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

Emmanuel Oluwadare Balogun,[a,b] Daniel Ken Inaoka,[a] Yasutoshi Kido,[a] Tomoo Shiba,[a] Takeshi Nara,[c] Takashi Aoki,[c] Teruki Honma,[d] Akiko Tanaka,[d] Masayuki Inoue,[e] Shigeru Matsuoka,[e] Paul A. M. Michels,[f] Shigeharu Harada[g]* and Kiyoshi Kita[a]*

[a]Department of Biomedical Chemistry, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, [b]Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria, [c]Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, Tokyo 113-8421, Japan, [d]Systems and Structural Biology Center, RIKEN, Tsurumi, Yokohama 230-0045, Japan, [e]Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan, [f]Research Unit for Tropical Diseases, de Duve Institute and Laboratory of Biochemistry, Université Catholique de Louvain, Avenue Hippocrate 74, B-1200 Brussels, Belgium, and [g]Department of Applied Biology, Graduate School of Science and Technology, Kyoto Institute of Technology, Sakyo-ku, Kyoto 606-8585, Japan

Correspondence e-mail: harada@kit.ac.jp, kitak@m.u-tokyo.ac.jp

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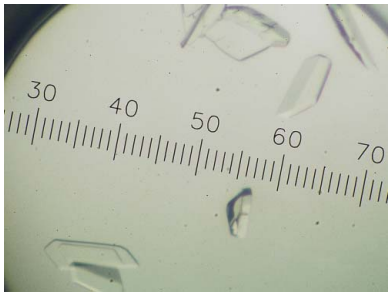

In the bloodstream forms of human trypanosomes, glycerol kinase (GK; EC 2.7.1.30) is one of the nine glycosomally compartmentalized enzymes that are essential for energy metabolism. In this study, a recombinant *Trypanosoma brucei gambiense* GK (rTbgGK) with an N-terminal cleavable His_6 tag was overexpressed, 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 belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 63.84, b = 121.50, c = 154.59 Å. The presence of two rTbgGK molecules in the asymmetric unit gives a Matthews coefficient (V_M) of 2.5 $Å^3$ Da^{-1}, corresponding to 50% solvent content.

1. Introduction

Human African trypanosomiasis (HAT) is a neglected haemoparasitic 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 (World Health Organization, 2006). The human pathogens for the disease are *T. brucei gambiense* and *T. b. rhodesiense*, which cause West and East African trypanosomiasis, respectively, with animals serving as their reservoirs (Njiokou *et al.*, 2006), while the animal pathogens include *T. b. brucei*, *T. vivax*, *T. congolense* and *T. evansi* (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 meningoencephalitic 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 owing to resistance, high cost and cases of toxicity have been reported (Brun *et al.*, 2001). Therefore, the need to search for new, safer, affordable and more effective drugs with a broader spectrum of action cannot be overemphasized.

Interestingly, the bloodstream forms (BSFs) of these parasites possess several 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 needs (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) has been reported to cause parasite 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). In addition, trypanosomes contain a unique glycerol kinase (GK) in their glycosomes. Unlike the host GK, which only catalyses 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 n*M* (Minagawa *et al.*, 1997) compared with 10 µ*M* for the previously discovered TAO inhibitor SHAM (Njogu *et al.*, 1980). However, *in vitro* and *in vivo* experiments have revealed that AF-induced or SHAM-induced killing of trypanosomes is considerably enhanced when they are co-administered with 5 m*M* 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 to be mediated *via* an expected mass-action-induced inhibition of GK by the added glycerol, thereby blocking the anaerobic ATP generation of glycolysis in the parasites. Unfortunately, this nonphysiologically high concentration of glycerol required for co-administration with AF is toxic to 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). To date, prokaryotic GKs have been the most widely studied. Of the eukaryotes, structural information is only available on *Plasmodium falciparum* GK, but GK is not essential for growth of the asexual blood stages in this organism (Schnick *et al.*, 2009). Kinetic studies also revealed a striking difference between the GKs of trypanosomes and those of other organisms (Kralova *et al.*, 2000). In *T. b. brucei* GK is encoded by five identical tandemly arranged genes (Colasante *et al.*, 2006) and plays an essential role in the survival of the parasite, especially in the absence of oxygen or in the presence of TAO inhibitors (Minagawa *et al.*, 1997), owing to its ability to catalyze the reverse reaction leading to the production of ATP required by the parasites. One may wonder whether the ability of the trypanosomal GK to catalyze the reverse reaction, in contrast to the human enzyme, is purely a consequence of the compartmentalization in glycosomes of the former or whether structure-based catalytic differences also make a contribution. We therefore perceive the parasite GK to be an interesting subject for structural investigation in terms of fundamental enzymology as well as drug-target exploitation. Here, we report the preliminary X-ray diffraction analysis of GK from *T. b. gambiense*, which may lead us to the design of parasite-specific GK inhibitors that spare the host enzyme. Since *T. b. brucei* TAO has also been crystallized recently (Kido *et al.*, 2010), X-ray structure analysis of both enzymes will aid us in the search for a new generation of chemotherapeutic agents against BSFs.

