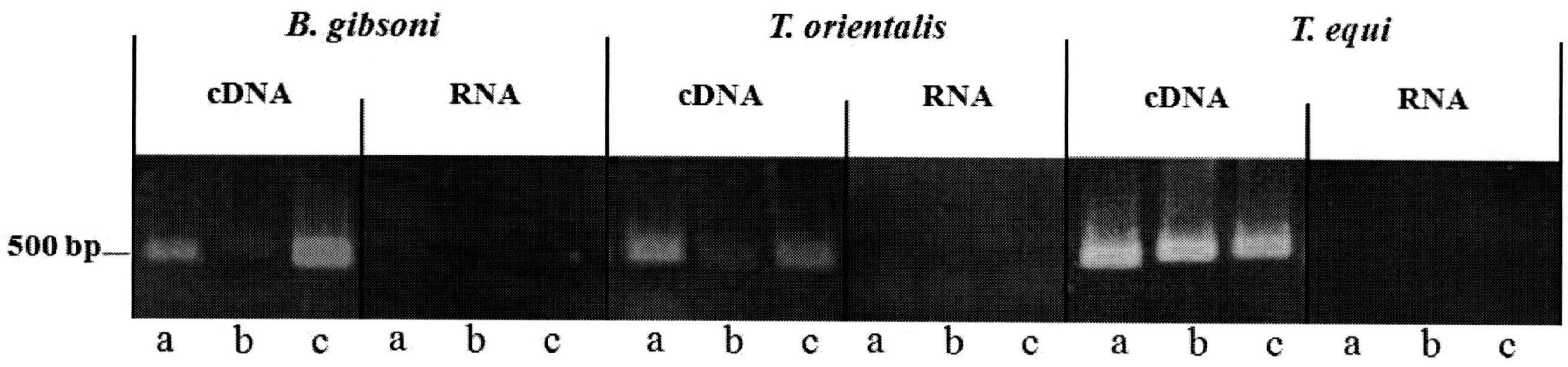

FIG. 3.

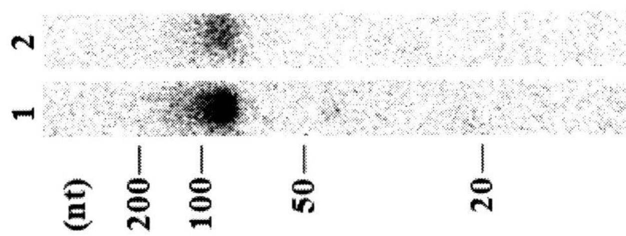

FIG. 4.

