Differential Kinetic Activities of Glycerol Kinase among African Trypanosome Species: Phylogenetic and Therapeutic Implications

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ABSTRACT. African trypanosome species are causative agents for sleeping sickness in humans and nagana disease in cattle. Trypanosoma brucei can generate ATP via a reverse reaction with glycerol kinase (GK) when alternative oxidase (AOX) is inhibited; thus, GK is considered to be a crucial target for chemotherapy combined with AOX. However, the energy metabolism systems of African trypanosome species other than T. brucei are poorly understood. Thus, GK genes were surveyed from genome databases and cloned by PCR from T. vivax and T. congolense. Then, recombinant GK proteins (rGK) of T. vivax, T. congolense and T. brucei were expressed and purified. Kinetic analysis of these rGK proteins revealed that the $K_m$ values of T. congolense rGK for ADP and G-3-P substrates were lower than those of T. vivax and T. brucei. The expression level of GK molecules was highest in T. congolense cells and lowest in T. vivax cells. Based on these results, effective combination dosages of ascofuranone, a specific inhibitor of AOX, and glycerol, an inhibitor of the GK reverse reaction, were determined by using in vitro-cultured trypanosome cells.

KEY WORDS: ascofuranone, glycerol, glycerol kinase, phylogenetic analysis, trypanosome.

African trypanosome species cause sleeping sickness in humans and nagana disease in cattle. The bloodstream forms of the parasites in mammalian hosts are totally dependent on glycolysis for energy production. In the glycolytic pathway, two moles of ATP are synthesized from one mole of glucose. Reducing equivalents produced during glycolysis are reoxidized by mitochondrial alternative oxidase (AOX) [1, 2, 4].

Since trypanosomes escape elimination by the host immune system by varying their cell surface antigens, including repeated changes of the variant surface glycoprotein, production of effective vaccines against them is very difficult. For this reason, anti-trypanosomal chemotherapy is currently a major strategy against African trypanosomiasis. Since mammalian hosts do not possess AOX, it is a promising target for chemotherapy [11]. Indeed, we previously found that ascofuranone (AF), a specific inhibitor of AOX, has a strong trypanocidal effect [9]. However, trypanosome cells can convert glycerol-3-phosphate (G-3-P), which accumulates inside glycosomes when AOX is inhibited, into glycerol via a glycerol kinase (GK) reverse reaction with a coupling synthesis of ATP. Trypanosomes can survive by excreting glycerol out of glycosomes, but the net synthesis of ATP is decreased to one mole per mole of glucose. Therefore, for chemotherapy to be effective, it must target both GK and AOX [9, 15]. Indeed, in the case of Trypanosoma brucei, glycerol, which inhibits the GK reverse reaction in a product-inhibition manner, markedly enhanced the trypanocidal effect of AF [9, 15].

GXK has been purified from glycosomes of T. brucei bloodstream forms and used for a preliminary biochemical analysis and kinetic studies [7, 10]. Detailed kinetic studies of the GK reverse reaction have been performed with T. brucei recombinant GK proteins (rGK) [8, 13]. Although GKS have been identified and their forward activity—the conversion of glycerol into G-3-P at the expense of ATP—has been reported from various species, the physiological roles of the GK reverse reaction were reported only for trypanosome species.

In terms of African food problems, livestock infections by other trypanosome species, such as T. vivax and T. congolense, are much more significant than infections by T. brucei. However, few studies have examined the metabolic pathways of those species. Thus, although a similar respiratory system with that of T. brucei has been proposed for T. vivax and T. congolense, the presence of the GK molecule itself in those species has not been reported. In the present study, glycerol kinase cDNAs of T. vivax and T. congolense were cloned and compared with that of T. brucei. Then, the rGKS of the three species were expressed and purified. Kinetic studies of the rGK reverse reactions were subsequently performed by rapidly monitoring the produced ATP molecules. Furthermore, based on the obtained results, effective trypanocidal dosages of AF and glycerol were determined for each in vitro-cultured trypanosome species.
MATERIALS AND METHODS

Parasites: Bloodstream forms of T. brucei (ILTat1.4), T. vivax (IL1392) and T. congolense (IL1180) were used in this study. The strains were supplied by the International Live-

stock Research Institute and maintained in HMI-9 growth media [5] at 37°C.

