論文の内容の要旨

論文題目 X-ray crystal structure analysis of glycerol kinase from African human trypanosomes for anti-trypanosomal drug design

和訳 アフリカトリパノソーマの薬剤標的タンパク質「グリセロールキナーゼ」の X 線結晶構造解析

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1. Introduction

Trypanosoma brucei gambiense (*T. b. gambiense*) and *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) are generally referred to as African human trypanosomes. They are blood parasites that cause the disease called Human African Trypanosomiasis (HAT), also known as sleeping sickness. HAT is a potentially fatal but neglected disease that is endemic in sub-Saharan Africa. In this region, about 70 million people are at risk; 300,000 people are presently infected, and about 30,000 humans are annually infected. This disease is racially unbiased, as over 200 cases of imported HAT have been reported in various European countries. The parasites are transmitted by insects called tsetse flies, during their blood meal. The insect form is known as procyclic forms (PCFs), while the animal form is called blood stream forms (BSFs).

Upon infecting the human host, African trypanosomes live extracellularly in the blood stream, and are able to escape the host defense mechanisms by a strategy that is known as antigenic variation. Using this mechanism, they easily and repeatedly change their surface coat, hence loose recognition by the host antibody. This evasion of the adaptive immune responses contributes to parasite virulence, and has frustrated all efforts towards vaccine development;

hence, management of the disease by chemotherapy remains the only reasonable hope towards a solution to this devastating disease. Unfortunately, only four drugs (suramin, pentanidine, melarsoprol, and eflornithine) are available for treatment of HAT. All of them are no longer safe and effective because problems such as narrow spectrum, treatment failures due to resistance, high cost, and toxicities. Therefore, there is urgent need to discover and design new, safer, and affordable more effective drugs with a broader action spectrum.

. To design a good drug, it is important to identify and validate molecular targets in the parasites that are essential for their growth but absent, not essential, or structurally different from the host molecule. In other words, taking advantage of differences between the parasite and the humans will lead to the discovery of a good drug that is not toxic to the host. Looking closely at their energy generation mechanisms reveal some differences:

Trypanosomes (BSFs)	**Humans**
- Rudimentary mitochondria – no Krebs cycle and oxidative phosphorylation enzymes	- Fully functional mitochondria – have Krebs cycle and oxidative phosphorylation enzymes
- Rely solely on glycolysis for ATP synthesis	- ATP is produced from both glycolysis and mitochondria
- Glycolysis is compartmentalized into glycosomes	- Glycolysis occurs in the cytoplasm
- Mitochondria contains alternative oxidase (AOX)	- Mitochondria lacks AOX
- Cells **die** when AOX is inhibited	- AOX inhibitors have **no** effect
- Glycerol kinase (GK) have both forward and reverse activity i.e Glycerol + ATP → glycerol 3-phosphate + ADP glycerol 3-phosphate + ADP → Glycerol + ATP	- GK activity is forward only i.e Glycerol + ATP → glycerol 3-phosphate + ADP
- GK is essential and participate in anaerobic glycolysis	- GK is not involved in anaerobic glycolysis

Our laboratory found ascofuranone to be so far, the most excellent inhibitor of AOX; however, its full potential at curing animals that were experimentally infected with trypanosomes requires co-administration with 5 mM glycerol. This amount of glycerol is physiologically too high and toxic to the animals. Our group has identified and validated GK to be the target of glycerol. Although GK, in conjunction with AOX, is thus a promising target for chemotherapy, an effective and selective trypanosome GK inhibitor has yet to become available. In fact, there is no known inhibitor of any GK.

It has long been recognized that knowledge of the three-dimensional (3-D) structures of protein targets using X-ray crystallography, has the potential to accelerate the discovery and design of new drugs. It is in view of all the above that I design this work to utilize structure-based (*in silico* screening) and activity-based (high-throughput screening) approaches for the design of good compounds that will discriminatorily inhibit parasite GK.

2. Result and Discussion

GK gene (*gk*) from both *T. b. gambiense* and *T. b. rhodesiense* were cloned into pET151/D-TOPO vector and sequenced. The nucleotide sequence, which our group has deposited to the GenBank (accession Nos. AB517984 and AB517985, respectively) revealed that GKs of *T. b. gambiense* and *T. b. rhodesiense* are same at amino acid level. The cloned *gk* of *T. b. gambiense* was transformed into, JM109 (DE3 + pRARE2) *E. coli* strain for protein expression. The expressed His_6-tagged protein was purified to homogeneity by a combination of affinity chromatography on Ni-NTA, and gel filtration on superdex 200. Approximately 150 mg of active (31.7 μmol/min/mg) pure protein (single band on SDS-PAGE) was obtained from a 10 L culture of transformants. The enzyme was characterized, and used for crystallization experiments.