2. Materials and methods

2.1. Cloning and expression of TbgGK

Complementary DNA (cDNA) libraries were prepared from stocks of the bloodstream forms of *T. b. gambiense* (IL2343) and *T. b. rhodesiense* (Tbr; IL1501J21) using Toyobo reverse transcriptase. The cDNAs 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, with *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 and 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 was 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 using the dye-terminator method with an ABI Prism310 genetic analyzer (Applied Biosystems). The nucleotide sequence of *gk* revealed that the Tbg and Tbr GKs were exactly identical at the protein level; hence, Tbg *gk* was picked and used in 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 an LB plate containing 100 µg ml^{-1} carbenicillin and 50 µg ml^{-1} chloramphenicol were selected and grown aerobically in LB medium containing the same concentrations of antibiotics.

The expression conditions were optimized for the amount and the activity of GK in the cytosolic fractions using activity measurements and SDS–PAGE by varying the concentration of the expression inducer isopropyl β-D-1-thiogalactopyranoside (IPTG), the temperature and the post-induction time before transformant harvest. The best yield was achieved with 25 µ*M* 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 m*M* EDTA, 5 m*M* $MgSO_4$, 0.5 m*M* $NADP^+$, 50 m*M* glucose, 2 m*M* ADP, 10 m*M* glycerol 3-phosphate and one unit of hexokinase and glucose-6-phosphate dehydrogenase), TbgGK was added at 300 K. Using the 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 a Jasco V-660 spectrophotometer.

2.3. Purification of recombinant TbgGK

For large-scale preparation, the transformant was grown at 293 K in 10 l LB medium for 8 h after induction and was harvested by centrifugation at 10 000*g*. The *E. coli* pellet was washed twice in 50 m*M* Tris–HCl buffer pH 7.6 containing 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) and was resuspended in 300 ml lysis buffer [100 m*M* phosphate buffer pH 6.8, 300 m*M* NaCl, 10 m*M* $MgSO_4$, 0.1 m*M* 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 the 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 onto an Ni–NTA Agarose column (Qiagen; 1.5 × 15 cm) pre-equilibrated with 100 m*M* phosphate buffer pH 6.8 containing 20 m*M* imidazole, 300 m*M* NaCl, 10 m*M* $MgSO_4$ and 1%(*v*/*v*) glycerol. After washing the column with 100 ml of the same buffer, rTbgGK was eluted with 500 ml of buffer containing a linear gradient of 20–500 m*M* 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 the next purification step. 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 Biosciences) equilibrated with 100 m*M* phosphate buffer pH 6.8 containing 0.3 *M* NaCl and 1%(*v*/*v*) glycerol. 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 by SDS–PAGE and fractions containing highly pure rTbgGK were pooled. After buffer exchange to 10 m*M* MOPS buffer pH 6.8, 10 m*M* $MgSO_4$ and 1%(*v*/*v*) glycerol, the purified rTbgGK was concentrated to about 10 mg ml^{-1} for crystallization experiments. The addition of $MgSO_4$ and glycerol was crucial for preservation of the rTbgGK activity. The concentration of rTbgGK was estimated using the calculated molar extinction coefficient at 280 nm (ε_{280} = 81 080), giving an A_{280} of 1.0 for the pure rTbgGK solution at 0.74 mg ml^{-1}.