cDNA cloning and plasmid preparation: The T. brucei

GK nucleotide sequence (GenBank accession number AF132295) was used to search for GKs in the T. vivax and T. congolense genome databases with the NCBI blast search program. GK cDNAs were amplified by PCR with the fol-

lowing primers: for T. brucei, 5'-atgaagtacgtcggatccattg-3' forward and 5'-ctacaactttgcccacttc-3' reverse; for T. congolense, 5'-atgaagtacggttagtcatggctg-3' forward and 5'-tataga gcttcacccggttcgatgg-3' reverse; and for T. vivax 5'-atgaagtacggttagtcatggctg-3' forward and 5'-ctacaaccgcgtcactc-3' reverse. Amplified cDNAs were cloned into the pET-15b expression vector (Novagen) so that the recombi-

nant GK proteins could be expressed in Escherichia coli cells.

Protein expression and purification: E. coli cells harboring rGK protein expression vectors were grown at 37°C, and expression was induced with 0.4 mM IPTG when the OD600 reached 0.5. Three hours after induction, cells were har-

vested and sonicated. The rGK was purified by using the MagneHis Protein Purification System (Promega) according to the manufacturer’s protocol.

Gel electrophoresis and western blotting: Proteins were separated on SDS-polycrylamide gels and transferred onto PVDF membranes that were probed with polyclonal antibod-

ies to rGK of T. brucei, as previously described [14].

Enzyme assay: The assay for the reverse reaction of rGK proteins was performed in 0.1 M MOPS buffer (pH 6.8) containing 1 mM EDTA, 5 mM MgSO4, 2 mM ATP-free ADP (Apollo Scientific) and 10 mM G-3-P (Sigma). The level of ATP synthesized by rGK was measured with a lumino-

meter; and luciferin was used as a substrate of luciferase together with ADP and G-3-P. The Km and Vmax values for ADP were determined at concentrations of ADP between 0.1 and 1 mM. The Km and Vmax values for G-3-P were determined at concentrations of G-3-P between 0.1 and 5 mM. To determine the kinetic parameters, 1 µg of recombinant protein was used for each assay. Each assay was performed at least four times in each point. The kinetic parameters were determined from the results of three inde-

pendent freshly prepared samples.

Combination trials of glycerol and ascofuranone for in vitro-cultured trypanosomes: Culture-adapted bloodstream forms of three strains, ILTat1.4, IL1392 and IL1180, were used. The in vitro-cultured cells were dispensed to each well of 96-well plates at the concentration of 10⁴ cells/well with AF and/or glycerol. Total assay volume of each well was 100 µl. The assays were performed with serially diluted AF (0–1 mM) for T. vivax, T. congolense and T. brucei in the presence of 0, 5 or 50 mM glycerol. For T. congolense, the assay was also performed with serially diluted glycerol (0–240 mM) in the presence of 4 nM AF. Mini-

mum necessary dosages of AF and glycerol that could elim-

inate each in vitro-cultured strain were determined 24 hr after the addition of AF and/or glycerol. For each trypano-

some species, the results were confirmed by three assays with independently prepared trypanosome cells.

Phylogenetic analysis: The phylogenetic relationships among full-length encoding regions of all aligned GK sequences were examined with the maximum likelihood method by using an exhaustive tree search with JTT-F+ model (phyML application [3]).

RESULTS

Cloning and characterization of the GK cDNAs of T. vivax and T. congolense: To better understand the energy metabolism systems of T. vivax and T. congolense, we cloned the full-length GK cDNAs of these two species by PCR (Fig. 1). Both of the cDNAs were 2092nt and had an open reading frame (ORF) of 512 amino acids; the calculated molecular masses were 56.4 kDa for T. vivax GK and 56.2 kDa for T. congolense GK. Both of the deduced amino acid sequences were highly similar to T. brucei GK (AF132295) (T. vivax, 82%; T. congolense, 89%). The nucleotide sequences were submitted to the DDBJ sequence database and are available under accession number AB377226 (T. vivax) and AB127973 (T. congolense).