A total of 576 crystallization conditions from commercially available screening kits were tested by sitting drop vapour diffusion with protein sample of 5 mg/ml concentration. Tiny crystals were obtained by a conditions that contain 2.5 - 5 % PEG 6000, at 293 K. This condition was optimized, and single crystals suitable for X-ray diffraction experiments were obtained with 30 % (w/v) PEG 400 in HEPES buffer, pH 7.0. X-ray diffraction data were collected at λ = 1.000Å under cryocooled conditions (100 K) on BL41XU beam line at Spring-8 (Harima, Japan) and BL17A beam line at Photon Factory (Tsukuba, Japan). The cryoprotectant used was 40 % (w/v) PEG 400. Data were collected for ligand-free and ligand-bound GK. The ligands used were glycerol, glycerol 3-phosphate, ATP, ADP, and 4-nitrophenylphosphate; whose crystals diffracted X-ray to resolutions of 2.4, 2.8, 2.3, 2.7, and 2.7 Å, respectively. The ligand-free crystals diffracted to a resolution of 2.9 Å. Diffraction data were processed and scaled with *HKL-2000* software package. Analyses of the symmetry and systematic absences in the recorded diffraction patterns revealed that while the crystals of glycerol, ATP, and ADP bound TbgGK belong to the monoclinic $P2_1$ space group, those of ligand-free, and g3p- bound TbgGK belongs to the orthorhombic space group $P2_12_12_1$.

Structure of glycerol-bound TbgGK was solved by molecular replacement method with the *MOLREP* program from the CCP4 suite using the refined coordinates of GK from *P. falciparum* (PDB code: 2w41, 40% amino acid sequence identity). A promising solution with a homodimer structure was obtained. Using the solution of the molecular replacement, the structure of the various ligand-bound GK has been solved; adjustments and refinements of the various structures are done by repeated cycles of COOT and REFMAC programs.

The solved structure of TbgGK revealed that the enzyme is a homodimer, which is formed by a somewhat strong association of 2 monomer chains A and B where the dimer

interface is made up of an antiparallel beta sheet and 3 alpha helices that are contributed by each of the monomers (Fig 1). Each monomer is made up of 2 domains (I and II), glycerol binding site is in domain I.

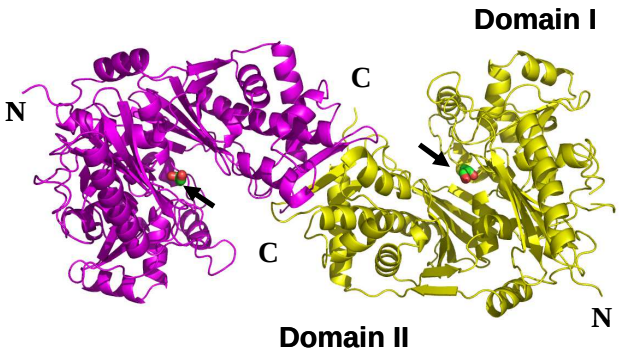

Figure1: Structure of glycerol-bound TbgGK coloured by chains. Arrows show glycerol in the active site of the enzyme. N and C represent the terminals of each monomer.

When the various forms of TbgGK were superposed, a difference is active site conformation was observed between the ligand-free and glycerol bound forms. In the ligand-free form a loop closes up the active site, the loop conformation was maintained by a disulphide linkage between cysteine residues at positions 278 and 319 (C278 and C319). Since the disulphide bond was absent in the glycerol-bound form, it was probably reduced during glycerol binding. To investigate the relevance of this disulphide bond and its reduction on the biological activity of TbgGK, I performed activity measurements in the presence and absence of 5 mM reducing agents (Dithionite or Tricarboxyethylphosphine, TCEP). The enzyme activity was 2 and 7 folds higher respectively, in the presence of reducing agents. This result was confirmed by site-directed mutagenesis, during which C278, C319, or both were mutated to alanine or serine. Activity of serine mutants were also about 7 - 8 folds higher than the wild type enzyme, and they were unresponsive to the presence of reducing agents. The alanine mutant activity was not

significantly higher than wild type enzyme, suggesting a strict positional requirement for polar residues.

Notable distinctive feature of the TbgGk is the presence of 2 loops at the active site, where they seem to affect the active site conformation. And also the presence of an unusual ADP / ATP binding site that is about 30 Å away from the active site groove. Interestingly, the unique loops and ADP / ATP binding sites are formed by amino acid residues that are not found at the corresponding segment of human GK therefore, the design of compounds that bind this loop may be potent TbgGK-specific inhibitors. The residues that bind ADP in the active site are also different from that of other GKs (Fig. 2), this unique binding result in orienting the ADP proximally (3.4 Å) to phosphoryl group of g3p. This could be the reason for the reverse-ability of trypanosome GK. In *P. falciparum* where GK lacks reverse capability, the second phosphoric acid group of ADP is over 8 Å away from phosphate of g3p, hence the lack of proximity necessary for catalysis.

