmajor2 (ATAAGAGGAGAGAGAGGTCAGG). All of the products were cloned into the vectors pUC18 or pGEM-T and sequenced (outsourced to MWG Biotech) to confirm the gene sequence. The full-length *L. mexicana* TKT was amplified using the primers pTKTLmex1 (TCACACACAAGCCATATGGCCTCC) and pTKTLmex2 (GCGCCCTCGAGCCTTACATCTTGC) that contain the restriction sites *Nde*I and *Xho*I respectively (shown underlined in the preceding sequences). The 2 kb TKT gene product was cloned into the vector pET-16b (Novagen) and sequenced. The predicted amino acid sequence was determined using Vector Nti (Informax).

Expression and purification of recombinant TKT

Escherichia coli strain BL21(DE3) containing the expression plasmid for *L. mexicana* TKT (pET16blmtkt) was grown in LB (Luria–Bertani) containing ampicillin (100 µg/ml), and the culture was induced with 0.4 mM isopropyl β-D-thiogalactoside at 15 °C for 16 h. The induced culture was harvested by centrifugation at 5000 *g* for 30 min at 4 °C, and lysed by sonication (Soniprep150; MSE) in 0.1 M Tris/HCl, pH 8.0/0.5 M NaCl, containing a proteinase inhibitor cocktail (Sigma). The sonicated homogenate was centrifuged for 30 min at 12 000 *g*. The cell-free extract containing soluble TKT was loaded on to the BioCAD 700E Workstation nickel-ion column at a flow rate of 5–10 ml/min. The column was washed in PBS buffer [50 mM sodium phosphate (pH 8.0)/300 mM NaCl] containing 0.5 mM imidazole, and the flow-through was collected (approx. 18 ml). A second wash using PBS containing 20 mM imidazole was used to remove tightly bound *E. coli* proteins from the column (approx. 15 ml). To elute the bound protein, a gradient of 20–500 mM imidazole in PBS was used over 10 column-volumes. Fractions (10 × 1.7 ml) were collected during the elution. PBS containing 500 mM imidazole was run through the column for a further five column-volumes. The absorbance at 280 nm was recorded throughout the purification procedure. The protein purification product was analysed by BioCAD trace and SDS/PAGE (8 % gels). The eluted recombinant TKT was pooled and dialysed overnight against 100 mM Tris/HCl buffer, pH 7.5.

Crystallization and data collection

Crystals of *L. mexicana* TKT were obtained by vapour diffusion. The protein solution was concentrated to 7 mg/ml and incubated with 5 mM ThDP and 1 mM MgCl₂. Of the protein solution, 3 µl was mixed with 3 µl of mother liquor [0.1 M sodium citrate (pH 6.0)/0.2 M ammonium acetate containing 30 % poly(ethylene glycol) (*M_r* ≈ 4000)]. Hanging drops were equilibrated against the mother liquor at 20 °C, and rod-shaped crystals, 0.5 mm long and 0.05 mm thick, were obtained after 2–3 weeks.

Table 1 Data collection and refinement statistics

Numbers in parentheses refer to the highest resolution shell. r.m.s.d., root-mean-square deviation.

Parameter	Value
Data collection	
Resolution (Å)	25.0–2.22 (2.39–2.22)
Unique reflections	61 529
Redundancy	3.95
R _{sym} (%)	10.8 (29.6)
(I/σI)	5.4 (25.4)
Completeness (%)	95.7 (85.5)
(B) Wilson Plot (Å ²)	22.8
Refinement	
Resolution (Å)	25.0–2.22 (2.24–2.22)
Number of reflections	
Work set	58 396
Test set	3096
R _{work} (%)	17.0 (22.8)
R _{free} (%)	21.3 (29.0)
Bond length r.m.s.d (Å)	0.005
Bond angle r.m.s.d. (°)	1.24
Ramachandran Plot	
Percentage in most favoured regions	87.5
Percentage in additional allowed regions	11.9
Number of protein atoms	10 085
Number of solvent atoms	911
Number of ligand atoms (Ca ²⁺ , TPP)	54
Average B-factors (Å ²)	
Protein	20.9
Solvent	30.1
Ligand atoms	25.4
r.m.s.d. between the two subunits in the asymmetric unit (Å)	0.1

A native diffraction data set was collected at 0.934 Å (1 Å ≡ 0.1 nm) wavelength at 100 K on beam-line ID14-1 at the European Synchrotron Radiation Facility (Grenoble, France) with an ADSC Q4R CCD detector. No cryoprotection was required. The data were integrated with Mosfilm [20] and scaled with Scala [21], and further processing was done within the CCP4 suite of programs [22] (Table 1). The crystals belong to space group P212121, with cell dimensions of *a* = 76.80 Å, *b* = 120.44 Å and *c* = 139.15 Å and contain two monomers per asymmetric unit.