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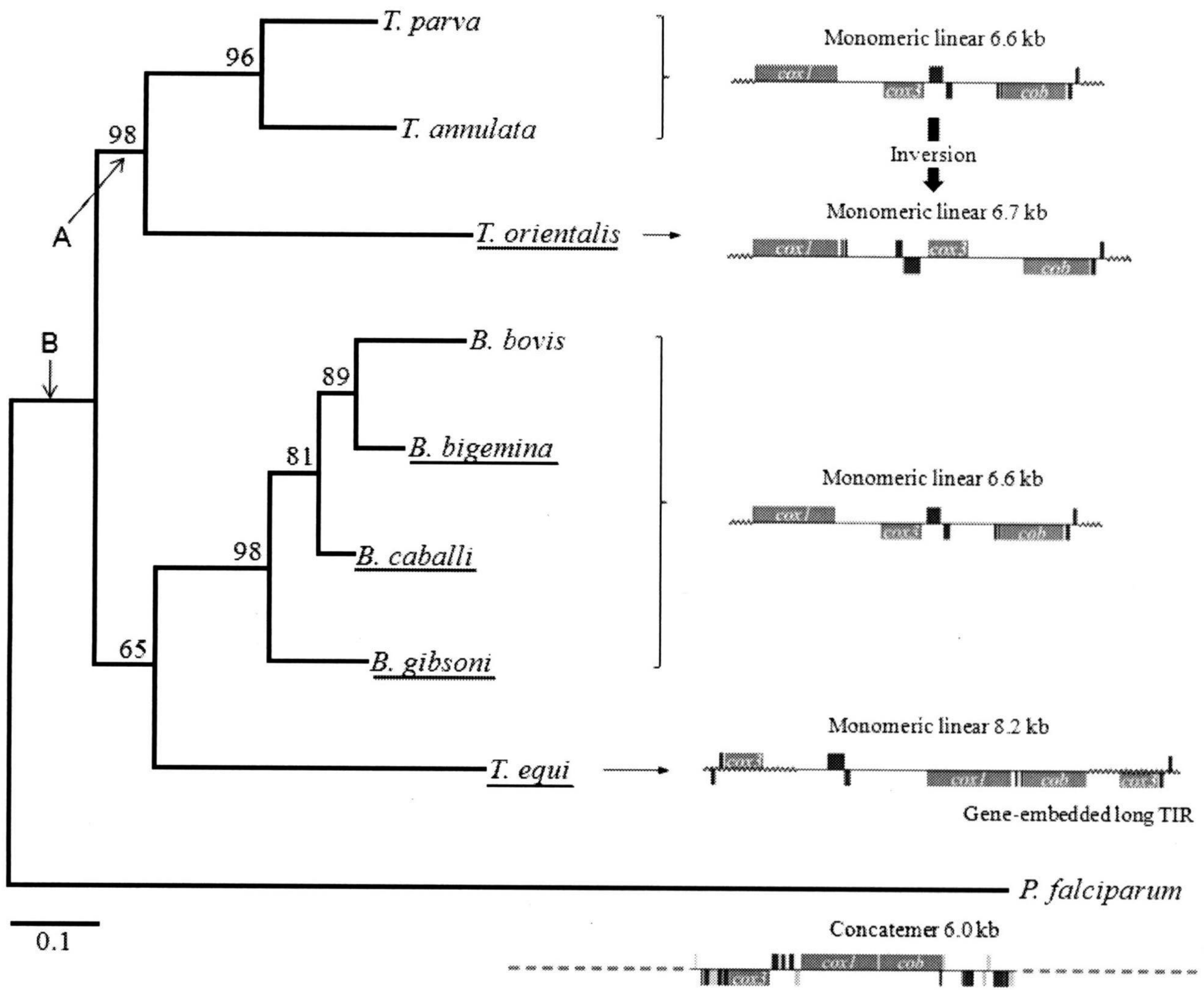

FIG. 5.

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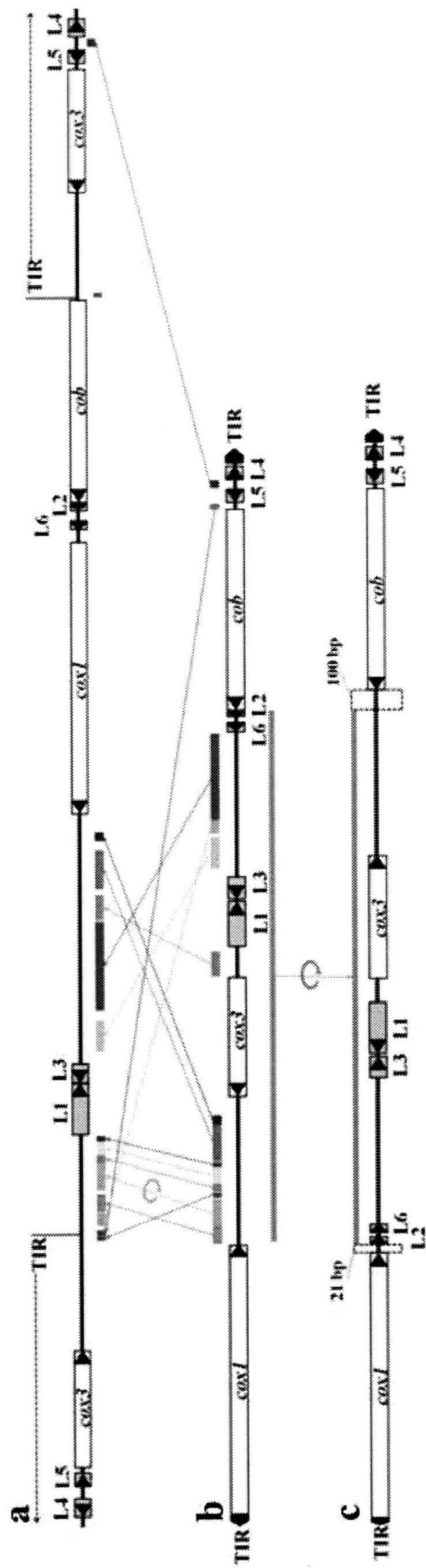

