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# The kinetic properties of hexokinases in African trypanosomes of the subgenus *Trypanozoon* match the blood glucose levels of mammal hosts

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ABSTRACT

We hypothesize that the hexokinases of trypanosomes of the subgenus *Trypanozoon* match the blood glucose levels of hosts. We studied the kinetic properties of purified hexokinase in *T. equiperdum* (specific activity = 302 U/mg), and compare with other members of *Trypanozoon*. With ATP ($K_m$ = 104.7 μM) as phosphate donor, hexokinase catalyzes the phosphorylation of glucose ($K_m$ = 24.9 μM) and mannose ($K_m$ = 8.8 μM). With respect to glucose, mannose and inorganic pyrophosphate respectively are a competitive, and a mixed inhibitor of hexokinase. With respect to ATP, both are mixed inhibitors of this enzyme. In *T. equiperdum*, hexokinase shows a high affinity for glucose. Pleomorphism—transformation of trypanosomes from a multiplicative to a non-multiplicative form—results in a self-limited growth stabilizing glucose consumption. It delays the death of the host, thus prolonging its exposure to tsetse flies. When glucose levels descend, top-down regulation allows trypanosomes to survive through the expression of alternative metabolic pathways. It accelerates the death of the host, but helps trypanosome density to increase enough to ensure transmission without tsetse flies. Pleomorphism, and a hexokinase with a high affinity for glucose, are two main adaptive traits of *T. b. brucei*. The latter trait, and a strong top-down regulation, are two main adaptive traits of *T. equiperdum*. For trypanosomes living in glucose-rich blood, a hexokinase with a high affinity for glucose would unnecessarily harm hosts. This may explain why the human parasites, *T. b. gambiense* and *T. b. rhodesiense*, possess hexokinases with a low affinity for glucose.

## 1. Introduction

Adaptations are often evident in enzymes that are upstream in a metabolic pathway, or that are in the branching points of metabolic pathways (Wright and Rausher, 2010; Olson-Manning et al., 2013). Hexokinase is a prime example of how both conditions can be met. It is the glucose-phosphorylating enzyme, thus it initiates both the glycolysis pathway, leading to ATP production (main function), and the pentose phosphate pathway, leading to NADPH production; it is also the mannose-phosphorylating enzyme, thus it initiates the sugar nucleotide biosynthesis pathway, leading to glycoprotein production (Alton et al., 1998; Cárdenas et al., 1998; Sharma et al., 2014).

African trypanosomes of the subgenus *Trypanozoon* include five species and subspecies that infect mammals. *T. brucei brucei*, occurring throughout Sub-Saharan Africa, and *T. b. rhodesiense*, occurring in eastern and southern Africa, cause nagana, mainly in ruminants. In addition, *T. b. rhodesiense* causes acute sleeping sickness in humans (Katunguka-Rwakishaya, 1996; Moloo et al., 1999; Van den Bossche et al., 2005; Anderson et al., 2011; Majekodunmi et al., 2013; Franco et al., 2014). *T. b. gambiense*, occurring in western and central Africa, causes chronic sleeping sickness in humans: it is responsible for 98% of the cases of human tripanosomiasis in Sub-Saharan Africa; wild mammals are minor reservoirs (Franco et al., 2014). *T. evansi* and *T. equiperdum*, which are widespread across the tropics and subtropics of the world, respectively cause surra and dourine in domestic and wild mammals, but some cases of human infection are known (Zablotskij et al., 2003; Desquesnes et al., 2013).

The three subspecies of *T. brucei* are termed pleomorphic because they exist as “slender” (asexually multiplicative) and “stumpy” (non-multiplicative) forms in mammalian blood, and as a procyclic (sexual) form in the endemic Sub-Saharan tsetse flies, which are their vectors (Franco et al., 2014). *T. evansi* and *T. equiperdum* are termed monomorphic because they exist only as the “slender” form in mammalian blood. *T. evansi* is transmitted mechanically in blood micro-volumes carried in the mouthparts of several genera of hematophagous flies, whereas *T. equiperdum* is transmitted as a venereal disease without the participation of insect vectors (Zablotskij et al., 2003; Desquesnes et al., 2013). Lack of dependence on tsetse flies may have been a crucial trait

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for trypanosomes of the subgenus *Trypanozoon* to spread out of their native Sub-Saharan Africa (Lun and Desser, 1995).