Comparison of the GK sequences of T. vivax and T. congolense with those of other species: The deduced amino acid sequences of both GK cDNAs were aligned with those of other Trypanosoma species, Leishmania major, and E. coli. There were 158 residues that were conserved among all try-

panosomes and E. coli (data not shown). The amino acid residues for the binding sites of Mg2+, ADP and glycerol, which were determined by a structural study of E. coli GK [9], were highly conserved in the trypanosomatid Gks (Fig.

1).

In the phylogenetic analysis of GK molecules, the entire amino acid sequences of each GK were used. T. vivax and T. congolense were placed at a distance from each other. T. congolense GK was more closely related to T. brucei GK and T. gambiense GK, which were placed at the branch leading to the T. vivax GK as a sister group. This phylogenetic reconstruction was strongly supported by high bootstrap proportion (BP) values of greater than 99 (Fig. 2).

Expression and purification of recombinant glycerol kinase of T. vivax and T. congolense: In a previous study, trypanosome species were classified into three groups based on their metabolic products during glucose breakdown [12]. The glycerol-excretion levels of T. vivax and T. congolense were lower than that of T. brucei but higher than that of T. cruzi. Thus, T. vivax and T. congolense were categorized in the same intermediate glycerol-excretion group [12]. The results of our phylogenetic analysis of GK molecules, however, were inconsistent with the previous report; our analy-

sis indicated that the potential GK activities of T. vivax and T. congolense would be different from each other, even
though these species both excrete the same level of glycerol. To evaluate the hypothesis, His (6 X His)-tagged rGK of T. vivax, T. congolense and T. brucei were expressed in E. coli and purified by nickel particles. Equal levels of rGK expression were confirmed by SDS-PAGE (Fig. 3). The three rGKs possessed very similar molecular weights of approximately 50 kDa, which was consistent with their calculated molecular masses and confirmed by western blot analysis with trypanosome GK-specific antibody (data not shown).

Kinetic analysis of recombinant GK proteins from three trypanosome species: To analyze the kinetic properties of the rGK reverse reaction, we established a novel luciferase-linked method in which ATP molecules that were produced by the rGK reverse reaction with G-3-P and ADP as substrates were used for the subsequent reaction of luciferase, and the obtained luminescence was measured with a luminometer (Fig. 4A).

The calculated $K_m$ values of T. vivax, T. congolense and T. brucei by Lineweaver-Burk plot (Fig. 4B) are summarized in Table 1. The $V_{max}$ values of T. congolense rGK were 6.51 ± 2.14 µmol/min/mg for ADP and 0.19 ± 0.05 µmol/min/mg for G-3-P. These values are higher than the corresponding values for other species, suggesting that the GK reverse reaction activity of the T. congolense was higher than those of T. vivax and T. brucei. T. vivax rGK had the lowest $V_{max}$ values for both substrates; 2.37 ± 1.29 µmol/min/mg for ADP and 0.12 ± 0.00 µmol/min/mg for G-3-P. Thus, the GK reverse reaction activity of the T. vivax appeared to be lower than those of T. congolense and T. brucei.
In addition, the $K_m$ value of *T. congolense* GK for ADP was the lowest among the three species and that of *T. vivax* was highest. Similar results were obtained for the other substrate, G-3-P. These results supported our hypothesis that the GK activities of *T. vivax* and *T. congolense* were different at the enzymatic level.

**Comparison of the expression levels of GK proteins in the bloodstream forms of the three trypanosome species:** To compare the GK protein expression levels in the bloodstream forms of *T. vivax*, *T. congolense* and *T. brucei* species, we performed a western blot analysis with specific anti-GK antibody (Fig. 5A), and the levels of GK protein expression were visualized as a relative intensity chart (Fig. 5B). While the GK proteins were detected at a similar molecular weight size as a single band, their expression levels were different. The expression levels of GK proteins in *T. congolense* and *T. brucei* were similar, but the expression level in *T. vivax* was lower than the others (less than 20%).

