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Hexokinase regulation in the hepatopancreas and foot muscle of the anoxia-tolerant marine mollusc, *Littorina littorea*

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ABSTRACT

Hexokinase from the hepatopancreas and foot muscle of *Littorina littorea* undergoes stable modification of its kinetic and structural properties in response to prolonged oxygen deprivation. In the hepatopancreas, a reduction in the K_m glucose for hexokinase from the anoxic animal suggests a more active enzyme form during anoxia. Conversely, in the foot muscle, an increase in K_m ATP and a decrease in V_{max} for anoxic snail hexokinase were consistent with a less active enzyme form during anoxia. In either case, the molecular basis for the stable modification of hexokinase kinetics is reversible phosphorylation. The activation of endogenous PKC and AMPK increased the K_m glucose for anoxic hepatopancreas hexokinase to a value that was similar to the control K_m glucose. Alternatively, stimulation of endogenous PKA, PKG, and CamK for control foot muscle hexokinase increased the K_m ATP to a value similar to that seen for the anoxic enzyme form. In both tissues, activation of endogenous phosphatases reversed the effects of protein kinases. Dephosphorylation and activation of hepatopancreas hexokinase during anoxia may allow for increased shunting of glucose-6-phosphate into the pentose phosphate pathway, thereby producing reducing equivalents of NADPH needed for antioxidant defense upon tissue re-oxygenation. Conversely, phosphorylation and inhibition of foot muscle hexokinase during anoxia may reflect the decreased need for glucose oxidation during hypometabolism.

1. Introduction

Hexokinase (HK; E.C. 2.7.1.1) is a critical enzyme within all cells that catalyzes the first reaction of glycolysis:

$$D-\text{glucose} + ATP \rightarrow D-\text{glucose}-6-\text{phosphate} + ADP$$

The phosphorylation of glucose, a key energy source, decreases the intracellular pool of free glucose, which permits facilitated glucose entry into cells. By catalyzing the ATP-dependent phosphorylation of glucose to yield glucose-6-phosphate (G6P), hexokinase controls the first committed step of glucose metabolism and initiates all major pathways of glucose utilization. G6P is a key branch-point metabolite since it can enter a variety of pathways such as glycolysis, the pentose phosphate pathway (PPP), as well as glycogen synthesis.

The integral role of hexokinase in carbohydrate matabolism makes this enzyme a potentially important regulatory point within the cell, especially within organisms that experience extreme environmental stress. One such organism, *Littorina littorea*, are intertidal marine snails that commonly experience extreme oxygen deprivation and require a drastic reorganization of cellular processes, including metabolism, in order to survive. Low oxygen conditions result from low tides as well as environmental factors that cause the marine snails to close their operculum (shell opening), which include predation, high salinity, and the presence of water soluble toxins (Truchot and Duhamel-Jouve, 1980; de Zwaan and Putzer, 1985). This high level of oxygen deprivation necessitates drastic physiological and metabolic changes within the snail to survive without oxygen. These changes include a decrease in metabolic rate to <10% of the normoxic rate and a dramatic reorganization of ATP utilization to shutdown non-essential processes and maintain those that are critical to survival (de Zwaan et al., 1991; van den Thillart et al., 1992; Storey, 1993; Churchill and Storey, 1996). Previous studies on *L. littorea* carbohydrate metabolism indicate that numerous enzymes involved in glucose oxidation are converted to a less active form by reversible phosphorylation (Russell and Storey, 1995). However, little is known about the regulation of hexokinase, and this study aims at determining the kinetic and regulatory properties of this enzyme within the foot muscle and hepatopancreas of *L. littorea*.

