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Glycolytic adjustments in tissues of frog *Rana ridibunda* and land snail *Helix lucorum* during seasonal hibernation

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ABSTRACT

The present work aimed to contribute to the understanding of the adaptation of the glycolytic pathway in tissues of frog *Rana ridibunda* and land snail species *Helix lucorum* during seasonal hibernation. Moreover responses of glycolytic enzymes from cold acclimated *R. ridibunda* and *H. lucorum* were studied as well. The drop in Po_2 in the blood of hibernated frogs and land snails indicated lower oxygen consumption and a decrease in their metabolic rate. The activities of glycolytic enzymes indicated that hibernation had a differential effect on the glycolysis in the two species studied and also in the tissues of the same species. The activity of L-LDH decreased significantly in the skeletal muscle and heart of hibernated *R. ridibunda* indicating a low glycolytic potential. Similar biochemical responses were observed in the same tissues during cold acclimation. The continuous increase in the activities of glycolytic enzymes studied, except for HK, might indicate a compensation for the impacts of low temperature on the enzymatic activities. In contrast to *R. ridibunda*, the activities of the enzymes increased and remained at higher levels than those of the prehibernation controls indicating maintenance of glycolytic potential in the tissues of hibernating land snails.

1. Introduction

During winter in temperate regions, hibernators as water frogs and snails have to deal with low and sometimes subzero temperatures. Metabolic depression is a common response of water frogs and land snails to low temperature in general and as well as during hibernation (Storey and Storey, 1990; Boutilier et al., 1997; Guppy and Withers, 1999). The biochemical and physiological responses leading to a hypometabolic state have been studied extensively in freezing tolerant frog species (Storey and Storey, 1986, 1987, 1988). It has been shown recently that the metabolic depression in frogs hibernating under hypoxic water is accompanied by a significant decrease in the aerobic capacity of their skeletal muscle, as indicated by a reduction in the activity of key enzymes of the TCA cycle and of the electron-transport chain (St-Pierre and Boutilier, 2001). The LDH activity of the skeletal muscle of overwintering frogs was also much lower than in prehibernation controls, supporting the idea of a decreased flux through the glycolytic pathway during hypoxic hibernation (i.e. the so-called 'reversed' Pasteur effect; Hochachka and Somero, 2002; Donohoe and Boutilier, 1998). Similarly, hibernation in land snails during winter is characterized by a marked decrease in their metabolic rate. However, compared with frogs, little is known about the biochemical and physiological responses of *Helix lucorum* to hibernation.

Several works have emphasized that metabolic depression can have quite separate and distinctly different effects on different tissues. As reported by Flanigan et al. (1991), metabolic depression at the whole-animal level might not be reflected equally in all tissues. For example while the *in vitro* respiration rate of intact skeletal muscle from aestivating frogs is reduced compared with controls, no such reduction occurs in the intestine, liver, skin or fat (Flanigan et al., 1991). Moreover, a reduction in the activities of glycolytic enzymes has been observed during long-term aestivation in several species of terrestrial snails and the frog *Neobatrachus pelobatoides*. The activities of hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and lactate dehydrogenase (LDH) were reduced in the foot muscle of aestivating snails compared with controls (Brooks and Storey, 1990). Conversely, in the kidney, heart and hepatopancreas of aestivating snails no reduction in the activity of LDH was found (Stuart et al., 1998b). The liver of aestivating frogs had lower activities of aldolase and glyceraldehyde-3-phosphate dehydrogenase compared with controls, but the activities of both

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these enzymes remained unchanged during aestivation in the ventricle, gastrocnemius and brain (Flanigan et al., 1990).

In the present study, we sought to determine whether changes in the activities of several key enzymes of the glycolytic pathway are down-regulated during hibernation in *Rana ridibunda* and *H. lucorum*. In the central and south of Europe the *Rana esculenta* complex occurs (Graf and Polls-Pelaz, 1989). The latter involves the parental species *R. ridibunda* Pallas 1771, *Rana lessonae* Camerano 1882 and the hybridogen *R. esculenta* Linnaeus 1758 (Berger, 1967, 1968). Compared to the other two frog species, *R. ridibunda* is poorly tolerant to hypoxia (Turner and Nopp, 1979; Plenet et al., 2000a,b), less tolerant to freezing, while it has no cryoprotective system (Voituron et al., 2003, 2005). Despite the differences in the habitat and wintering behavior among the three frog species mentioned above little is known about their physiological and biochemical responses during seasonal hibernation. In the present work we studied the glycolytic adjustment of *R. ridibunda*. Additionally we studied the glycolytic adjustment of land snail species. The species used, *H. lucorum*, is found throughout the mainland of Greece especially in the northern regions where its ecology and biology have been studied (Staikou et al., 1988). In the north of Greece snails of this species hibernate during winter. Hibernation starts at the beginning of November and is terminated by the end of March.