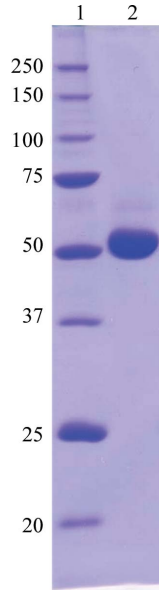

Figure 1
12.5% SDS–PAGE gel stained with Coomassie Brilliant Blue R-250 showing the apparent homogeneity of the purified rTbgGK. Lane 1, molecular-weight markers (kDa); lane 2, rTbgGK purified by Ni–NTA affinity chromatography and Superdex 200 gel filtration.

2.4. Crystallization and X-ray diffraction data collection

Crystallization conditions were initially screened at 277 and 293 K using the sitting-drop vapour-diffusion method in a 96-well Corning CrystalEX microplate with conical flat bottom (Hampton Research). A 0.5 µl droplet containing about 10 mg ml^{-1} rTbgGK dissolved in 10 m*M* MOPS buffer pH 6.8, 10 m*M* $MgSO_4$ and 1%(*v*/*v*) glycerol was mixed with an equal volume of reservoir solution and the droplet was allowed to equilibrate against 100 µl reservoir solution. In the initial screening experiment, commercially available screening kits from Hampton Research (Crystal Screen, Crystal Screen II, Grid Screen Ammonium Sulfate, 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 heavy protein precipitates and the screening was unsuccessful. Screening was then carried out using a 5 mg ml^{-1} rTbgGK solution and twice-diluted reservoir solutions. Out of 290 conditions screened, tiny crystals and their aggregates appeared at 277 and 293 K from reservoir solutions containing 2.5–5%(*w*/*v*) PEG 6000 in the pH range 6.0–8.0. The conditions were further optimized by varying the buffer pH (5.6–8.4), the molecular weight of the 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 using a reservoir solution consisting of 30%(*w*/*v*) PEG 400 and 100 m*M* HEPES pH 7.0 within 24 h.

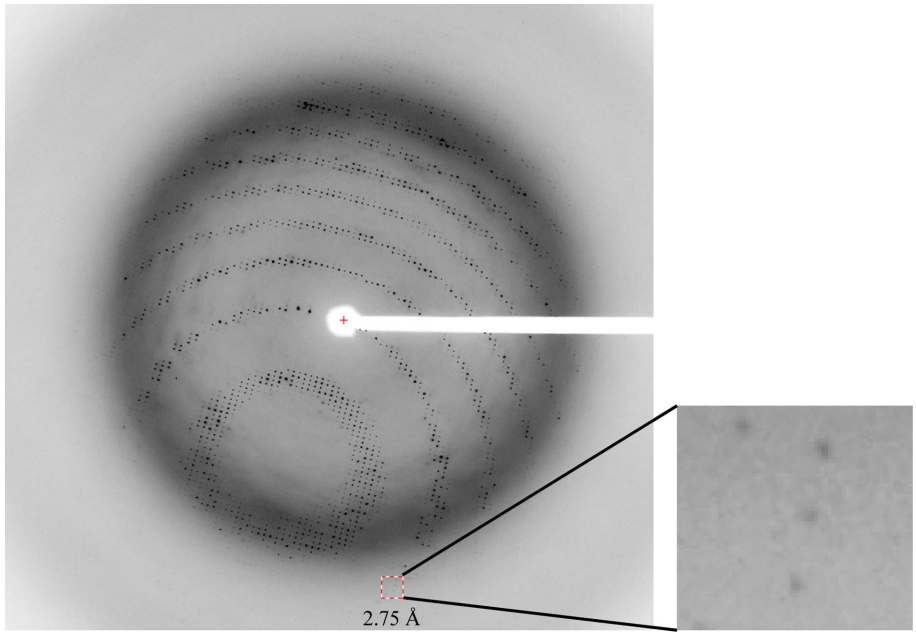

Figure 2
A typical X-ray diffraction pattern of an rTbgCK crystal. The detector edge corresponds to 2.4 Å resolution and an enlarged image of the indicated area around 2.75 Å resolution is shown. The exposure time was 1 s, with an oscillation angle of 1.0°.