Phasing, model building and refinement

The structure was solved by molecular replacement using the program EPMR [23] with a monomer of yeast TKT [PDB (Protein Data Bank) accession number 1TRK] as the search model. Searching for two monomers using a population size of 300 and 50 generations in the resolution interval 15–4 Å yielded a correct solution. Refinement was performed with the use of the CNS program package [24]. After an initial rigid-body refinement, this refinement consisted of anisotropic scaling, bulk-solvent correction, simulated annealing, conjugate gradient minimization and isotropic B-factor refinement against the maximum likelihood target. Non-crystallographic symmetry restraints were used, except for some residues in crystal contacts.

Model geometry was analysed with Procheck [25]. Further details of the refinement statistics are given in Table 1.

Model building, structure comparisons and structure alignments using default parameters were accomplished using the program O [26]. Figures were produced using the programs O [26], Bobscript [27] and Raster3D [28]. The coordinates and structure factors have been submitted to the PDB (accession code 1R9J).

Enzyme assays

The TKT assay was based on the coupled assay [29] utilizing R5P and Xu5P as the substrates. The reaction mixture contained (per ml): Tris/HCl, pH 7.5, 100 mM (Fisons); D-R5P, 10 mM; D-Xu5P, 2 mM; ThDP, 10 μM; MgCl₂, 1.2 mM; NADH, 100 μM; triosephosphate isomerase, 1 unit; and glycerol-3-phosphate dehydrogenase, also 1 unit. The reaction was initiated by the addition of the enzyme/cellular fraction. The standard control lacks the substrate R5P, thus measuring background NADH oxidation. The linear rate was measured as the absorbance change (AU)/s. One unit is defined as the amount of enzyme that catalyses the formation of 1 μmol of glyceraldehyde 3-phosphate/min. The specific activity is expressed as units/mg of protein. The kinetic parameters were determined with all the components of the reaction in excess, except for R5P, where concentrations from 0 to 100 mM were examined.

HK (hexokinase) was determined in the presence of 2 mM 6-phosphogluconate, subtracting the activity obtained in its absence, by a modification of a method described previously [30]: the assay mixture contained 0.05 M triethanolamine, pH 7.5, 5 mM MgCl₂, 0.5 mM NADP, 2.0 mM glucose, 0.25 mM ATP and 1 unit of glucose-6-phosphate dehydrogenase.

GPI (glucose phosphate isomerase) was determined by a modification of a method described previously [7]: the assay mixture contained 0.05 M triethanolamine, pH 7.5, 5 mM MgCl₂, 0.5 mM NADP, 2 mM fructose 6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase.

ICDH (isocitrate dehydrogenase) was also determined by a modification of a method described previously [7]: the assay mixture contained 0.05 M triethanolamine, pH 7.5, 5 mM MgCl₂, 0.5 mM NADP and 2.5 mM isocitrate.

PK (pyruvate kinase) and ALAT (alanine aminotransferase) were determined according to procedures described in [31] and [32] respectively, and PEPCK (phosphoenolpyruvate carboxykinase) was determined by the CO₂ fixation reaction on phosphoenolpyruvate [33].

Subcellular localization

The subcellular localization of TKT was studied by using three complementary experimental approaches, namely (a) digitonin extraction of intact cells; (b) subcellular fractionation by differential centrifugation; and (c) ultracentrifugation in isopycnic sucrose gradients.