To obtain energy from host blood glucose, "slender" trypanosomes of the subgenus *Trypanozoon* are fully dependent on their glycolytic capability (Marshall, 1948; Ryley, 1956; Grant and Fulton, 1957; Bringaud et al., 2006; Moreno et al., 2015; Moreno and Nava, 2015). This fact has caused trypanosomal glycolytic enzymes to be the subject of much attention as potential targets for chemotherapeutic drugs (Bakker et al., 1997; Bakker et al., 1999a, 1999b; Kotsikorou et al., 2006; Chambers et al., 2008a). Knowledge of the variability in the kinetic properties of hexokinases within and among members of *Trypanozoon* is limited. In particular, nothing is known on this topic for the monomorphic members of the subgenus. This is surprising given that hexokinase is the most upstream enzyme in three major metabolic pathways, and given that the kinetic properties of enzymes are fundamental to understand the rate and specificity of metabolic processes.

Hexokinase is assumed to be similar in molecular weight in the bloodstream form of all members of *Trypanozoon* (Risby and Seed, 1969). In *T. brucei*, hexokinase is a hexamer, with a native molecular weight of 295 KDa, and a subunit molecular weight of 50.3 KDa (Misset et al., 1986). In *T. brucei* (both bloodstream and procyclic), hexokinase is composed of two forms, hexokinase 1 (HK1) and hexokinase 2 (HK2), sharing 97.7% of their amino acid identity (Colasante et al., 2006). HK1 uses glucose, mannose, and fructose as substrates, and is not inhibited by inorganic pyrophosphate (PPi). HK2 lacks activity, but its presence causes the inhibition of HK1 by PPi (Morris et al., 2006; Chambers et al., 2008b). Although hexokinase lacks a classic regulation, it has been proposed that HK2 might have a regulatory role (Nwagwu and Opperdoes, 1982; Chambers et al., 2008b).

In this study, we present the first data on the kinetic properties of the hexokinase of *T. equiperdum*, which we compare with similar data available for pleomorphic *T. brucei* (Seed and Baquero, 1965; Nwagwu and Opperdoes, 1982; Hara et al., 1997; Morris et al., 2006; Chambers et al., 2008b, 2008c). We seek to establish whether the kinetic properties of hexokinases in members of *Trypanozoon* are tuned to 'match' (Bar-Even et al., 2011) the blood glucose and mannose concentrations of their hosts.

## 2. Materials and methods

Experiments complied with Venezuelan ("Ley para la Protección de la Fauna Doméstica Libre y en Cautiverio", Articles 52–55), and European legislation (Directive 2010/63/EU) for the protection of research animals. Our stock of *T. equiperdum* (TeAp-N/D1, previously known as TEVA1, Perrone et al., 2009; referred to as *T. evansi* in previous studies, Sánchez et al., 2015), was originally isolated from cattle-ranch horses in the Venezuelan Llanos region (Moreno et al., 2013).

Parasites were cultured in rats, trypanosomes were purified from rats, and a glycosome-rich pellet was purified from trypanosomes as in a previous study (Moreno and Nava, 2015). For resuspension of the glycosome-rich pellet, 4 ml were used of pH 6.5 40 mM MOPS (3-(N-morpholino) propanesulfonic acid) buffer containing 2 mM $MgSO_4$, 50 mM NaCl, and a protease inhibiting cocktail (57 μM PMSF, 100 μM TLCK, 10 μM leupeptin and 1 mM benzamidine). Solubilization of the glycosome membranes was performed as in the previous study (Moreno and Nava, 2015). Centrifugation at 33,000*g* for 15 min of the mixture so obtained produced the supernatant used in subsequent procedures.

One unit (U) of enzymatic activity is defined as the quantity of enzyme required to catalyze the formation of 1 μmol/min of product. Spectrophotometrical assay (340 nm) of hexokinase was carried out at ~25 °C in a final volume of 1 ml (Misset and Opperdoes, 1984). In the case of glucose as a substrate, the catalysis was coupled to the glucose 6-phosphate dehydrogenase reaction, with $NADP^+$ reduction being detected. In the case of mannose, the catalysis was coupled to the pyruvate kinase and lactate deshydrogenase reactions, with NADH oxidation being detected.

