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Hysteresis and positive cooperativity as possible regulatory mechanisms of *Trypanosoma cruzi* hexokinase activity

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ABSTRACT

In *Trypanosoma cruzi*, the causal agent of Chagas disease, the first six or seven steps of glycolysis are compartmentalized in glycosomes, which are authentic but specialized peroxisomes. Hexokinase (HK), the first enzyme in the glycolytic pathway, has been an important research object, particularly as a potential drug target. Here we present the results of a specific kinetics study of the native HK from *T. cruzi* epimastigotes; a sigmoidal behavior was apparent when the velocity of the reaction was determined as a function of the concentration of its substrates, glucose and ATP. This behavior was only observed at low enzyme concentration, while at high concentration classical Michaelis–Menten kinetics was displayed. The progress curve of the enzyme's activity displays a *lag* phase of which the length is dependent on the protein concentration, suggesting that HK is a hysteretic enzyme. The hysteretic behavior may be attributed to slow changes in the conformation of *T. cruzi* HK as a response to variations of glucose and ATP concentrations in the glycosomal matrix. Variations in HK's substrate concentrations within the glycosomes may be due to variations in the trypanosome's environment. The hysteretic and cooperative behavior of the enzyme may be a form of regulation by which the parasite can more readily adapt to these environmental changes, occurring within each of its hosts, or during the early phase of transition to a new host.

1. Introduction

Chagas disease affects 9 million people in Latin America and also a few hundreds of thousands of persons in some other countries around the world [1]. *Trypanosoma cruzi*, the causal agent of this potentially fatal disease, belongs to the protist group of Kinetoplastea. The parasite has three different stages within its life cycle. As it is transmitted cyclically between a mammal and an invertebrate host, it encounters diverse environments [2]. These parasites are characterized by the compartmentalization of some of their key metabolic pathways in peroxisome-like organelles named glycosomes. Specifically, the major part of glycolysis takes place within the glycosomes, including the first step in this catabolic route of glucose, catalyzed by hexokinase (HK) [3–5]. This enzyme has been considered a potential drug target, because it generates glucose 6-phosphate, the common substrate for glycolysis and the pentose-phosphate pathway, metabolic processes that are important to produce ATP and NADPH, respectively, for the cell. HK is expressed in all *T. cruzi* life-cycle stages [6–8].

HK has been biochemically characterized in many organisms, eukaryotes and prokaryotes, and for most of them, it has been reported that its activity is regulated by various metabolites through different mechanisms. The specific activity and kinetic behavior of HK has also been described both for *T. cruzi* and for some other Kinetoplastea, like *Trypanosoma brucei* and *Leishmania mexicana* [9–13]. Contrary to HK of many other organisms, the activity of the enzyme from *T. cruzi* and related parasites is not regulated by its products glucose 6-phosphate and ADP, but PPi was identified as having a regulatory effect on HK of trypanosomatids, although in each species in a different manner [10,12–14].

Furthermore, it has been shown that HKs of yeast and wheat germ display a hysteretic behavior [15–21]. These enzymes respond slowly (with regard to some of their kinetic characteristics) to a rapid change in the concentration of a ligand, either a substrate

Abbreviations: HK, hexokinase; PPi, inorganic pyrophosphate; GEF, glycosome-enriched fraction.

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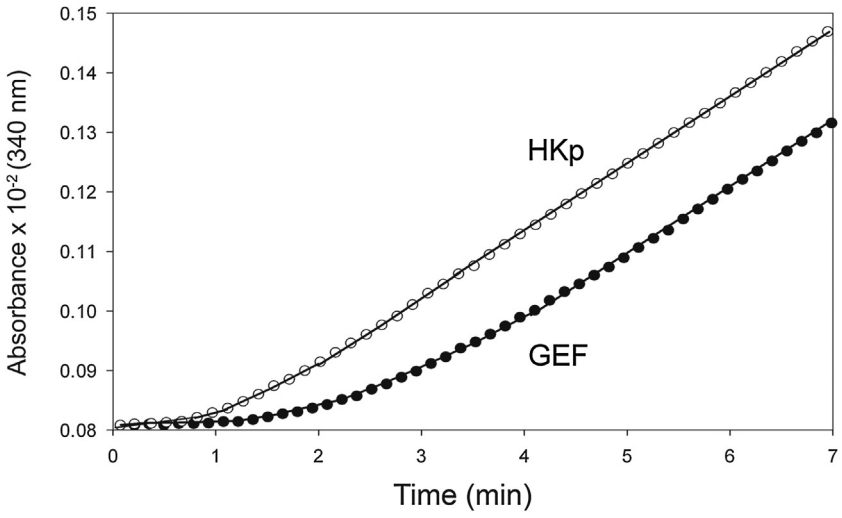