For digitonin-extraction experiments, promastigotes (10 mg, wet weight, corresponding to 1.64×10^8 parasites) were extracted with digitonin (0–2.5 mg/ml) in a buffer solution containing 25 mM Tris/HCl, pH 7.6, 0.25 M sucrose, 1 mM EDTA and 0.25 mM tosyl-lysylchloromethane (TLCK) (TSEB buffer), in a final volume of 0.5 ml. The suspensions were incubated at 25 °C for 5 min and centrifuged at 18 000 *g* for 2 min in an Eppendorf centrifuge at room temperature. The supernatants (S1) were separated immediately after centrifugation, and the pellets were washed with 0.5 ml of TSEB buffer. After centrifugation under identical conditions, the supernatants were discarded, and the pellets were resuspended in 0.5 ml of TSEB buffer containing digitonin (2.5 mg/ml) and disrupted in a Branson 450 Sonifier (4 pulses of 20 s each, at 60 % of maximum power). The homogenate was centrifuged at 18 000 *g* for 2 min, and the supernatant (S2) was kept. The activities of TKT, the cytosolic marker PK, the glycosomal marker PEPCK, the cytosolic and glycosomal marker GPI and the mitochondrial matrix markers ALAT and ICDH were determined in all S1 and S2 fractions. ICDH was inhibited by digitonin, and could not be used as a marker in this experiment. Total activity for each enzyme at a given digitonin concentration

was taken as the sum of the activities in S1 and S2. The percentage of total activity present in S1 was plotted as a function of digitonin concentration.

For differential centrifugation, promastigotes (1.5 g, wet weight) were mixed with 2.25 g of silicon carbide (Crystalon™) and ground in a mortar in an ice-bath until approx. 90% of the parasites were disrupted, as judged from microscopical observation (usually at approx. 15–30 s). The paste was suspended in TSEB buffer, and centrifuged for 3 min at 100 *g* at 4 °C to remove the abrasive. The supernatant was centrifuged at 1000 *g* for 10 min at the same temperature, and the pellet, containing intact cells, large debris, nuclei and flagella, was discarded. The supernatant was then centrifuged for 10 min at 14 500 *g*. The pellet (large plus small granules) was kept, and the supernatant was centrifuged for 1 h at 105 000 *g*. The pellet (microsomal fraction) and the final supernatant (containing both the cytosol and contents leaking from disrupted organelles) were kept. Both pellets were washed once in TSEB buffer, the supernatants were discarded, and the pellets were resuspended in TSEB buffer and carefully homogenized in a Potter–Elvehjem homogenizer. Latency of TKT and HK activities in the particulate fractions was measured in reaction mixtures made isotonic with sucrose, by determining them in either the absence or the presence of 0.2% (v/v) Triton X-100.

Isoopycnic sucrose gradient ultracentrifugation was performed using an NVT 65 rotor in a Beckman Optima™ XL-100K ultracentrifuge. Gradients (0.25–2 M sucrose, with a 2.5 M cushion) were formed in polyallomer tubes (16 × 67 mm). Half of the large-plus-small-granule fraction, obtained as above for differential centrifugation, was layered on top of the gradient, the tubes were sealed, and then centrifuged for 3 h at 290 000 *g*. Fractions (0.4 ml) were collected with the aid of a peristaltic pump. Density was calculated from the determination of sucrose concentrations in the fractions by refractometry. TKT, HK and the mitochondrial matrix marker ICDH were assayed in all the fractions, as described above. The results were plotted as frequency (the fraction of enzyme activity in each tube with respect to the total activity recovered) as a function of density.

RESULTS

TKT enzyme activity in *L. mexicana* promastigotes

The specific activity of TKT in cell-free extracts of *L. mexicana* promastigotes was determined as 58.75 ± 11.75 nmol/min per mg of cell protein, which compares with a previously published value of 12.4 nmol/min per mg of cellular protein [7]. The specific activity recorded in the present study was higher in comparison with the previously recorded value, and could be due to the difference in substrates used to assay the enzyme, or to differences in the growth phase of the parasite. The specific activity of TKT in procyclic form *T. brucei* was 50.1 ± 10.8 nmol/min per mg of cell protein [8].