FIG. 6.

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Overproduction, purification, crystallization and preliminary X-ray diffraction analysis of *Trypanosoma brucei gambiense* glycerol kinase

Acta Crystallographica (accepted on Dec 29, 2009)

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Running title: Crystallization of glycerol kinase from Human African Trypanosomes

Category: *crystallization communications*

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Synopsis

Glycerol kinase from human African trypanosomes has been purified and crystallized for X-ray structure analysis.

Abstract

In the blood-stream forms of human trypanosomes, glycerol kinase (GK) (EC 2.7.1.30) is one of the nine glycosomally-compartmentalized enzymes essential for their energy metabolism. In this study, an N-terminal cleavable His_6-tagged recombinant *Trypanosoma brucei gambiense* GK (rTbgGK) was overexpressed, purified to homogeneity, and crystallized by the sitting-drop vapour diffusion method using PEG 400 as a precipitant. A complete X-ray diffraction data set to 2.75 Å resolution indicated that the crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters of a = 63.84, b = 121.50, c = 154.59 Å. The presence of two rTbgGK molecules in the asymmetric unit gives a Matthew's coefficient (V_M) of 2.5 $Å^3Da^{-1}$, corresponding to 50% solvent content.

Keywords: Human African trypanosomiasis; *Trypanosoma brucei gambiense*; drug design; glycerol kinase.

1. Introduction

Human African Trypanosomiasis (HAT) is a neglected hemo-parasitic disease caused by species of the protozoan genus *Trypanosoma,* and transmitted by tsetse flies. Over 20,000 new cases are reported annually, it is also a threat to 60 million human lives (WHO, 2001). Human pathogens for the disease are *Trypanosoma brucei gambiense* and *T. b. rhodesiense* that cause West and East African Trypanosomiasis, respectively, and animals serve as their reservoirs (Njiokou *et al.*, 2006), while the animal pathogens are *T. b. brucei, T. vivax, T. congolense,* and *T. evansi etc.* (Stevens & Brisse, 2004). HAT occurs in two forms, an acute form caused by *T. b. rhodesiense* and a chronic form caused by *T. b. gambiense*. Both agents of the disease present an early haemolymphatic stage and a late meningo-encephalitic phase; and are deadly at the second stage if left untreated. Unfortunately, only a few drugs are available, and problems such as narrow spectrum, treatment failures due to resistance, high cost, and cases of toxicities have been reported about them (Brun *et al.*, 2001). Therefore, the need to search for new, safer, affordable and more effective drugs with a broader action spectrum cannot be overemphasized.

Interestingly, blood-stream forms (BSFs) of these parasites possess some structural and metabolic features that are absent in the mammalian hosts. Such distinctive features, which provide valid drug targets include compartmentalization of their glycolysis into microbody-like organelles called glycosomes, their sole dependence on glycolysis for their energy need (Haanstra *et al.*, 2008) and the presence of a rudimentary mitochondrion that houses an indispensable cytochrome-independent alternative oxidase (AOX) (Chaudhuri *et al.*, 2006). AOX is not found in the host, and its inhibition by salicylhydroxamic acid

(SHAM) or ascofuranone (AF) was reported to cause parasites death as a result of impaired ATP metabolism (Minagawa *et al.*, 1997; Michels *et al.*, 2000; Hannaert *et al.*, 2003; Guerra *et al.*, 2006; Yabu *et al.*, 2006; Singha *et al.*, 2008). Lastly, trypanosomes contain a glycerol kinase (GK), which is also present in the glycosomes. Unlike the host GK, which catalyses only the forward reaction, *i.e.* ATP-dependent glycerol phosphorylation, trypanosomal GK can also catalyze the reverse reaction (Kralova *et al.*, 2000).