**In vitro combination trials of AF and glycerol for each trypanosome species:** We previously demonstrated that glycerol, the product of the GK reverse reaction, remarkably enhanced the trypanocidal effect of AF in *T. brucei*-infected mice; oral treatment with 100 mg/kg AF cured *T. brucei*-infected mice when it was combined with 3 g/kg glycerol [15]. For *T. vivax*, oral treatment of 100 mg/kg AF could cure *T. vivax*-infected mice without glycerol [16]. However, for *T. congolense*, oral treatment with 300 mg/kg AF and 3 g/kg glycerol using the same protocol with Yabu et al. [15] could not cure *T. congolense*-infected mice, and the trypanocidal effect of 300 mg/kg AF was not significantly enhanced by 3 g/kg glycerol (Suzuki et al. unpublished data).

In the present study, we demonstrated that the enzymatic properties and expression levels of GKS were different among *T. vivax*, *T. congolense* and *T. brucei*. Although similar levels of glycerol excretion had been previously reported for the bloodstream forms of *T. vivax* and *T. congolense*, the GK activity seemed to be higher in *T. congolense* than in *T. vivax*. Therefore, we hypothesized that an increased amount of glycerol would enhance the anti-*T. congolense* effect of AF. To evaluate this hypothesis, we performed combination trials of AF and glycerol towards cultured *T. vivax*, *T. congolense* and *T. brucei*. We found that 5 mM glycerol significantly enhanced the trypanocidal effect of AF for *T. congolense* (Table 2; a minimum necessary concentration of AF that could eliminate *T. congolense* growth was decreased from 250 nM without glycerol to 8 nM with 5 mM glycerol), but its effect on *T. vivax* was relatively weak (Table 2; a minimum necessary concentration of AF that could eliminate *T. vivax* growth was decreased from 125 nM without glycerol to 15 nM with 5 mM glycerol). Furthermore, an increased concentration of glycerol from 5 mM to 50 mM caused additional enhancement of the trypanocidal effect of AF for *T. congolense* (Table 2; a minimum necessary concentration of AF that could eliminate *T. congolense* growth was decreased from 8 nM with 5 mM glycerol to 4 nM with 50 nM glycerol), while it caused little enhancement for *T. vivax* (Table 2).

These results indicate that an increased glycerol concentration enhanced the trypanocidal effect of AF for *T. congolense* in a dose-dependent manner. Since 4 nM AF caused

![Fig. 2. Unrooted tree based on GK protein phylogeny. The best tree of the maximum likelihood method is shown. The entire amino acid sequences of each GK were used in the analysis. Bootstrap proportion values are shown at the internal branches. The bar denotes 0.1 substitutions per site.](image-url)

![Fig. 3. Expression and purification of recombinant GK proteins of *T. brucei*, *T. vivax* and *T. congolense*. Purified rGK proteins of *T. brucei*, *T. vivax* and *T. congolense* were subjected to SDS-polyacrylamide gel electrophoresis (6 µg of purified recombinant protein per lane). A single band of approximately 50 kDa was detected in each lane.](image-url)
complete elimination of *T. congolense* growth in the presence of 50 mM glycerol, the minimum effective concentration of glycerol for the elimination of *T. congolense* growth was further determined by serial dilution of glycerol in the presence of the same AF concentration. Resultantly, 30 mM glycerol could completely eliminate the growth of cultured *T. congolense* cells in the presence of 4 nM AF.

**DISCUSSION**

Since no physiological function of GK reverse reaction has been reported for mammalian hosts, the unique reverse reaction of GK in trypanosomes is a crucial target for effective chemotherapy together with AOX [9, 15]. However, few studies have examined the metabolic pathways of the *T. vivax* and *T. congolense* species, which cause livestock infections that are severely impacting the food shortage in Africa. In the present study, we focused on the GK molecules of *T. vivax* and *T. congolense* and compared their kinetic properties in the reverse reaction and their expression levels in the bloodstream forms with those of *T. brucei*. Then, we determined the minimum necessary dosages of AF and glycerol to eliminate the growth of those *in vitro*-cultured trypanosome species.