Cloning of a TKT gene

PCR was used to amplify part of the TKT gene from *L. mexicana* using oligonucleotides designed on the basis of the so-called TKT box and the ThDP box of this protein (Figure 1A). The 5' region was amplified from cDNA using reverse transcriptase–PCR with the 5' spliced leader sequence. The 3' region was cloned when the 3' region from the *L. major* TKT sequence became available in the *Leishmania* genome database, using oligonucleotides based on this *L. major* sequence. Using oligonucleotides from the 5' flanking region of the *L. major* TKT gene, we then amplified 750 bp upstream of the *L. mexicana* gene from genomic DNA. Com-

Figure 1 Schematic representation of the structure of the gene with arrows showing the location of the primers used to amplify the gene (A), and Southern blot of *L. mexicana* TKT (B)

(A) The so-called TKT-box (TKT), the thiamin pyrophosphate box (ThDP) and the spliced leader sequence (SL) are all indicated. (B) Southern blot showing that the *L. mexicana* TKT is a single copy gene. Restriction sites are indicated in (A). *EcoRI* and *XhoI* do not cut the TKT gene.

parison of this genomic flanking sequence with the splice leader containing cDNA sequence revealed that an AG splice acceptor site lies 145 bp upstream of the initiation codon (Figure 1A). Figure 1(B) shows that the *L. mexicana* TKT is a single copy gene per haploid genome. Low-stringency Southern blotting failed to reveal any closely related genes in the *L. mexicana* genome, indicating that there is a single TKT isoform in these cells.

The TKT from *L. mexicana* predicted from the cloned gene contains 671 amino acids with a monomer molecular mass of 71.8 kDa (ProtParam tool; ExPASy). Forty-one invariant residues in TKTs from all sources [34] are present in the *L. mexicana* TKT protein sequence. The sequencing of the TKT gene revealed the predicted protein to contain the conserved ThDP and TKT motifs, common to all TKT proteins (Figure 2). All of the residues implicated in binding substrate or coenzyme by crystallographic analysis of the *S. cerevisiae* enzyme (reviewed in [6]) are conserved in the cognate positions of the *L. mexicana* enzyme. A type-1 PTS (peroxisome-targeting signal), SKM, was shown to be present at the C-terminus of the protein, suggesting a possible glycosomal localization for the enzyme.

Expression, purification and characterization of recombinant TKT

Recombinant TKT was expressed as described in the Materials and methods section. The temperature at which the cells were induced was critical for the expression of soluble recombinant TKT protein. At 37 °C, the protein was not expressed at all. At 25 °C, TKT was expressed, but it was totally insoluble (results not shown). At 15 °C, on the other hand, approx. 50% of the over-expressed protein was present in the soluble fraction.

The purified *L. mexicana* TKT protein, with an apparent molecular mass of 70 kDa, was eluted from the metal-chelate chromatography column at imidazole concentrations between 400 and 500 mM. Fractions containing the eluted recombinant TKT were pooled and dialysed overnight against 100 mM Tris/HCl buffer, pH 7.5, and used for kinetic characterization.

The eluted protein was tested for activity using R5P and Xu5P as substrates, and had a specific activity of 1.65 units/mg of protein. A percentage (5–8%) of the total protein loaded on to the column

Substrate inhibition was seen during the kinetic analysis, with > 50 mM R5P (results not shown) inhibiting the reaction, as seen previously in TKT from *S. cerevisiae* (baker's yeast; [35,36]), and is attributed to the mechanism of action where both substrates enter the catalytic crevice sequentially. A pronounced lag phase at the beginning of the reaction noted for the *S. cerevisiae* enzyme is also seen in the *Leishmania* enzyme. ThDP is lost during dialysis for the leishmanial enzyme, as for the *S. cerevisiae* enzyme, but not the *E. coli* TKT A, so it was essential to add cofactor to the assay mix for purified enzyme. The enzyme was most efficiently stored at -80°C without glycerol.

Crystal structure of *L. mexicana* TKT

The final model of TKT from *L. mexicana* consists of a dimer with 669 amino acid residues, one ThDP and one calcium ion per subunit and, in total, 911 water molecules. The final R-factor to $2.22\text{ \AA}$ is 17.0%, while R_{free} , obtained from 5.3% of the reflections, is 21.3%. The average B-factor for all atoms in the model is $21.7\text{ \AA}^2$, in good agreement with the value obtained from the Wilson plot ($22.8\text{ \AA}^2$). In the Ramachandran plot, 87.5% of the non-glycine and non-proline residues are in the most favoured region, but three residues in each subunit, Phe-104, Asp-423 and Ser-347, are in the disallowed region. However, the densities for these Ramachandran outliers are very clear, and their unusual conformation is thus a genuine feature of the structure. The last two residues in each subunit are not visible in the electron density map.