Our laboratory has found AF to be an excellent inhibitor of trypanosomal AOX (TAO), its K_i against TAO is 2.38 nM (Minagawa *et al.*, 1997) as compared to 10 μM for the earlier discovered TAO inhibitor, SHAM (Njogu *et al.*, 1980). However, *in vitro* and *in vivo* experiments revealed that AF- or SHAM-induced killing of trypanosomes is considerably enhanced when co-administered with 5 mM glycerol (Fairlamb *et al.*, 1977; Van der Meer & Versluijs-Broers, 1979; Minagawa *et al.*, 1997; Yabu *et al.*, 2006). This synergistic effect of glycerol is most likely mediated *via* an expected mass action-induced GK inhibition by the added glycerol, thereby blocking the anaerobic ATP generation of glycolysis in the parasites. Unfortunately, this unphysiologically high concentration of glycerol required for co-administration with AF is toxic for the host. Although GK, in conjunction with TAO, is thus a promising target for chemotherapy, an effective and selective parasite GK inhibitor has not yet become available.

GK is ubiquitous in archaea, bacteria, and eukaryotes; where it belongs to the sugar kinase/heat shock protein 70/actin superfamily (Hurley, 1996). So far, prokaryotic GKs are the most widely studied. Of the eukaryotes, only structural information of *Plasmodium falciparum* GK is available, but in this organism, GK is not essential for

growth of the asexual blood stages (Schnick, *et al.*, 2009). Also, kinetic studies revealed a striking difference between GK of trypanosomes and that of other organisms (Kralova *et al.*, 2000). In *T. b. brucei*, GK is encoded by five identical tandemly-arranged genes that encode GK (Colasante *et al.*, 2006), and plays an essential role for the parasites' survival, most especially in the absence of oxygen or in the presence of their TAO inhibitors (Minagawa *et al.*, 1997) due to it's ability to catalyze the reverse reaction, leading to the production of ATP, required by the parasites. One may wonder if the ability of the trypanosomal GK to catalyze the reverse reaction, in contrast to the human enzyme, is only due to the compartmentalization in glycosomes of the former, or if structure-based catalytic differences also contribute to it. We therefore perceive the parasite GK to be an interesting subject of structural investigation in terms of fundamental enzymology as well as drug target exploitation. We hereby report a preliminary X-ray diffraction analysis of GK from *T. b. gambiense*, as this may lead us to the design of parasite-specific GK inhibitors that spare the host enzyme. Since *T. b. brucei* TAO has been crystallized recently (Kido *et. al.*, in press), the X-ray structure analysis of both enzymes will aid us in the search of a new generation of chemotherapeutic agents against the BSFs.

2. Materials and Methods

2.1. Cloning and expression of TbgGK

Complementary DNA (cDNA) libraries were prepared from stocks of bloodstream forms of *T. b. gambiense* (IL2343) and *T. b. rhodesiense* (Tbr) (IL1501J21) using TOYOBO reverse transcriptase. The cDNA served as templates for the amplification of

their GK encoding genes (*gk*) by PCR using 5'-CACCATGAAGTACGTCGGATCCATT-3' and 5'-CTACAACTTTGCCCACTTCGTCCTC-3' as forward and reverse primers respectively, using *PfuUltra* II Fusion HS DNA polymerase (Stratagene). The amplicons were gel-purified using the TOYOBO gel purification method. Plasmid constructs were obtained by cloning the blunt-ended gene into the pET151/D-TOPO plasmid vector (Invitrogen) by a ligation-independent cloning procedure. Cloning in this vector leads to the addition of an N-terminal tag containing a His_6 sequence, a V5 epitope, and a tobacco etch virus (TEV) protease cleavage site (for removal of the fused 4 kDa tag) to the expressed recombinant protein.