The amino acid residues that are predicted to be necessary for the binding with Mg\(^{2+}\), glycerol and ADP were conserved in GKs among *T. vivax, T. congolense* and *T. brucei* (Fig. 1). However, the kinetic properties of the three rGK molecules were quite different (Table 1), though removing His-tags might help obtain more precise kinetic properties of them. Thus, the enzymatic properties of GK molecules might be determined by undefined regulating domain(s) in the molecule: non-conserved regions could be important for the accessibility of the substrate [6, 14].
In the previous study that classified trypanosome species by their end products during glucose breakdown, both *T. vivax* and *T. congolense* were classified in the same intermediate glycerol-excretion group [12]. However, according to the phylogenetic analysis of GK molecules in the present study, *T. congolense* GK is closer to *T. brucei* GK, which was previously classified in the high glycerol-excretion group, than to *T. vivax* GK (Fig. 2). Moreover, the cellular GK activity in *T. vivax* appears to be lower than that in *T. congolense* GK because of its higher $K_m$ values to substrates and its lower expression level in the cell. Thus, the glycerol-excretion level also appears to be different between *T. vivax* and *T. congolense*. This inconsistency in the two studies may be explained by environmental factors, such as oxygen concentration and temperature that may affect the metabolic processes in each type of trypanosome. Alternatively, glycolysis may occur at a more rapid rate in *T. congolense* cells than in *T. vivax* cells under normal condition in which AOX is not inhibited. Resultantly less amount of G-3-P might accumulate in glycosomes of *T. congolense* cells than *T. vivax* cells under normal condition and thus glycerol-excretion levels may not be significantly different between those species regardless of their different GK properties. In either case, the energy metabolic system including GK and AOX of each trypanosome species should be elucidated in depth to develop effective chemotherapies against each trypanosome species.

An increased glycerol concentration enhanced the trypanocidal effect of AF against cultured *T. congolense* in a dose-dependent manner. Thirty mM glycerol caused complete growth-elimination of cultured *T. congolense* cells in the presence of 4 nM AF. Thus, for the treatment of *T. congolense*-infected animals, increasing the amount of glycerol may be the first choice for treatment together with AF. However, an oral treatment of 300 mg/kg AF and 3 g/kg glycerol could not cure *T. congolense*-infected mice (Suzuki et al. unpublished data). Since 3 g/kg glycerol is a relatively large amount for oral administration, it may be impractical to increase the dosage of glycerol for oral treatment. Thus a novel glycerol-administration route other than oral treatment should be developed. Alternatively, the development of a GK-specific inhibitor would be important for the practical use of AF to treat *T. congolense*-infected cattle. We anticipate that our rapid assay system of reverse GK activity will be a valuable method for high-throughput screening of GK reverse reaction-specific inhibitors.

The relatively lower concentration of AF (125 nM) caused complete growth-elimination of cultured *T. vivax* cells without glycerol (Table 2). In addition, an increased concentration of glycerol from 5 mM to 50 mM did not enhance the trypanocidal effect of AF against cultured *T. vivax* cells (Table 2). Thus the necessary dosage of glycerol is considered to be low for the treatment of *T. vivax*-infected animals together with AF. Indeed as mentioned above, oral treatment of 100 mg/kg AF could cure *T. vivax*-infected mice without glycerol [16].

![Fig. 5. Expression levels of GK proteins in bloodstream forms of three trypanosome species. A. Whole cell extracts (5 µg) of *T. brucei*, *T. vivax* and *T. congolense* bloodstream forms were separated by electrophoresis and transferred onto a PVDF membrane that was probed with 2000-fold diluted anti-*T. brucei* rGK sera. B. The relative intensity of each band (A) is represented in the bar graph.](image-url)

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### Table 1. Enzymatic properties of recombinant GK proteins

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<tr>
<th>Species</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (µmol·min⁻¹·mg⁻¹)</th>
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<tr>
<td></td>
<td>ADP</td>
<td>G-3-P</td>
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<tr>
<td><em>T. brucei</em></td>
<td>0.37 ± 0.07</td>
<td>1.13 ± 0.32</td>
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<tr>
<td><em>T. congolense</em></td>
<td>0.24 ± 0.04</td>
<td>1.09 ± 0.32</td>
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<tr>
<td><em>T. vivax</em></td>
<td>0.69 ± 0.24</td>
<td>1.37 ± 0.17</td>
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a), b) Values were determined by three independent experiments. c) G-3-P; L-glycerol 3-phosphate.
Finally, obtained profiles for GK molecules may not directly correspond with those in vivo trypanosome cells, since we used culture-adapted trypanosome strains in the present study. Thus analysis using isolated trypanosomes from infected animals would be important for further validation of GK molecules as a combined target for chemotherapy.

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