2. Methods and materials

2.1. Animals

Marine snails, *L. littorea*, originated from Nova Scotia, Canada, and were purchased from a local supplier in Ottawa on May 2007. These snails were submerged in a container with 30 L of aerated, full-strength seawater (1000 mOsm/L made using Instant Ocean Sea Salt; salinity confirmed with a buoyancy meter). The container was held at 9 °C in

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an incubator with constant darkness. No food was given to the snails, but the water was changed once every 2 days and dead snails were removed. Experiments to gather aerobic control (sampled directly from the seawater) and 24 h anoxic snails were conducted following a 7-day acclimation period.

For anoxia exposure, 25 snails were placed in each of several sealed jars that possessed two gas ports. A small amount of deoxygenated (1 cm deep) water was placed in the bottom of the containers and they were all held on ice while being bubbled with N_2 gas for 30 min. Following this period, the lids for the containers were tightened and sealed with parafilm and a N_2 gas line was connected to one of the ports. N_2 gas was continuously bubbled through the water in the jars for 20 min, after which the N_2 line was removed and the ports closed. The containers were then returned to the 9 °C incubator for a 24 h anoxia exposure.

For sampling of anoxic snails, a container of snails was removed from the incubator, placed on ice and the nitrogen gas tubing was reattached to one of the ports and the container was continually gassed. Snails were then quickly removed from the jar and dissected immediately. A hammer was used to crack and remove the outer shell and then hepatopancreas and foot muscle were excised and frozen in liquid nitrogen. Tissues were stored at −80 °C.

2.2. Sample preparation

Frozen samples of both foot muscle and hepatopancreas were homogenized 1:5 w:v in cold homogenization buffer containing 50 mM Tris–HCl, pH 7.5, 10% v:v glycerol, 10 mM 2-mercaptoethanol, 2.5 mM EDTA, and 2.5 mM EGTA. The buffer components were optimized with respect to the effects of sodium fluoride (NaF) and β-glycerol phosphate (β-GP) on enzyme activity and, after optimization, 25 mM NaF was also included in the homogenization buffer. A few crystals of phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, were added at the time of homogenization. Homogenates were centrifuged at 13,500*g* for 30 min at 4 °C, and the supernatant was decanted and held on ice.

2.3. Sephadex G-50 filtration of crude extracts

To properly assess HK properties, low molecular weight metabolites and ions needed to be removed from the enzyme preparation. This was achieved by Sephadex G-50 gel filtration. A 5-cm column of Sephadex G-50 in a 5-mL syringe barrel was equilibrated in homogenization buffer and centrifuged at 500*g* in a bench-top centrifuge for 2 min to remove excess buffer which was discarded. Then a 500-μL aliquot of supernatant was layered on top, the column was centrifuged again, as above; and the protein-containing eluant was removed and stored on ice.

2.3.1. Hexokinase assay and Kinetics

Hexokinase activity was assayed at 340 nm using a Thermo Labsystems Multiskan Spectrum Microplate Spectrophotometer. Optimum assay conditions for both tissues were 2 mM Mg · ATP (with 3.2 mM additional $MgCl_2$), 2 mM glucose, 0.5 mM NAD^+ and 0.25 U of G6PDH (from *Leuconostoc mesenteroides* recombinant, expressed in *Escherichia coli*; Sigma) coupling enzyme in 50 mM Tris–HCl assay buffer, pH 7.5. Reactions were initiated by adding 30 μL of enzyme extract to a 200-μL total reaction volume in the microplate well. Microplate Analysis (MPA) and Kinetics 3.51 computer programs (Brooks, 1992, 1994) were used to analyze the data. One unit of HK activity is defined as the amount of enzyme that produces 1 μmol NADH per minute at 22 °C (at room temperature). Activities are reported in mU/mg soluble protein. Soluble protein was quantified using the Coomassie blue dye binding method and the Bio-Rad prepared reagent with a standard curve of bovine serum albumin.

Effects of various ions on HK activity were assessed using Sephadex G-50 filtered extracts of hepatopancreas and foot muscle from control and 24 h anoxic snails. NaCl, KCl, and NH_4Cl were tested at concentrations up to 2 M using standard assay conditions at pH 8.0.