One Shot TOP10 *Escherichia coli* cells were transformed with the Tbg or Tbr *gk*-pET151/D-TOPO (plasmid construct) by heat shock. Colonies were grown on Luria-Bertani (LB) plates containing 100 μg ml^{-1} carbenicillin, positive clones carrying the inserted gene were confirmed by colony PCR and selected for liquid culturing in LB media for construct amplification. Plasmid extraction from the cultured TOP10 cells was achieved using a TOYOBO MagExtractor kit, and subjected to further confirmation by a combination of nested PCR and digestion with *Nco*I. Gene sequencing using the construct and designed sequencing primers was conducted by the dye-terminator method with an ABI Prism310 genetic analyzer (Applied Biosystems). The nucleotide sequence of the *gk* revealed that Tbg and Tbr GKs are exactly identical at the protein level; hence Tbg-*gk* was picked and used for this study. The recombinant plasmid was transformed into the JM109 (DE3 + pRARE2) *E. coli* strain (Novagen) for protein expression. Colonies of the transformants grown on a LB plate containing 100 μg ml^{-1} and 50 μg ml^{-1} of carbenicillin

and chloramphenicol respectively, were selected and grown aerobically in LB medium containing the same concentrations of the antibiotics.

The expression conditions were optimized for the amount and activity of GK in the cytosolic fractions using activity measurements and SDS-PAGE, by varying the concentration of the expression inducer isopropyl β-D-thiogalactopyranoside (IPTG), temperature, and post-induction time before transformant harvest. The best yield was achieved with 25 μM IPTG, growth at 293 K, and post-induction for 8 h.

2.2. Assay of GK activity

The TbgGK activity was assayed using the reverse reaction of TbgGK, glycerol 3-phosphate + ADP ---> glycerol + ATP. To 1.0 ml of the reaction mixture (1 mM EDTA, 5mM $MgSO_4$, 0.5mM $NADP^+$, 50mM glucose, 2mM ADP, 10mM glycerol 3-phosphate and 1 unit of hexokinase and glucose-6-phosphate dehydrogenase), TbgGK was added at 300 K. Using ATP produced by TbgGK, hexokinase converts glucose to glucose 6-phosphate, and finally glucose-6-phosphate dehydrogenase produces NADPH from glucose 6-phosphate and $NADP^+$. The rate of NADPH accumulation was spectrophotometrically monitored at 340 nm using the JASCO V-660 spectrophotometer.

2.3. Purification of recombinant TbgGK

For large-scale preparation, the transformant was grown at 293 K in 10 L of LB medium for 8 h after induction, and harvested by centrifugation at 10,000 g. The *E. coli* pellet was washed twice in 50 mM Tris-HCl buffer pH 7.6 containing 0.1 mM