The subunit of TKT from *L. mexicana* is built up of three consecutive domains, where the two first are similar in structure. In the dimer, the 2-fold-related active sites with the bound ThDP cofactors are located between the N-terminal domain of one subunit and the middle domain from the second subunit (Figure 3A). The C-terminal ends of the subunits make up the dimer contacts for the C-terminal domain. However, the last two residues in each subunit are not visible in the electron density map, and are thus probably protruding out into the solvent. The two crystallographically independent subunits are very similar, with a root-mean-square deviation of $0.17\text{ \AA}$ for all C atoms.

A structure comparison of the dimer (1340 C α atoms) of TKT from *L. mexicana* with TKT from *S. cerevisiae* (pdb-id 1TRK) [34] gives an overall root-mean-square deviation of $1.0\text{ \AA}$ for 1305 equivalent C α atoms and to maize (pdb-id 1ITZ) [18], $1.1\text{ \AA}$ for 1286 C α atoms. Overlaying of the subunits gives only slightly lower deviations, so both the subunit and the dimer organization are very similar for these enzymes. A few minor insertions and deletions at the surface of the protein distinguish the structures. The active sites, the calcium ion ligation and the ThDP binding are virtually identical in the three enzymes (Figure 3B). The only exception is that in the *L. mexicana* enzyme, one of the phosphates of ThDP hydrogen-bonds to a threonine side chain, Thr-29, which is not present in the other enzymes, where the corresponding residues are Ala-33 and Leu-41 respectively. In these TKTs, a corresponding hydrogen bond is formed with a water molecule. The maize enzyme is shorter than either the leishmanial or yeast enzymes, ending at the residue corresponding to position 652. The latter two enzymes have an extended C-terminus, but they are quite different in conformation between *L. mexicana* and yeast from residue 658 onwards, including the type-1 PTS of the leishmanial enzyme, which apparently protrudes from the surface of the enzyme in an accessible position. TKT from *L. mexicana* has 50.4% amino acid sequence identity with the enzyme from *S. cerevisiae* [34], and 47.7% with the maize enzyme [37], and it is thus not surprising that overall the crystal structures are very similar.

Figure 3 (A) Schematic view of the structure of TKT from *L. mexicana*, and (B) comparison of the active-site structures in TKTs from *L. mexicana*, *S. cerevisiae* and maize

In (A), the three domains of one subunit are coloured in different shades of blue, and in the other subunit they are coloured red. ThDP, bound between the first domain in one subunit and the second domain of the other subunit, is shown as a ball-and-stick model. (B) The active-site structures in TKTs from *L. mexicana* (colour coded by atom type), *S. cerevisiae* (in green) and maize (in cyan) are shown.

Subcellular localization

Carbohydrate catabolism in trypanosomatids involves an interaction between the glycosome, cytosol and mitochondrion. Compartmentation appears to be important for the regulation of the pathways [38]. TKT possesses a typical peroxisome targeting

Figure 4 Digitonin extraction of *L. mexicana* promastigotes

Digitonin treatment of the intact promastigotes and enzyme determinations were performed as described in the Materials and methods section.

sequence; thus it was considered of interest to study subcellular compartmentation of this enzyme.

Digitonin is able to solubilize cell components by selectively disrupting membranes, depending on their accessibility and sterol composition. The experiment shown in Figure 4 indicates that approx. 70 % of the TKT activity was extracted by low digitonin concentrations (up to 0.2 mg/ml), as was the cytosolic marker PK and the cytosolic component of GPI. The remaining 30 % of the TKT activity, on the other hand, was completely released at concentrations over 1.5 mg/ml, following a pattern similar to that of both the glycosomal component of GPI and the glycosomal marker PEPCK. The slopes of the segments of the three curves at digitonin concentrations higher than 0.2 mg/ml differ in the proportion of the fraction of each of these enzymes in the particulate compartment. HK, which has been reported to be very tightly bound in a glycosomal 'core', was only partially extracted, but the extracted component had a profile similar to that of the particulate TKT. ALAT has a dual localization [39], cytosolic and mitochondrial, and the latter fraction follows a distinctive extraction profile. The predominant mitochondrial activity was extracted at digitonin concentrations higher than 1 mg/ml, whereas a minor cytosolic component was extracted under the same digitonin conditions that released PK.