Also herein, I developed a new high throughput screening assay method for trypanosome GK by using 4-nitrophenylphosphate; its phosphate group is cleaved by TbgGK, and probably transferred to ADP. This assay method has been optimized for the screening of inhibitors that were predicted by *in silico* screening with the solved structures. The experimentally screened compounds were from The University of Tokyo's faculty of pharmaceutical sciences chemical compound library established by Professor Nagano. So far, about 50 hits have been found out of 2000 compounds. The hits were compounds that inhibited 50 % of TbgGK activity at 20 μM. Representatives of the hits were compounds with UT ID: T-103825, T-103826, and T-103843; which showed 70, 60, and 92 % inhibitory effects respectively. They contain diazinane ring. The best poses from the docking of these compounds

revealed that they are bound to the active site cleft, at or close to the ADP binding pocket (Fig. 3). Since the ADP binding mode in TbgGK is different from the human hosts, optimization of these compounds by structure-activity relationships will help us to obtain drug-like specific inhibitors of TbgGK.

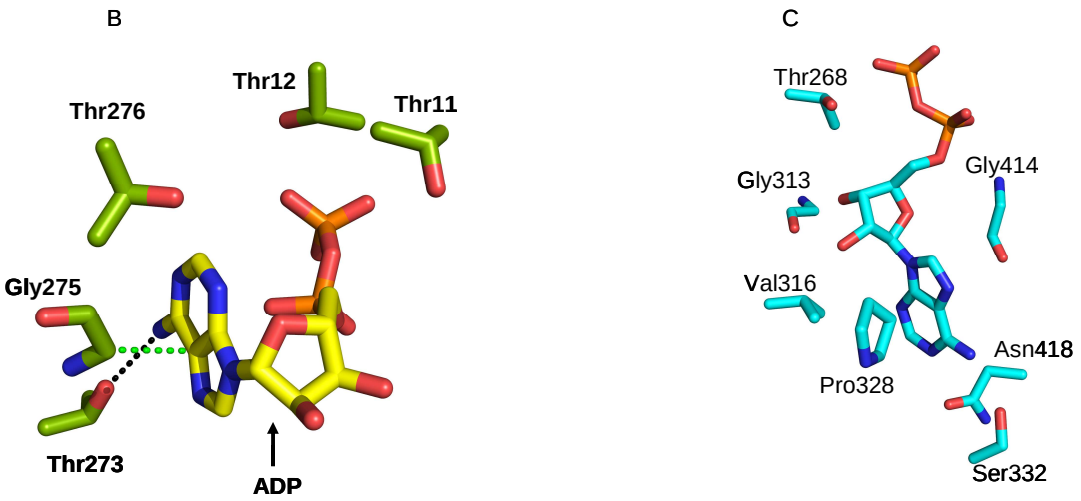

Figure 2**:** Active site ADP binding amino acid residues in TbgGK are different from the *P. falciparum* GK residues. (B) TbgGK amino acid residues that bind ADP, they are different from those for ADP binding in *P. faciparum* GK (C)

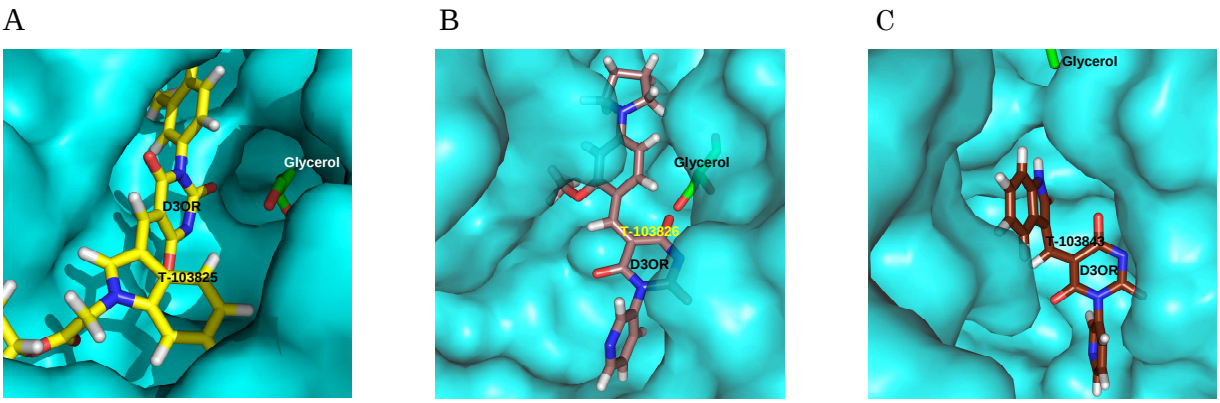

Figure 3: Best poses for the docked structures of some TbgGK inhibitors showing the proposed binding mode for (A) T-103825, (B) T-103826, and (C) T-103843. The compounds are bound to the ADP binding site. Diazinane-trione ring (marked “D3OR”) is common to them and seems to mediate their binding.