phenylmethylsulfonyl fluoride (PMSF) and resuspended in 300 ml of lysis buffer (100 mM phosphate buffer pH 6.8, 300 mM NaCl, 10 mM $MgSO_4$, 0.1 mM PMSF, 1 mg ml^{-1} lysozyme and 10 % (*v*/*v*) glycerol). The cell suspension was kept on ice for 30 min, passed twice through a French pressure cell operated at 140 MPa to break cells, and then subjected to centrifugation at 26,000 g to remove unbroken cells and inclusion bodies. The supernatant was further centrifuged at 146,000 g to remove residual undissolved material, and then applied to a Ni-NTA Agarose column (Qiagen; 1.5 × 15 cm) pre-equilibrated with 100 mM phosphate buffer pH 6.8 containing 20 mM imidazole, 300 mM NaCl, 10 mM $MgSO_4$ and 1 % (*v*/*v*) glycerol. After washing the column with 100 ml of the same buffer, rTbgGK was eluted with 500 ml of the buffer containing a linear gradient of 20 to 500 mM imidazole. Fractions containing active rTbgGK of higher purity as assessed by SDS-PAGE (Laemmli, 1970) were pooled, concentrated to approximately 40 mg ml^{-1} using a centrifugal ultrafiltration tube (Amicon Ultra-15, 30 kDa cutoff; Millipore), and stored at 253 K in the presence of 50% (*v*/*v*) glycerol until next purification. About 5 mg of the affinity-purified protein was further purified by gel-filtration chromatography using a Superdex 200 (1 × 30 cm) column (GE Healthcare Bio-sciences) equilibrated with 100 mM phosphate buffer pH 6.8 containing 0.3 M NaCl and 1 % (*v*/*v*) glycerol. The elution was carried out at a flow rate of 0.5 ml min^{-1} on a high-performance liquid chromatography (HPLC) instrument. Each fraction (0.5 ml) was analyzed on SDS-PAGE, and fractions containing highly pure rTbgGK were pooled. After buffer exchange for 10 mM MOPS buffer pH 6.8, 10 mM $MgSO_4$ and 1% (*v*/*v*) glycerol, the purified rTbgGK was concentrated to about 10 mg ml^{-1} for crystallization experiments. Addition of $MgSO_4$ and

glycerol was crucial for the preservation of the rTbgGK activity. Concentration of rTbgGK was estimated using the calculated molar extinction coefficient at 280 nm (ε_{280}=81,080), giving A_{280}=1.0 to the pure rTbgGK solution of 0.74 mg ml^{-1}.

2.4. Crystallization and X-ray diffraction data collection

Crystallization conditions were initially screened at 277 and 293 K by the sitting drop vapour-diffusion method in a 96-well Corning Crystal EX micro-plate with conical flat bottom (Hampton Research). A 0.5 μl droplet containing about 10 mg ml^{-1} rTbgGK dissolved in 10 mM MOPS buffer pH 6.8, 10 mM $MgSO_4$ and 1% (*v/v*) glycerol was mixed with an equal volume of reservoir solution, and the droplet was allowed to equilibrate against 100 μl of the reservoir solution. In the initial screening experiment, commercially available screening kits from Hampton Research (Crystal Screen, Crystal Screen II, Grid Screen A/S, Grid Screen PEG 6000, Grid Screen MPD and Quick Screen) and from Emerald BioStructures (Wizard Screen I and II) were used as the reservoir solutions. However, most of the conditions gave only protein precipitates and the screening was unsuccessful. Screening was then carried out using a 5 mg ml^{-1} rTbgGK solution and the reservoir solutions diluted twice. Out of 290 conditions screened, tiny crystals and their aggregates appeared at 277 K and 293 K from reservoir solutions containing 2.5-5% (*w/v*) PEG 6000 in the pH range of 6.0-8.0. The conditions were further optimized by varying the buffer pH (5.6-8.4), the molecular weight of PEG and its concentration (1-10% (*w/v*) for PEG 3350 and PEG 6000; 10-30% (*w/v*) for PEG 400). Finally, single crystals suitable for

X-ray diffraction experiments were obtained from a reservoir solution containing 30% (*w/v*) PEG 400 and 100 mM HEPES pH 7.0 within 24 h.

A crystal mounted in a nylon loop was transferred and soaked briefly in the reservoir solution containing 40% (*w/v*) PEG 400 and then flash-frozen at 100 K in a stream of nitrogen gas. X-ray diffraction experiments were performed at the BL41XU beam line (λ= 1.000 Å; a Rayonix CCD detector MX225HE, 100 K) of SPring-8 (Harima, Japan). A total of 180 images were recorded with an oscillation angle of 1.0°, an exposure time of 1 s per image, and a crystal-to-detector distance of 200 mm. The diffraction data were processed and scaled with the *HKL*-2000 software package (Otwinowski & Minor, 1997).