To investigate the possible glycosomal localization of the particulate TKT, first subcellular fractionation by differential centrifugation was performed. A large percentage (91 %) of TKT was recovered in the 105 000 *g* supernatant, which only contained 14 % of the total HK activity. The large plus small granule fraction, on the other hand, contained 79 % of the HK and only 9 % of the recovered TKT activity. The remainder of both enzyme activities was present in the microsomal fraction. The HK and TKT activities in the granular fraction presented a latency of 76 and 70 % respectively, showing that they were effectively at least partially contained in a membrane-bound organelle. The lower percentage of enzyme activities associated with particles in subcellular fractions as compared with the values obtained in the digitonin experiment is due to the partial disruption of organelles during the preparation and processing of the homogenate.

Isopycnic sucrose gradient ultracentrifugation of the granular fraction (Figure 5) showed that a substantial portion of the TKT

Figure 5 Ultracentrifugation of a large plus small granule fraction in an isopycnic sucrose gradient

Ultracentrifugation and enzyme determinations were performed as described in the Materials and methods section.

activity co-migrated with HK, at a density of 1.22 g/ml. The mitochondrial marker, ICDH, peaked at a density of 1.17 g/ml. Both TKT and ICDH also presented a soluble component (centred at a density of 1.08–1.09 g/ml), most likely arising from damaged organelles.

DISCUSSION

As part of a systematic study into the enzymes of the PPP in trypanosomatids, a TKT gene was cloned from *L. mexicana* and the protein expressed in *E. coli* was purified, characterized and the crystal structure was determined. The translated protein contained features specifically characteristic of TKT proteins. A ThDP box, known to be conserved in all ThDP-dependent enzymes [40] and crucial for both the binding of ThDP to TKT and activation of the enzyme [41,42], is present in the sequence between amino acid positions 144 and 183. A TKT box, specific to the enzyme and

known to form part of the active site, is present in the *L. mexicana* TKT sequence between amino acid residues 465 and 493. The conserved residues that form the TKT box are directly involved in the cofactor and donor (aldehyde) substrate binding [43]. All key residues that have been identified by crystallography or site-directed mutagenesis in yeast to play key roles in substrate and cofactor binding [6] are conserved. As expected from the strong sequence conservation, the crystal structure for the *L. mexicana* TKT is highly similar to the enzymes from *S. cerevisiae* and maize, with the notable exception of the C-terminus, where a type-1 PTS sequence is found on the leishmanial enzyme.

Expression of the recombinant *L. mexicana* protein was strongly temperature-dependent. Reducing the induction temperature to 15 °C did stimulate production of approximately half of the total protein in soluble form, possibly because the rate of protein expression is reduced, creating favourable conditions for the correct folding of the protein. The turnover number determined for the purified recombinant *L. mexicana* TKT was 8.4 s⁻¹. Even when compared with other TKTs (with turnover numbers in the range of 20–200 s⁻¹), this is extraordinarily low. The sequence of the expressed protein was unaltered compared with that predicted from the gene present in *L. mexicana* according to the electron density map, and since several independent PCR products of the gene gave the same sequence it is unlikely that PCR-based alterations to gene sequence affected activity. The presence of the hexahistidine tag could influence activity in spite of its being spatially discrete from the catalytic site of the enzyme, although this has not been determined experimentally. It is also possible that the enzyme is adversely affected during purification on the nickel column, since reducing agents were not included during that preparation. Other measured biochemical characteristics are similar to other TKTs. For example, the reaction showed a distinctive lag phase before reaching the steady state (results not shown). The *L. mexicana* TKT K_m for R5P is 2.75 mM, which is similar to the *E. coli* K_m for R5P of 1.4 mM. Substrate inhibition occurred with R5P concentrations exceeding 50 mM.

The subcellular localization of the PPP in trypanosomatids is of particular interest. Many of the enzymes of the glycolytic scheme are present within glycosomes, membrane-bound organelles related to peroxisomes [1].