Buffers used (Good et al., 1966) are: Buffer A, a pH 6.5 solution containing 50 mM Tris-HCl, 50 mM NaCl, 2 mM $MgSO_4$, and 1 mM dithiotreitol; MOPS buffer; Buffer B, a pH 6.5 40 mM MOPS buffer containing 2 mM $MgSO_4$; MES buffer; and Tris-HCl buffer.

### 2.1. Purification of hexokinase

#### 2.1.1. Protein precipitation

Solid ammonium sulfate was slowly added to the supernatant from 0% to 40% with constant stirring for 1 h. The mixture was allowed to stand at 4 °C for 1 h, then centrifuged at 10,000*g* for 20 min, obtaining a pellet that was discarded. The procedure was repeated, but with solid ammonium sulfate added from 40% to 80%, obtaining a new pellet, which was resuspended in Buffer A. The mixture was placed in a dialysis sack and kept at 4 °C overnight in 1 l of Buffer A, with constant stirring. Finally, hexokinase activity was measured in the dialyzed mixture.

#### 2.1.2. Ion exchange chromatography A

The hexokinase-active dialyzed mixture was passed through a phosphate cellulose column (2.5 × 20 cm) equilibrated with Buffer B, and 0.05 M NaCl. Then, the column was washed with Buffer B until the absorbance (280 nm) of the effluent dropped to near zero. A 0.05–0.40 M NaCl linear gradient in Buffer B (total volume, 400 ml), at a flow rate of 42 ml/h, was used to eluate hexokinase. Fractions (1 ml) coming out from the column were collected and assayed. Those with high hexokinase activity were pooled and diluted in Buffer B until a 0.12 M NaCl concentration was reached in the mixture.

#### 2.1.3. Ion exchange chromatography B

The above mixture was passed through a second cellulose phosphate column (2.5 × 20 cm) equilibrated with Buffer B, and 0.12 M NaCl. The column was washed with Buffer B as above. A 0.12–0.25 M NaCl linear gradient in Buffer B was used as above to eluate hexokinase. Fractions (1 ml) coming out from the column were collected and assayed. Those with high hexokinase activity were pooled, and concentrated using solid ammonium sulfate. The pellet so obtained was resuspended in Buffer A. The mixture was placed in a dialysis sack and kept at 4 °C overnight in 1 l of Buffer A, with constant stirring. Hexokinase activity was measured in the dialyzed mixture. Finally, the purification steps were analyzed by means of SDS-PAGE, and western blot analysis, to verify that hexokinase was being purified (Rosenberg, 2005).

### 2.2. Determination of pH and ionic strength optima of purified hexokinase

For the pH optimum, the following mixture of buffers was used: MES (pH 5.5–6.5); MOPS (pH 6.5–7.5); and Tris-HCl (pH 7.5–8.5); final concentration of 0.1 M for each buffer; 2 mM $MgSO_4$ were added to the buffer mixture. For the ionic strength optimum, pH 7.5 0.1 M MOPS buffers ranging from 0 to 0.5 M NaCl were used. Both assays involved the incubation of purified hexokinase for 5 min, with the reaction being subsequently triggered by adding its substrates (glucose, ATP).

### 2.3. Kinetic analysis and inhibition of purified hexokinase

The Michaelis-Menten constant, $K_m$, is the concentration of substrate to which an enzyme reaches 50% of its maximum catalytic activity. This percentage was chosen based on the fact that substrate concentrations in living tissues are typically far below the saturation point (Gunderson and Stillman, 2014). The $K_m$ constant is a measure of the relative affinity of an enzyme for its substrate: the lower the $K_m$ the higher the affinity, and viceversa (Fields et al., 2015). $K_m$ constants are dependent on cofactors, buffers, temperature, pH, and on whether they have been purified or not (Copeland, 2000; Ringe and Petsko, 2008; Bar-Even et al., 2011). Therefore, we refer to apparent $K_m$ constants,