2. Materials and methods

2.1. Animals and experimental design

Adult *R. ridibunda* frogs were collected from the surroundings of Thessaloniki in October 2006. They were transferred to the laboratory within the same day and they were put into plastic tanks measuring 50 cm height, 80 cm wide and 1.50 m length. The plastic tanks contained soil and water in order to simulate the ponds where the frogs were collected from. The plastic tanks were placed outdoors so that frogs were exposed to natural conditions of light and temperature. Tanks were covered to prevent flooding and animals drowning during rainy periods. Frogs were fed with larvae of *Tenebrio molitor* everyday until the first 5–7 days of November. Feeding was stopped as frogs started to be less active and remained longer in the water. From the first days of November (2006) until the middle of March (2007) animals were removed at regular periods and were killed by double pithing. Animals were rapidly dissected open and a blood sample was removed as described by Stewart et al. (2004) for the determination of partial pressure of oxygen (Po_2) (see below). Then the heart and skeletal muscle (gastrocnemius) tissues were dissected, freeze-clamped between aluminum tongs cooled in liquid nitrogen and ground under liquid nitrogen. Tissue powders were stored at −80 °C until measurements of enzymatic activities and the levels of L-lactate.

To examine the effects of ambient temperature on the activities of glycolytic enzymes from active frogs, a plastic tank containing a number of animals was put into a cool room at 5 °C and maintained under these conditions for 15 days. Individuals were drawn after 1, 5, 10 and 15 days of acclimation at 5 °C, tissues were dissected and treated for the measurements of enzymatic activities as described above.

The snails (*H. lucorum*) used for the experiments described in this paper were collected in October 2006 from a population in the vicinity of Edessa, in northern Greece. Adult snails (35–42 mm) were transferred to the laboratory where they were put in large glass boxes, filled with soil derived from the area from where snails were collected. The boxes were placed outdoors so that snails were exposed to natural conditions of light and temperature. The boxes were covered to prevent flooding and animals drowning during rainy periods. Water was added periodically to ensure that soil was kept moist and the ambient humidity was maintained above 80%. Snails were fed fresh lettuce leaves everyday by the first 5–7 days of November. Feeding was stopped as soon as snails started to burrow into the ground. From the first days of November (2006) until the middle of March (2007) snails were removed at regular periods and immediately their haemolymph was sampled as described by Pedler et al. (1996) for the determination of Po_2. Then foot muscle and mantle tissues were dissected, freeze-clamped between aluminum tongs cooled in liquid nitrogen and ground under liquid nitrogen. Tissue powders were stored at −80 °C until measurements of enzymatic activities and the levels of D-lactate.

To examine the effects of ambient temperature on the activities of glycolytic enzymes from active snails a number of snails maintained at 20 °C were put into a cool room at 5 °C and maintained under these conditions for 15 days. Individuals were drawn after 1, 5, 10 and 15 days of acclimation at 5 °C, tissues were dissected and treated for the measurements of enzymatic activities as described above.

Frogs and snails were sampled in the middle of the day and ambient temperature was recorded at the day of sampling.

2.2. Collection of blood and haemolymph and determination of Po_2

Blood sampling from frogs closely followed the methods of Stewart et al. (2004). Briefly, each frog was double-pithed and the heart exposed via a ventral midline incision. A heparinized (100,000 units/L ammonium heparin in Ringer's solution) tuberculin syringe was used to withdraw a 0.4-ml blood sample anaerobically for determination of Po_2.

Haemolymph collection from snails was performed as described by Pedler et al. (1996). In brief, after removing a small section of shell to expose the pericardium, haemolymph was collected after puncturing the heart with a needle fitted to a syringe, previously equilibrated with pure nitrogen. The Po_2 was determined in snails' haemolymph and frogs' blood by use of a Clarke type oxygen electrode (E5047) in the gas cuvette of the BMS3/MK2 Blood Micro System. All determinations were performed at temperatures corresponding to those recorded at sampling occasions.

2.3. Preparation of tissues homogenates for the determination of enzymatic activities

Glycolytic enzyme assays were adapted from those described by Storey and Storey (1984) and Stuart et al. (1998a). Briefly, samples of frozen tissue powders (200–500 mg) were rapidly weighed and homogenized (1:5, wt/vol) in ice-cold 50 mM imidazole–HCl (pH 7.0) containing 100 mM sodium fluoride (NaF), 5 mM EDTA, 5 mM EGTA, 15 mM 2-mercaptoethanol and 0.1 mM PMSF added just prior to homogenization, using a Polytron PT10 homogenizer (3 periods, 20 s each time). After centrifugation (15,000 *g*, 4 min, 4 °C), the supernatant was removed and passed through a 5-ml column of Sephadex G-25 equilibrated in 40 mM imidazole–HCl buffer (pH 7.0) containing 5 mM EDTA, 15 mM 2-mercaptoethanol, and 20% glycerol to remove metabolites of low molecular mass (Helmerhorst and Strokes, 1980). The column was centrifuged in a desktop centrifuge at 2000 *g* for 1 min, and the supernatant was used for the determination of enzyme activity. Enzyme activities (expressed as μmol/min g wet weight) were determined at 20 °C using a Hitachi 150-20 recording spectrophotometer with water-jacketed cell. Assays were carried out in duplicate and rates of reactions involving NAD or NADH were followed at 340 nm (millimolar extinction coefficient ε_{340}=6.22). In addition, to examine the effects of low temperature on enzymatic activities from hibernated frogs and snails, samples of frozen tissue powders from animals collected at the end of December 2006 were treated as described above and the enzymatic activities determined at 5 °C, which corresponds to the ambient temperature measured at the same periods of animal collection. All enzymes were assayed for 10 min in 50 mM imidazole–HCl pH 7.0 in a final assay volume of 1 ml. Specific assays conditions were as follows.