Various plant TKTs contain chloroplast targeting sequences and have been localized to the chloroplast [9], where this enzyme plays a key role in the Calvin cycle. Mammalian cells are thought to have three sets of PPP enzymes: one is cytosolic, another is located in the endoplasmic reticulum [6], while a third set is found in the peroxisomes [11,12]. The PPP enzyme 6-phosphogluconolactonase has recently been cloned and sequenced from *T. brucei*, and was shown to contain a C-terminal peroxisomal targeting tripeptide, AKF [14]. Subcellular localization experiments showed that 10% of 6-phosphogluconolactonase activity was associated with the glycosomal fractions. Glucose-6-phosphate dehydrogenase in *T. brucei* was also partially localized to the glycosome [15], although a typical PTS could not be identified in the sequence of this enzyme [14]. These data suggest that the oxidative branch of the PPP may function in the glycosomes and the cytosol in *T. brucei*, as suggested previously [2]. Peroxisomal targeting sequences can have varying ability to target proteins to peroxisomes and glycosomes depending upon their context [44], and this mechanism appears to be used to ensure a dual localization of proteins that presumably play roles in both the peroxisomal/glycosomal compartment and the cytosol. The *L. mexicana* TKT protein sequence has a C-terminal PTS, suggesting that it too may be glycosomal or may have a bi-compartmental distribution. The results of the subcellular localization experiments reported herein clearly indicate a dual localization for the leishmanial TKT,

primarily in the cytosol, with a significant glycosomal component. Given that only of the order of 10% of TKT localizes to the glycosome, and given that the volume of the cell occupied by glycosomes is not far short of 10% [1], immunofluorescence microscopy cannot be used to generate meaningful data on subcellular localization of this enzyme.

This is the first paper to report that an enzyme of the non-oxidative branch of the PPP is located in a peroxisomal compartment. The latter component might be more quantitatively important in other parasite stages, such as the intracellular amastigote, which has not been investigated. The C-terminus of the leishmanial protein also differs markedly from that of other species, and the C-terminus is exposed at the surface of the enzyme, ensuring that the PTS tripeptide is available to interact with the glycosomal uptake machinery. It is currently uncertain how a PTS-1 type motif can carry a proportion, but not all, of a protein to the glycosome. However, the same phenomenon occurs for glucose-6-phosphate dehydrogenase [14] and phosphoglucose isomerase [45], and in *T. brucei* different PTS-1 variants carried differing proportions of firefly luciferase to the glycosome [44]. Possibly the context of the signal is important, and it is possible that flexibility in the tail, whereby the signal is mobile between a buried and exposed position, would drive this process. In the case of the *L. mexicana* TKT, the PTS sequence just protrudes from a crevice within the enzyme, and this might offer an explanation as to its variable efficacy as a signal.

A role for TKT within the glycosome has yet to be determined. However, given that the oxidative branch dehydrogenases appear to be crucial for the production of NADPH for reductive biosyntheses in this compartment, it is necessary for the cell to dispose of the phosphorylated carbohydrate intermediates of the pathway. Pentose phosphate cycle activity, to regenerate glycolytic intermediates, would offer the means of dealing with accumulated products of the oxidative branch of the PPP. This could be of particular value, given that 6-phosphogluconate is a potent inhibitor of phosphoglucose isomerase; thus the accumulation of this metabolite must be prevented within the glycosome [2].

Genome database searching has provided several putative TKT protein sequences from trypanosomatids. These sequences were aligned with the *L. mexicana* TKT in order to determine if they too had a peroxisome-targeting sequence. Putative *L. major* and *T. brucei* TKT amino acid sequences also contain a PTS at the C-terminal of the protein (results not shown).

In summary, the main findings of this research show that the *L. mexicana* TKT has a PTS at the C-terminus that lies on the outside of the protein, and is therefore available for transporting this protein into the glycosome. Subcellular localization studies show that a significant quantity of TKT activity is associated with glycosomes, and therefore this is the first study to suggest there may be a non-oxidative PPP present within this organelle. This may be of importance with regard to chemotherapeutic intervention, with rational drug design relying on the identification of new biochemical targets that are present only in the pathogen. The PPP is known to be up-regulated in proliferative cells and tumour tissue, possibly due to an increase in nucleic acid requirement [46]. Oxythiamine, a competitive inhibitor of TKT, has been shown to partially inhibit tumour cell proliferation [46]. The PPP has been shown to be more active in *Leishmania* when the cell is under oxidative stress [3], therefore suggesting that targeting the PPP may prevent the parasite from protecting itself against the host's natural defence.

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