Fig. 1. The progress curve of *T. cruzi* hexokinase activity. The HK activity was monitored continuously and plotted as absorbance change *versus* time. Results obtained for two different HK samples assayed have been plotted, the purified native HK (A) and the glycosome-enriched fraction (GEF) (B), used at protein concentrations of 1 and 5 μg ml^{-1}, respectively.

or effector. Such slow changes, defined in terms of their rate relative to the overall catalytic reaction, result in a lag in the response of the enzyme to the changed ligand level. Hysteresis can be caused by conformational changes, dissociation–addition reactions or a displacement of one substrate by another [22,23]. The substrate acts to either shift the equilibrium toward a pre-existing additional state or to induce the presence of a new one. Hysteresis can occur in both oligomeric and monomeric enzymes, producing a transition that happens slowly at the timescale of the measurements. It has been proposed that slow transitions in conformation may play a more dynamic role in the function of enzymes than often appreciated, underlining the complexity of motions in enzyme structures at different timescales [22,24]. The possible relation between hysteresis of enzymes and their role in the regulation of complex metabolic processes has been interpreted in terms of the fact that the slow response of the hysteretic enzyme to changes in ligand levels will lead to time-dependent buffering of some metabolites, and the importance of this phenomenon for distributing fluxes through pathways that utilize common intermediates or that contain multiple branch points [22,24].

In this paper, we give evidence of the hysteretic behavior of *T. cruzi*'s HK, as a possible regulatory mechanism. This enzyme shows a slow kinetic response as a consequence of variations in glucose, ATP and enzyme concentrations and a cooperative behavior associated to enzyme concentrations. This phenomenon, defined as a delay of the product accumulation rate in time, was present in each of the enzyme samples analyzed, both purified native HK preparations and a glycosome-enriched cell fraction. As such hysteretic characteristic has been reported for enzymes that have important roles in buffering fluctuations of substrate levels, we propose that, in the case of *T. cruzi* HK, it might be associated to a regulatory effect inherent to the parasite's metabolism.

2. Materials and methods

2.1. Parasites

Epimastigotes of *T. cruzi* strain EP (isolated from a patient with Chagas disease in its acute phase in 1967) were cultured at 28 °C in LIT medium (liver infusion-tryptose) supplemented with 5% inactivated fetal bovine serum as previously described [25]. Parasites were harvested in the exponential phase of growth, at an optical density at 600 nm of 0.8.

2.2. Purification of the T. cruzi hexokinase from a glycosome-enriched fraction obtained by subcellular fractionation

Subcellular fractionation by differential centrifugation was performed after abrasion of a dense trypanosome suspension with silicon carbide as described previously [26], providing the small granular pellet that is a 'glycosome-enriched fraction' (GEF). Purification of native HK was performed as reported previously [12], yielding the enzyme in homogeneously pure and active state.

2.3. Enzymatic assays and data analysis

HK activity was assayed by measuring the formation of NADPH by coupling the phosphorylation of glucose to the reduction of $NADP^+$ by glucose-6-phosphate dehydrogenase (G6PDH), at 340 nm and 25 °C using a Hewlett-Packard 8452 diode array spectrophotometer. The assay was performed in a 1 ml cuvette containing 240 mM triethanolamine, pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 4 mM D-glucose, 0.72 mM $NADP^+$ and 2 U ml^{-1} G6PDH (type XXIV from *Leuconostoc mesenteroides*, Sigma). The components in the cuvette were mixed for 30 s prior to the registration of the enzyme activity. The K_m values for glucose and ATP were determined by varying the concentration of glucose and ATP each between 5 and 400 μM. For these measurements two fractions were used, purified HK (HKp) and the GEF, each at three different protein concentrations: 1, 6 and 17 μg ml^{-1} (HKp), and 2, 6 and 12 μg ml^{-1} (GEF). The data of the substrate-dependent HK activity were fitted by a curve given by the Hill equation using nonlinear regression [27]. The *lag* phase in the product appearance was described as follows by the Neet equation [23]:

$$V(t) = \mathrm{Vss} + (\mathrm{Vi} - \mathrm{Vss})e^{-kt}$$

where $V(t)$ is the velocity at any given time, Vi the initial velocity, Vss the velocity at steady state (after the *lag* phase), and k the apparent rate constant for the transition. The lag period (τ) was determined according to the method of Neet and Ainslie [23], as $1/k$. The effect of hydrogen ion concentrations on the *lag* phase time was measured using 25 mM MES (2-(N-morpholino) ethanesulfonic acid) and Tris–HCl buffer, varying the pH between the values