Urea denaturation of hepatopancreas and foot muscle HK was evaluated using crude tissue extracts that were incubated with urea at concentrations from 0 to 2 M in homogenization buffer, pH 8.0, for 24 h at 22 °C. After incubation, remaining HK activity was measured under standard assay conditions.

2.4. DEAE-Sephadex elution profiles

DEAE-Sephadex columns (5 cm × 1 cm) were equilibrated in column buffer (25 mM Tris–HCl, 5% v:v glycerol, 5 mM 2-mercaptoethanol, 12.5 mM NaF, 1.25 mM EDTA, 1.25 mM EGTA, pH 7.0). A 300-μL aliquot of supernatant was added to the column, which was then washed with 10 mL of the same buffer to remove unbound proteins. Then the column was eluted with a linear gradient of 0–2 M KCl in 40 mL of column buffer. Extracts from control and 24 h anoxic hepatopancreas tissues were run separately; however, foot muscle control and anoxic tissue were combined and run simultaneously on the same DEAE column. Approximately 60 fractions were collected with a Gilson FC203B fraction collector. HK activity was assayed under optimal assay conditions using 50 μL from each fraction.

2.5. Western blot analysis of HK phosphorylation state

L. littorea foot muscle and hepatopancreas HK was partially purified using affinity chromatography. More specifically, 1 mL of crude foot muscle or hepatopancreas extracts (prepared as described above) were added on top of a blue-agarose column (3 cm × 1 cm, h × d) pre-equilibrated in the column buffer. HK was then eluted with a 0–1 M KCl gradient. The fraction with the highest activity was then kept on ice until they could be prepared to be run on SDS resolving gels.

Partially purified control and anoxic HK samples were mixed 1:1 (v:v) with SDS loading buffer (100 mM Tris buffer, pH 6.8, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, and 10% v:v 2-mercaptoethanol) and boiled for 5 min, cooled on ice, and frozen at −20 °C.

SDS resolving gels (8% v/v acrylamide, 400 mM Tris, pH 8.8, 0.1% w/v SDS, 0.2% w/v ammonium persulfate (APS), 0.04% v/v TEMED) were prepared with a 5% stacking gel (5% acrylamide, 190 mM Tris, pH 6.8, 0.1% w/v SDS, 0.15% w/v APS, 0.1% v/v TEMED). Partially purified HK samples were loaded onto these gels and separated electrophoretically in SDS–PAGE running buffer (25 mM Tris-base, 190 mM glycine, and 0.1% w/v SDS) at 180 V for 45 min. Spectra™ Multicolor Broad Range Protein Ladder (3 μL) was added to one lane of every gel to act as molecular weight markers. Following electrophoresis, proteins were electroblotted onto polyvinylidienedifluoride (PVDF) membranes (Millipore) by wet transfer. Electroblotting was performed at room temperature for 1.5 h at 160 mA under transfer buffer (25 mM Tris, pH 8.5, 192 mM glycine, and 20% v/v methanol).

Following protein transfer, PVDF membranes were incubated overnight at 4 °C with a phospho-serine primary antibody (Calbiochem) diluted at 1:1000 in Tris-buffered saline solution with Tween-20 (TBST; 20 mM Tris-base, 140 mM NaCl, 0.05% Tween-20) with a small amount of sodium azide added. After the overnight incubation, membranes were washed with TBST three times for 5 min each, which was followed by incubation with the anti-rabbit secondary antibody conjugated with horseradish peroxidase (Bioshop Canada) at a dilution of 1:4000 in TBST. Membranes were incubated at room temperature for 30 min and then washed three times for 5 min each time with ddH_2O. A signal was then detected using enzymatic chemiluminescence (ECL), initiated by the addition of 600 μL of hydrogen peroxide and 600 μL of luminol reagent to the membrane's surface for several seconds. The mixture was then poured off and the chemiluminescence was detected ChemiGeniusBioimaging System (Syngene, MD, USA).