X-ray diffraction experiments were performed under cryocooled conditions (100 K) on the BL41XU (λ = 1.000 Å; Rayonix MX225HE CCD detector) and BL44XU (λ = 0.900 Å; Bruker DIP-6040 detector system) beamlines at SPring-8 (Harima, Japan) and the BL17A (λ = 1.000 Å; ADSC Quantum 270 detector) beamline at the Photon Factory (Tsukuba, Japan). A crystal mounted in a nylon loop was transferred to and soaked briefly in reservoir solution containing 40%(*w*/*v*) PEG 400 and then flash-frozen at 100 K in a stream of nitrogen gas. 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 seven point differences when compared with the *gk* sequence from *T. b. brucei* (TREU927; accession No. XM_822408); only one of these differences (T212 in *T. b. brucei* to C in *T. b. gambiense* and *T. b. rhodesiense*) resulted in a change of a single amino acid (Phe71 in TbbGK to Ser71 in TbgGK and TbrGK) in 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 accession Nos. AB517984 and AB517985, respectively.

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 purified enzyme with a specific activity of 31.7 µmol min^{-1} mg^{-1} was obtained from a 10 l culture. The rTbgGK protein eluted from the Superdex 200 column with a retention time corresponding to a molecular weight of about 119 kDa, indicating that the enzyme exists as a homodimer in solution.

In a screening of 290 crystallization conditions, crystals of rTbgGK were obtained using PEGs as precipitant. After optimization of the crystallization conditions, the best crystals, which diffracted X-rays to a resolution of 2.75 Å (Fig. 2), were grown at 293 K using a reservoir solution containing 30%(*w*/*v*) PEG 400 and 0.1 *M* HEPES buffer pH 7.0. The crystals attained typical dimensions of about 0.25 × 0.1 × 0.05 mm in 2 d (Fig. 3). Analysis of the symmetry and systematic absences in the recorded diffraction patterns revealed that the crystals of rTbgGK belonged 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 in the 50.0–2.75 Å resolution range with an R_{merge} of 5.5%. The data-collection and processing statistics are shown in Table 1.

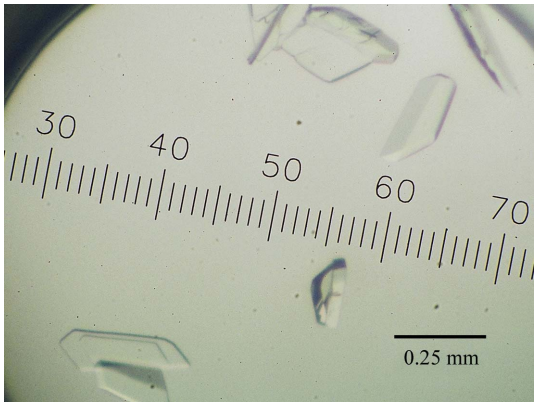

Figure 3
Crystals of rTbgGK obtained by the sitting-drop vapour-diffusion method using PEG 400 as a precipitant.

Table 1
Diffraction data statistics for the crystal of rTbgGK.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 63.84, b = 121.50, c = 154.59
V_M† ($Å^3$ Da^{-1})	2.5
Solvent content† (%)	50
X-ray source	BL41XU, SPring8
Wavelength (Å)	1.000
Temperature (K)	100
Resolution (Å)	50–2.75 (2.85–2.75)
Total reflections	135987
Unique reflections	31848
Completeness (%)	97.1 (95.7)
$R_{merge}(I)$‡ (%)	5.5 (46.1)
$\langle I/\sigma(I)\rangle$	18.4 (3.0)

† Assuming the presence of two molecules in the asymmetric unit. ‡ $R_{merge}(I) = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is their average.

An attempt to solve the structure using the molecular-replacement method with the *MOLREP* program (Vagin & Teplyakov, 1997) from the *CCP*4 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 to 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 molecular-replacement solution, 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 at the University of Tokyo is also under way.

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

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