3. Results and discussion

Gene sequence analyses for the cDNAs of *T. b. gambiense* and *T.b. rhodesiense gk*s revealed a total of 7 point differences when compared to the *gk* sequence for *T. b. brucei* (TREU927; Accession number XM_822408), and only one difference (212-T in *T. b. brucei* to C in *T. b. gambiense* and *T. b. rhodesiense*) resulting in a change of a single amino acid (Phe71 in TbbGK to Ser71 in Tbg and TbrGK) out of the 512 amino acid residues of TbgGK. The nucleotide sequence data for the cDNAs of *T. b. gambiense* and *T. b. rhodesiense gk*s have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB517984 and AB517985, respectively.

The His_6-tagged rTbgGK with 545 amino acid residues (60.4 kDa) was overexpressed and purified to homogeneity by a combination of Ni-NTA affinity chromatography and Superdex 200 gel filtration chromatography (Fig. 1). About 80 mg of

purified enzyme with specific activity of 31.7 μmol/min/mg was obtained from a 10 L culture. The rTbgGK protein eluted from the Superdex 200 column at a retention time corresponding to a molecular weight of about 119 kDa, indicating that the enzyme exists as a homodimer in solution.

Out of the 290 crystallization conditions screened, crystals of rTbgGK were obtained using PEGs as precipitants. After the optimization of crystallization conditions, the best crystals, which diffract X-rays to a resolution of 2.75 Å (Fig. 3), could be grown at 293 K using the reservoir solution containing 30% (*w/v*) PEG 400 and 0.1 M HEPES buffer pH 7.0. Crystals attained a typical size of about 0.25 × 0.1 × 0.05 mm in 2 d (Fig. 2). Analyses of the symmetry and systematic absences in the recorded diffraction patterns revealed that the crystals of rTbgGK belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters; a = 63.84, b = 121.50, c = 154.59 Å. Assuming the presence of two rTbgGK molecules (2 × 60.4 kDa) in the asymmetric unit, the V_M value was calculated to be 2.5 $Å^3$ Da^{-1} with an estimated solvent content of 50% (Matthews, 1968); these values are within the range commonly observed for protein crystals. A total of 135,987 observed reflections recorded on 180 images were merged to 31,848 unique reflections from 50.0 to 2.75 Å resolution with an R_{merge} of 5.5%. The data collection and processing statistics are shown in Table 1.

An attempt to solve the structure using the molecular replacement method with the *MOLREP* program (Vagin & Teplyakov, 1997) from the CCP4 suite (Collaborative Computational Project, Number 4, 1994) was carried out using the refined coordinates of GK from *P. falciparum* (PDB code: 2w41, 40% amino acid sequence identity with

rTbgGK; Schnick *et al.*, 2009). A promising solution with a homodimeric structure was obtained (correlation coefficient and R factor of 0.406 and 51.6%, respectively). Using the solution of the molecular replacement, the structure is being subjected to refinement. In parallel with the refinement, we are now trying to obtain crystals of rTbgGK complexed with ligands including substrates and substrate analogues. *In silico* screening of potential inhibitors from a compound library of the Chemical Biology Research Initiative, the University of Tokyo is also underway.

It should be noted that the amino acid sequence of TbgGK is identical to that of TbrGK and shows only one difference from that of the TbbGK. Therefore, inhibitors of TbgGK should also be effective against other trypanosome GKs. Since TbgGK provides a greater potential for the primary target of chemotherapy, the detailed structures of TbgGK complexed with inhibitors will help structure-based drug design aimed at African Trypanosomiasis.

Acknowledgment

We thank all staff members of the beam line BL41XU at SPring-8 for their help with X-ray diffraction experiments. This work was supported by a grant from the Targeted Proteins Research Program (TPRP) and was supported in part by a grant-in-aid for Creative Scientific Research (18GS0314 to KK) from the Japan Society for the Promotion of Science, and a grant-in-aid for scientific research on Priority Areas (18073004 to KK) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. EOB is

supported by a Japanese Government Scholarship from the Ministry of Education, Science, Culture, Sports, and Technology.

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